TITLE: Cyclical cervical function in the mare involves remodelling of collagen content, which is correlated with modification of oestrogen

AUTHORS: Campbell, M L H; Peachey, L; Callan, L J; Wathes, D C; De Mestre, A M

JOURNAL: Animal Reproduction Science

PUBLISHER: Elsevier

PUBLICATION DATE: 12 September 2019

DOI: https://doi.org/10.1016/j.anireprosci.2019.106192

Cyclical cervical function in the mare involves remodelling of collagen content, which is correlated with modification of oestrogen receptor 1 abundance

Campbell, M. L. H.[a*], Peachey, L[1], Callan, L[2], Wathes, D. C. [a], and de Mestre, A. M[b].

The Royal Veterinary College, Hawkshead Lane, North Mymms, Herts, AL9 7TA, UK

[a]Department of Pathobiology and Population Sciences

[b]Department of Comparative Biomedical Sciences

[*]Corresponding author: mcampbell@rvc.ac.uk

[1]Present address: University of Bristol, Langford House, Langford, North Somerset BS40 5DU

[2]Current address: Ivybank Veterinary Clinic, 2 Low Green Road, Irvine, KA128RA

Declarations of interest: none

ABSTRACT

This study was conducted to elucidate mare cervical dilation mechanisms by testing two hypotheses: (i) the proportion of collagen staining in histological samples of mare cervices and (ii) the abundance of hormone receptors in the equine cervix differ with stage of the oestrous cycle and site within the cervix. Tissues and jugular vein blood samples were collected from 15 mares. Collagen content was assessed using Masson's Trichome staining. Receptor abundance was assessed using RT-PCR, qRT-PCR and immunohistochemistry. In sub-epithelial stroma, there was less collagen during the follicular than luteal phase, in the caudal- ($P = 0.029$), mid- ($P = 0.0000$) and cranial ($P = 0.001$) cervical tissue. In the deep stroma, there was less collagen staining during the follicular stage in the mid- ($P = 0.004$) and cranial- ($P = 0.041$) cervical regions. There were *PTGER2*, *PTGER3*, *PGR* and *ESR1* mRNA transcripts in the cervix. A greater proportion of cells were positive for ESR1 protein during the follicular phase in sub-epithelial ($P = 0.019$) and deep ($P = 0.013$) stroma. The abundance of ESR1 in the epithelium was negatively correlated with collagen staining in sub-epithelial ($P = 0.007$) and deep ($P = 0.005$) stroma. The results of the study provide new information about the cervical biology of mares by increasing the knowledge about collagen content and the relationship between collagen content and ESR1 protein abundance during the oestrous cycle which indicates the ESR1 receptor is a candidate for involvement in control of cervical dilation.

Keywords

Mare; Cervix; Oestrogen receptors; Collagen remodelling; Prostaglandin receptors

1. Introduction

The cervix functions as a variable barrier between the environment and the uterus. The cervix of mares is composed of longitudinally arranged folds that are continuous with the uterine folds, made up of thick circular smooth muscle containing elastic fibre, highly folded mucosa and epithelium containing many mucus-secreting cells and some kinocilia (Huchzermeyer et al., 2005). Histologically, it is more glandular at the cranial end, and contains columnar or cuboidal epithelium in the mid-cervix, and squamous epithelium at the caudal end. Results of studies in women indicate that there is a majority of cervical tissue (90%) composed of extra cellular matrix (ECM), and the remainder of smooth muscle cells (House et al., 2009). The ECM consists predominantly of collagen and also proteoglycans, hyaluronon, elastins and water (House et al., 2009). In the non-gravid rat, the distribution of collagen differs in different parts of the cervix, with the largest amount located at the caudal end (Barone et al., 2012).

The cervix of the mare undergoes characteristic changes in gross anatomy associated with different stages of the estrous cycle (Greenhof and Kenney,1975, Katila, 2012). During oestrus, the mucous membranes become congested, the folds are prominent, and cervical secretions are pronounced. There is relaxation of the cervix and movement of the cervix into a more ventral position, facilitating relatively easy access through the cervical lumen during ejaculation or insemination for semen transiting into the uterus. Cervical relaxation during oestrus is also a prerequisite for clearance from the uterus of the normal substances resulting from the physiological inflammatory reaction that occurs as a consequence of mating. During dioestrus, the mucous membranes are less congested, cervical folds less pronounced, and the cervix becomes firmer and protrudes caudally, thus functioning as a barrier between the vagina and uterus (Lieux, 1970).

Failure of cervical dilatation during oestrus is a recognised phenomenon in mares (Katila, 2012). It is clinically significant as it can inhibit the passage of semen into the uterus

following insemination or natural mating, and can also compromise fertility by inducing post-breeding endometritis due to inhibition of egress of inflammatory fluid from the uterus (LeBlanc et al., 1994; Bucca et al., 2008; Reilas et al., 2016). Failure of cervical dilation can also be associated with congenital malformations or adhesions of the cervix caused, for example, by previous dystocia. Many mares in which cervices fail to dilate during oestrus (most commonly older, nulliparous mares) do not have any gross anatomical abnormalities. These mares are typically described in the literature and clinically as having "fibrotic" cervices (Pycock, 2004; Troedsson, 2006; LeBlanc, 2012). Even though there is frequent use of this term, there is a dearth of evidence that the failure of cervical relaxation during oestrus is associated with abnormal quantities of collagen or fibrous tissue within the cervix.

Much of what we know about the mechanisms regulating cervical relaxation relate to preparation for parturition (Leppert, 1995), which involves a reduction in collagen and glycosaminoglycans and increases in water content, hyalurononan and metalloproteinases (Soh et al., 2012). Less is known about cervical re-modelling at different stages of the oestrous cycle of domestic mammals. In ewes, cervical relaxation during oestrus is associated with changes in the extracellular matrix of cervical tissues (Kershaw-Young et al., 2010). In the bitch, cervical patency is positively correlated with extent of collagenase activity, and there is negative correlation between cervical patency and collagen concentration (Tamada et al., 2012). Cervical dilation (either during oestrus or prior to parturition) is associated with increased synthesis of prostaglandin E_2 (PGE_2) in ewes (Leethongdee et al., 2010), cows (Mizrachi and Shemesh, 1999), and bitches (Kunkitti et al., 2011). There is a stimulation of cervical dilation as a result of the actions of PGE_2 by inducing collagen fibres to separate and disperse as reflected by an increased inter-fibrillary distance, thus reducing the tensile strength (Feltovich et al., 2005) and inducing smooth muscle relaxation and GAG synthesis (Kershaw-Young et al., 2009). The biosynthetic pathways which when stimulated result in an

increase in PGE_2 production appear to be induced by oestrogen in ewes (Kershaw-Young et al., 2009) and bitches (Kunkitti et al., 2011), possibly via oxytocin actions. The actions of oxytocin are greater when there are relatively greater oestrogen concentrations (Kershaw et al., 2007). In the cervix of cattle, there are increased concentrations of PGE_2 at oestrus that are induced by a local increase in the number and/or function of FSH receptors (Mizrachi and Shemesh, 1999).

The results of these studies indicate that a failure of collagen re-modelling rather than abnormal quantities or distribution of collagen *per se* is more likely the cause of inadequate cervical dilation. Although failure of cervical dilation is a recognised clinical problem, research on hormonally-controlled mechanisms for cervical dilation in the mare is sparse. Further, the changes in collagen content during the estrous cycle have not been quantified. Identifying the mechanisms of normal cervical dilation during oestrus in the mare is necessary to inform further studies in which the aim is to develop targeted therapies for failure of cervical dilation. Fernandes et al. (2017) compared the mRNA abundance of steroid and specific prostaglandin receptors of horses in the cervical mucosa and stroma during oestrus, dioestrus and late pregnancy. Abundance patterns for prostaglandin E2 type 3 receptor (PTGER3), along with how hormone receptor abundance correlates with cervical collagen modelling are not known. The research described in this paper, therefore, was conducted to (i) compare collagen content in the cervix of mares during the follicular and luteal phases of the oestous cycle, (ii) determine the presence of a range of candidate hormone receptors within the mare cervix and (iii) investigate correlation(s) between abundance of candidate receptors and collagen remodeling. Two hypotheses were tested:

(1) That the proportion of collagen staining in histological samples taken from the cervices of mares differs with stage of the oestrous cycle, and site within the cervix.

(2) That the abundance of steroid and prostaglandin receptors in the mare cervix differs with stage of the oestrous cycle and site within the cervix.

2. Materials and methods

2.1. Sample collection and storage

Tissues and jugular vein blood samples were collected from non-pregnant mares during the breeding season at an abattoir immediately following slaughter. The reproductive tracts were assessed by three experienced reproductive biologists. Mares with gross cervical, ovarian and uterine abnormalities were excluded. Mare age was confirmed using information recorded in the mare's passport and ranged from 4 to 18 years. The number and size of the follicles, corpora lutea and corpora haemorrhagica in both ovaries was recorded for each mare. An approximately equal proportion of stroma and mucosa was collected in duplicate samples from three sites of the cervix (uterine, mid and vaginal) and placed in RNA *later* solution (Ambion; Ambion Inc USA) and was subsequently stored at -20 °C. Additionally, a 2 x 2 cm tissue section was removed from the same three sites of the cervix and placed in 4% paraformaldehyde (Electron Microscopy Sciences 15710). Blood was centrifuged at 1200 rpm for 20 minutes and serum stored at -20 °C.

2.2. Progesterone ELISA and classification of stage of oestrous cycle

An ELISA assay was used to determine serum progesterone on all serum samples using a blood progesterone ELISA kit (Ridgeway Science Ltd, Gloucestershire, UK) according to the manufacturers' instructions. The ELISA absorbance was obtained using the Tecan Sunrise Absorbance Reader and analysed utilising the Magellan Software Version 6.5. Progesterone concentrations were quantified using a standard curve constructed from control samples provided in the kit. Results were further validated by radioimmunoassay as

previously described (Wathes et al., 1986), with inter- and intra-assay coefficients of variation of 10.3% and 6.6%, respectively. Mares were classified as being in the luteal phase of the oestrous cycle at the time of slaughter if there were serum progesterone concentrations of >4 ng/ml and the presence of at least one corpus luteum in the ovaries of the mare. Mares were classified as being in the follicular phase of the oestrous cycle if there was one or more follicles >35 mm present in the ovaries of the mare, an absence of a corpus luteum or corpus haemorrhagicum, and a serum progesterone concentration of <0.2 ng/ml (assay detection limit 0.16 ng/ml)). A total of 15 mares (out of a total of 32 mares with tissue collected) met one of these classification criteria, with nine mares defined as being in the luteal phase and six mares in the follicular phase at the time of slaughter. The number of mares from which there were tissues that contributed to each individual experiment is shown in the respective figure legends.

2.3. RNA isolation, cDNA synthesis and RT-PCR

The RNA was extracted from 100 mg of cervical tissue collected from the cranial, mid- and caudal cervical tissues using a RNeasy Fibrous Tissue Mini kit (Qiagen) following the manufacturers' protocol. Homogenization was achieved by initially using a pestle and mortar placed in liquid nitrogen to pulverise the tissue followed by passage through a QIA shredder column (Qiagen). The RNA concentrations were quantified using the NanoDrop spectrophotometer, utilising the ND1000 V 3.7.1 software. Total RNA was DNase-treated using DNase I amplification grade (Invitrogen). Initially there was strand cDNA synthesis on 500 ng total RNA in a total volume of 50 µl conducted using M-MLV Reverse Transcriptase (USB), OligoDTs (Invitrogen) as previously described (De Mestre et al., 2009) with subsequent storage at -20 °C. All subsequent RT-PCR and qPCR experiments were performed using 2 µl of cDNA (20 ng).

Primers for *oestrogen receptor 1 (ESR1), oestrogen receptor 2 (ESR2)*, *progesterone (PGR), prostaglandin E2 type 2 (PTGER2)*, *type 3 (PTGER3)* and *type 4 (PTGER4*), *luteinising hormone receptor* (*LHR*) and *follicle stimulating hormone receptor* (*FSHR*) were previously described (Rambags et al., 2008, Fernandes et al, 2017) or designed using Primer3 (http://fokker.wi.mit.edu/primer3/input.htm) and obtained from Invitrogen (Table I). Annealing temperature and primer concentrations were optimized (Table 1). The RT-PCR was performed as previously described (de Mestre et al., 2009) with *ACTB* used as a loading control. The PCR products were cloned and sequenced to confirm amplification of the correct target gene and products were separated using gel electrophoresis

2.4. Absolute quantitative RT-PCR

The qRT-PCR reactions were performed using a Qiagen QuantiFast SYBR Green PCR kit (Qiagen) to quantify the absolute copy numbers of ESR1 using a C-1000 thermal cycler and CFX-96 Real time system (BioRad) in a total volume of 20 µl. The PCR reactions were conducted for 38 cycles of 30 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C. A melting curve was calibrated from 60 to 95°C and there was confirmation of the generation of a single PCR product for all samples. The Ct of the no template controls were less than the sensitivity of the assay (did not amplify at 38 cycles). Standard curves were developed by performing 10-fold serial dilutions of the known concentrations of the PCR product for each gene and absolute copy number calculated as previously described (de Mestre et al., 2009) from the standard curve. Briefly, each set of oligonucleotides were assessed for generation of a single amplicon (Supplementary Figure 1). The amplicon was extracted from the gel using a Qiagen QIAquick Gel extraction kit (Qiagen) as described by the manufacturer and the extracted DNA quantified using NanoDrop spectrophotometer. Each cDNA sample was

normalised to 6000 copies of succinate dehydrogenase complex, subunit A (*SDHA*; reference gene).

2.5. Tissue embedding, sectioning and H and E staining

Fixed cervical tissue samples were stored for 7 days at 4 °C in 4% paraformaldehyde (Electron Microscopy Sciences). On day 7, these sample were dehydrated in graded alcohol before being further dehydrated, cleared and paraffin impregnated for 18 hours in the Tissue-Tek 'VIP' Processor. The samples were embedded in paraffin using the Tissue Tek Embedding Centre. Tissue sections were cut at 7 µm using a HM60 rotary microtome and floated onto SuperFrost Plus slides (VWR International). These were dried overnight at 42 °C and stored at room temperature. All tissue sections were initially stained with Haematoxylin and Eosin using standard techniques to verify the cellular structure of the cervical tissue. All stained sections were visualised and photographed using the Leica application suite with DM400B upright microscope.

2.6. Masson's Trichrome staining

Collagen content was assessed using Masson's Trichome staining on sections from the caudal, mid- and cranial tissues of the cervix, as previously described (Kershaw et al., 2007). Images of stained sections were obtained using a DM4000B upright microscope at 200 x magnification. The total red and blue staining per image was assessed in five different fields per slide using Volocity Software (Perkin Elmer). The ratio of blue to red staining and total blue staining in the deep stroma and the total blue staining in the sub-epithelial stroma were compared in the different cervical regions and between the stages of the oestrous cycle as subsequently described in this manuscript.

2.7. Immunohistochemistry

Immunohistochemistry for confirmation and localisation of ESR1 protein was conducted on duplicate serial sections (with one section used for isotype control labelling) of mid-cervical tissues. Based on the previous literature, the endometrial tissues obtained from a mare in the follicular phase of the oestrous cycle was selected as a positive control tissue for ESR1 abundance. Sections were deparaffinised and rehydrated in alcohol and were washed in deionised water for 10 minutes. For antigen retrieval, sections were processed in a microwave while the samples were submerged in 10 mM sodium citrate buffer (pH 6) for three periods of 5 minutes. After heating, the slides were allowed to cool for 20 minutes and washed ten times in deionised water. Endogenous peroxidase activity was quenched with 3% hydrogen peroxidase solution for 20 minutes followed by ten washings with deionised water and blocking was performed for 1 hour at room temperature in 3% bovine serum albumin (BSA). After blocking, slides were washed with 5 mM Tris Buffer Solution (pH 7.4) with Tween (TBST) and incubated at 4 °C overnight in a humidified chamber with a monoclonal mouse anti-human ESRα antibody (Dako, Clone 1D5) or monoclonal mouse anti-human ESRβ antibody (Dako, Clone PPG5/10) diluted 1:50 and 1:10, respectively, in antibody diluent previously described for use with horse tissues (Lunelli et al., 2013). Serial sections were alternatively incubated with a mouse IgG2a isotype control (negative control). Following incubation, slides were washed in TBST three times then the secondary antibody (Biotinylated Link, LSAB+System-HRP, Dako K0679) was added for 30 minutes and incubated in a humidified chamber at room temperature. The three TBST washings were repeated and streptavidin-HRP solution was applied to the sections for 20 minutes. There was subsequent addition of DAB chromagen to the sections and the slides were counter-stained with Mayer's Haematoxylin. For evaluation of immunoreactivity, the stained sections were visualised and photographed at different magnifications using the Leica application suite with the DM4000B

upright microscope (Leica Microsystems, Milton Keynes, UK). The number of positively stained cells in the epithelium and stroma as well as the total number of epithelial and stromal cells in the section were counted using Image J software (Abramoff et al., 2004).

2.8. Statistical analysis

Statistical analysis of collagen staining was performed in IBM SPSS Statistics Version 20 (IBM Corporation, Portsmouth, UK). All datasets were initially subject to normality testing and found to be normally distributed. A repeated measures two way ANOVA was used to compare the position within the cervix (caudal-, mid- or cranial-cervical tissues) and stage of oestrous cycle. Independent t-tests were performed to compare collagen content in the two stages of the oestrous cycle within the same region of the cervix. Statistical analysis of *ESR1* mRNA and protein abundance and the correlation coefficientwas determined using collagen staining utilising Prism 7 (GraphPad Software). *ESR1* mRNA and protein abundance was compared between luteal and follicular phases using an unpaired Student's t-test. Correlation coefficients between collagen staining and ESR1 protein abundances were determined using either a Pearson's correlation for data that were normally distributed (collagen content in deep stroma) or Spearman's test for data that were non-parametric (collagen content in sub-epithelium).

3. Results

3.1. Collagen content in the cervix

Tissue obtained from the cranial-, mid- and caudal cervical tissue was initially assessed by H and E to confirm that the sections were representative of the different regions of the cervix (Supplementary Figure 2). The vaginal cervix was lined by stratified squamous epithelium whilst the mid cervix was lined with simple columnar epithelium and had

structurally prominent tertiary folds. In the sub-epithelial stroma, there was less collagen staining during the follicular than luteal phase, in the cranial, (P = 0.029), mid- (P = 0.000) and caudal (P = 0.001) regions of the cervix (Figure 1A, C). Similarly, in the deep stroma there was less collagen staining during the follicular compared with the luteal phase in the mid- (P=0.004) and cranial- (P = 0.041) tissues of the cervix (Figure 1B, D). This difference was not significant in the caudal cervical tissue. There was no difference in collagen staining in the deep stroma or sub-epithelial stroma between regions of the cervix within stages of the oestrous cycle (Figure 1C, D). The mean age of mares in the two groups was not different between stage of the oestrous cycle (mean ± SEM for luteal phase group = 10 ± 1.3 years and follicular phase group = 12.5 ± 1.7 years). Furthermore, there was no correlation between mare age and collagen content within the luteal and follicular phase of the oestrous cycle (data not shown).

3.2. Determination of abundance of prostaglandin and gonadotrophin receptor mRNA

The RT-PCR results provided evidence that there was *PTGER2* and *PTGER3* mRNA transcripts in the mid-cervical tissue at all stages of the oestrous cycle (Figure 2A,B). No RT-PCR amplicons of the correct size could be reliably detected for *PTGER4* for the caudal-, mid- and cranial-cervical tissues or positive control tissue evaluated during the course of the study using three different sets of primers . The *LHR* mRNA transcript was detected in the mid-cervix tissue in three of six samples in the luteal phase and in none of the samples from mares that were in the follicular phase of the oestrous cycle at the time of slaughter. Positive control tissue (stallion testis) was also positive for *LHR* mRNA transcript. The *FSHR* mRNA transcript was not detected in any mid cervix tissue (Figure 2B) or any of the caudal- or cranial-cervical tissues evaluated (n = 22, data not shown) but was routinely detected in the positive control tissues, ovary and follicle (Figure 2B; data not shown).

3.3 Identification of steroid receptor mRNA

The RT-PCR data indicated there was *PGR* mRNA transcript in the mid-cervical tissues during all stages of the oestrous cycle (Figure 3A). Similarly, there was *ESR1* mRNA transcript in the mid-cervical tissue during all stages of the oestrous cycle, with the most intensely stained bands apparent in the follicular phase compared with the luteal phase. There was *ESR2* mRNA transcripts in the cervix of some but not all of the mares in both the follicular and luteal phases of the oestrous cycle. There was *ACTB* mRNA transcript in follicular and luteal samples (Figure 3A). The band intensities for *ESR2* were relatively less than those for both *ESR1* and *PR*. Abundances of mRNA transcripts for all three steroid receptors were similar among the cranial-, mid- and caudal-cervical tissues of the cervix (data not shown).

There was selection of *ESR1* from all the receptors evaluated for further studies as there was the greatest variation among stages of the oestrous cycle and the greatest abundance of *ESR1* mRNA transcript during the follicular phase of the oestrous cycle. Absolute quantitative RT-PCR analyses indicated there were 5,289 ± 1,355 (mean ± SEM) copies *ESR1* mRNA transcript in the mid-cervical tissue during the follicular phase of the oestrous cycle compared with 2,988 ± 1,060 copies in the luteal phase, a 1.8 fold greater abundance with this difference not being significant ($P = 0.20$; Fig. 3B).

3.4. Localisation of ESR1 using immunohistochemistry

Immunohistochemistry evaluations using an antibody against ESR1 indicated there was a distinct nuclear labelling of cells collected during the follicular and luteal phases of the oestrous cycle (Figure 4A). The ESR1 was localised within the epithelium and to a lesser extent the stroma of the cervix. There was a larger proportion of cells positive for ESR1 during the follicular compared with the luteal phase, both in the surface epithelium (2.0 fold,

P = 0.019; Fig. 4B) and stroma (2.3 fold, *P* = 0.013; Fig. 4C). The intense positive staining detected during the follicular phase was no longer present as the stages of the oestrous cycle advanced, with staining intensity becoming less and intermittent during the luteal phase. The ESR2 staining was also more intense in the nucleus of cells during the follicular and luteal phases, although there was no difference in intensity of staining for ESR2 between the two stages of the oestrous cycle (Supplementary Fig. 3).

3.5. Correlation between ESR1 protein abundance and collagen staining

The ESR1 receptor abundance in the epithelium was negatively correlated with collagen staining in both the sub-epithelial stroma (*P* = 0.007) and deep stroma (*P* = 0.005; Figure 5A, B). The ESR1 receptor abundance in the stroma was negatively correlated with collagen content in the sub-epithelium (*P* = 0.012), and in the deep stroma (*P* = 0.051; Fig 5C, D). During both stages of the oestrous cycle, there was no correlation between mare age and collagen content or mare age and ESR1 protein abundance.

4. Discussion

The findings in this study allow for quantification of changes in collagen content between the follicular and luteal phases of the oestrous cycle in the mare and allow for the calculation of a correlation coefficient for these changes and ESR1 protein abundance. The results of this study provide new information about the cervical biology of horses that will allow for further evaluation of the mechanistic pathways between ESR1 abundance and collagen content and, ultimately, what regulates cervical dilation. The reliance on slaughterhouse material for the present study is a limitation because there was no reproductive history available for each mare. The use of ELISA and radioimmunoassays for analyzing serum progesterone concentrations combined with visual inspection of the follicles

and corpora lutea on each ovary facilitated characterisation of the two stages of the oestrous cycle assessed, which enabled valid comparisons between mares in the follicular and luteal phases.

The histology of the regions of the cervix as defined in this study was consistent with descriptions from previous studies (Huchzermeyer et al., 2005; Katila, 2012). Collagen staining was markedly reduced in the follicular phase compared to the luteal phase in all areas of the sub-epithelial cervical stroma, and also in the mid- and cranial-deep cervical stroma. These findings indicate that, consistent with reports in the ewe (Kershaw-Young et al., 2010) and the bitch (Tamada et al., 2012), collagen remodelling and specifically the disruption of collagen fibrils may be an important aspect of cervical relaxation during oestrus in the mare. The reduction in collagen staining in the follicular compared with luteal phase was non-significant in the caudal-cervical tissues.

In contrast to studies in cows (Mizrachi and Shemesh, 1999), in the present study, there was no *FSHR* mRNA transcript detected in the mare cervix indicating this receptor does not have a function in regulation of cervical dilation in mares. There was *LHR* mRNA transcript detected, but only in half of the cervices collected from mares in the luteal phase of the oestrous cycle at the time of slaughter. The cause of this variation in abundance of *LHR* mRNA transcript among cervices of mares is not known and future *in vivo* experiments that allow for more precise assessment of the stage of the oestrous cycle may help elucidate the factors that regulate *LHR* mRNA transcript abundance in the mare cervix. The consistent detection of *PTGER2* mRNA transcript in the mare cervix during both the follicular and the luteal phases of the oestrous cycle in the present study is consistent with the results of Fernandes et al. (2017). There was no detection of *PTGER4* mRNA transcript in the mare cervices even though there were repeated attempts to do so. Fernandes et al (2017) reported that there was *PTGER4* mRNA transcript present in the cervix during both the luteal and

follicular phases. In the present study, there was also evaluation of *PTGER3* mRNA transcript and it was present during both the luteal and the follicular phases. The combination of results in the present study, including the new information about *PTGER3*, and those of Fernandes et al., (2017) indicate there is little variation in the abundance of prostaglandin receptors in the mare cervix during the oestrous cycle. This may indicate prostaglandin receptors are unlikely candidates as key regulators of cervical dilation in mares. Prostaglandin actions on the cervix cannot be discounted, however, because there may be regulatory effects through ligand availability.

In the present study, the abundance of *ESR1* and *ESR2* mRNA transcripts in the mare cervix during both the follicular and the luteal phases of the oestrous cycle is consistent with previously reported findings (Fernandes et al., 2017). High-affinity ESR1 and ESR2 receptors are also detected in the cervix of ewes (Falchi et al., 2012; Falchi and Scaramuzzi, 2013). In the present study, the abundance of *ESR2* mRNA transcript did not differ between stages of the oestrous cycle, and this was again consistent with the findings of Fernandes et al. (2017). The *ESR1* mRNA abundance, however, was not greater during the follicular than luteal phase of the oestrous cycle in the present study and again this finding is consistent with that of Fernandes et al. (2017). Of all the receptors investigated in the current study, *ESR1* was the only receptor where there was a difference in abundance during the two different stages of the oestrous cycle. This receptor, therefore, was selected for immunohistochemistry studies because it appeared to be the most likely candidate for involvement in cervical remodeling. Results indicated there was a proportion of cells that stained positive for the ESR1 receptor in both the epithelium and stroma of the cervix during the follicular compared to the luteal phase. The variable and patchy pattern of ESR1 protein abundance in the surface epithelium in the luteal phase indicates that the location of the biopsy site could significantly affect mRNA transcript detected and hence may explain why this difference in ESR1 protein was

not also detectable using qRT-PCR. Alternatively, these differences could be explained by post-transcriptional modifications of mRNA. It is likely that there is an increase in abundance of ESR1 receptor during the follicular phase as a mechanism of cervical relaxation which occurs normally in oestrous mares. The fact that there is a negative correlation between ESR1 receptor abundance in the cervical epithelium and stroma and the collagen staining of the cervical stroma indicated that the function of ESR1 may be to mediate collagen re-organisation.

Exactly how ESR1 receptors could be mediating collagen remodelling and thus cervical relaxation in the mare was not determined in the present study. Results of studies in other species indicate plasma oestradiol, which is relatively greater in concentration can have actions during the follicular phase in the mare (Noden et al., 1975) to enhance the *ESR1* abundance (Pinzone et al., 2004) in the cervix (Zhao et al., 1999). In ewes, once activated by oestradiol, ESR1 receptors translocate into the nucleus where these receptors function to regulate gene expression, including the enhancement of oxytocin receptor gene expression (Kershaw et al., 2007). Oestradiol can have a direct effect on cervical function by increasing synthesis of PTGS2 (Zhang et al., 2007), which in turn promotes the production of PGE_2 (Kershaw-Young et al., 2010). There are PGE_2 actions that induce collagen fibril dispersion (Feltovich et al., 2005), and there are also direct relaxing effects of PGE_2 on the smooth muscle of the cervix in ewes (Kershaw-Young et al., 2009). In the present study, there *PTGER2* and *PTGER3* mRNA transcript in the cervix during both phases of the oestrous cycle indicating that the target receptors are available for the PGE2 to act in the mare.

Even if the primary mechanism of cervical relaxation in mares is indeed a hormonally-regulated action, it is likely to be multifactorial and to include multiple hormones and receptors functioning in concert. It remains unclear, however, whether these are direct or

indirect functions. Oestrogen (via ESR1*)* could be having a direct effect on collagen remodeling. Such a mechanism, however, has not yet been elucidated for the actions of the oestrogens on the mare cervix. Results from research in other species and other tissues indicate this may be a possibility. For example, in mice oestrogen regulates genes involved in cervical collagen assembly (COL3A1 and COL5A2 and processing (PLOD2; Nallasamy et al., 2017). Similarly, in rats relatively greater systemic concentrations of oestrogen are associated with reduced synthesis of cross-linked collagen content in the vagina, through a reduction in abundance of ESR1 (Montoyo et al., 2015). Research on ESR1 positive breast cancer cells using a mouse model has also provided results indicating there is a dynamic relationship between oestrogen activity and extracellular matrix remodeling, through modification of collagen fibre alignment (Jallo et al., 2019).

Alternatively, oestrogen (via ESR1*)* could be exerting an indirect effect on collagen synthesis and degradation via other hormones and receptors for these hormones. Oxytocin receptors in the mare cervix, for example, are in a relatively greater abundance during the follicular phase of the oestrous cycle (Annandale et al 2018). Furthermore, topical application of oxytocin to the mare cervix induces an increase in the height, width and area of the cervix (Klewitz et al 2014). This response could possibility result from the relatively greater systemic oestrogen in oestrous mares could occur because of an increased in numbers or functionality of cervical *ESR1* receptors during the follicular phase to induce a 'signalling' function which affects collagen remodeling not directly, but via an oxytocin-mediated pathway.

Signalling through hormone receptors is likely to be only a partial explanation of what controls cervical relaxation in mares. Other possibly pathways, for example those involving inflammation or autonomic innervation systems (Di Tommaso et al., 2017), have also been suggested to be functional in other species (Gonzalez et al., 2009). In ewes, there is a

negative correlation between the concentration of matrix metalloproteinase-2 and cervical collagen (Rodrigues-Pinon et al 2015). All of these possible pathways have yet to be investigated in horses and would be useful studies for further investigation.

5. Conclusions

The results of the present study indicate there is a correlation between *ESR1* protein abundance in the mare cervix, which is enhanced in the follicular phase, and collagen content, which is reduced during the follicular phase of the mare oestrous cycle. Whilst the present study provides new information about the cervical biology of mares and results indicate *ESR1* is somehow involved in collagen remodeling during cervical dilation, the mechanisms of that involvement need to be further elucidated. Future experiments that explore the oestradiol - ESR1 - collagen pathways in clinical cases of failure of cervical dilation are warranted.

Acknowledgements

Zhangrui Chang for P4 RIA assays, Andrew Hibbert for microscopy, Daniel Hampshire and Victoria Cabrera-Sharp for technical assistance.

Funding

Laura Peachey was funded by a Paul Mellon Fund Fellowship

References

Abramoff, M.D., Magalhaes, P.J., Ram, S.J., 2004. Image Processing with ImageJ.

Biophotonics Int. 11, 36-42.

Annandale, A., Stroehle, R. M., Schulman, M. L., Sibeko-Matjila, K. P., Fosgate, G. T., Handler, J., Vemming, D. C., Clift, S. J. 2018. Influence of cycle stage, age and endometrial biopsy score on oxytocin receptor distribution and gene expression in the cervix and uterus of non-pregnant mare. Theriogenology 120, 1-9

Barone, W.R., Feola, A.J., Moalli, P.A., Abramowitch, S.D., 2012. The effect of pregnancy and postpartum recovery on the viscoelastic behavior of the rat cervix. J. Mech. Med. Biol. 12, 12500091-125000917.

Bucca, S., Carli, A., Buckley, T., Dolci, G., Fogarty, U., 2008. The use of dexamethasone administered to mares at breeding time in the modulation of persistent mating induced endometritis. Theriogenology 70, 1093-1100.

de Mestre, A.M., Miller, D., Roberson, M.S., Liford, J., Chizmar L. C., McLaughlin, K.E., Antzak, D.F., 2009. Glial cells missing homologue 1 is induced in differentiating equine chorionic girdle trophoblast cells. Biol. Reprod. 80, 227-234.

di Tommaso, S., Cavallotti, C., Malvasi, A., Vergara, D., Rizzello, A., De Nuccio, F., Tinelli, A., 2017. A Qualitative and Quantitative Study of the Innervation of the Human Non Pregnant Uterus. Curr. Prot. Pept. Sc. 18, 140-148.

Falchi, L., Scaramuzzi, R.J., 2013. The expression of ERalpha, OTR, cPLA(2), COX-2, and PPARgamma in the cervix of the ewe during the estrous cycle. Theriogenology 79, 40-47.

Falchi, L., Taema, M., La Clanche, S., Scaramuzzi, R.J., 2012. The pattern of cervical penetration and the effect of topical treatment with prostaglandin and/or FSH and oxytocin on the depth of cervical penetration in the ewe during the peri-ovulatory period. Theriogenology 78, 376-384.

Feltovich, H., Ji, H., Janowski, J.W., Delance, N.C., Moran, C.C., Chien, E.K., 2005. 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. 192, 753-760.

Fernandes, C.B., Loux, S.C., Scoggin, K.E., Squires, E.L., Troedsson, M.H., Esteller-Vico, A., Ball, B.A., 2017. Sex-steroid receptors, prostaglandin E2 receptors, and cyclooxygenase in the equine cervix during estrus, diestrus and pregnancy: Gene expression and cellular localization. Anim. Reprod. Sci.187, 141-151.

Gonzalez, J.M., Xu, H., Chai, J., Ofori, E., Elovitz, M.A., 2009. Preterm and term cervical ripening in CD1 Mice (Mus musculus): similar or divergent molecular mechanisms? Biol. Reprod. 81, 1226-1232.

Greenhoff G. R., Kenney R. M., 1975. Evaluation of reproductive status of non-pregnant mares. J. Am. Vet. Med. Ass. 167, 449- 458

House, M., Kaplan, D.L., Socrate, S., 2009. Relationships between mechanical properties and extracellular matrix constituents of the cervical stroma during pregnancy. Semin. Perinatol. 33, 300-307.

Huchzermeyer, S., Wehrend, A., Bostedt, H., 2005. Histomorphology of the equine cervix. Anat. Histol. Embryol. 34, 38-41.

Jallow, F., O'Leary, K.A., Rugowski, D.E., Guerrero, J.F., Ponik S.M., Schuler, L.A. 2019 Dynamic interactions between the extracellular matrix and estrogen activity in progression of ER+ breast cancer. Oncogene. 2019 Aug 12. doi: 10.1038/s41388-019-0941-0. [Epub ahead of print]

Katila, T. 2012. The Equine Cervix. Pferdeheilkunde. 28 (1), 35-38

Kershaw-Young, C.M., Khalid, M., McGowan, M.R., Pitsillides, A.A., Scaramuzzi, R.J., 2009. The mRNA expression of prostaglandin E receptors EP2 and EP4 and the changes in glycosaminoglycans in the sheep cervix during the estrous cycle.

Theriogenology 72, 251-261.

Kershaw-Young, C.M., Scaramuzzi, R.J., McGowan, M.R., Pitsillides, A.A., Wheeler-Jones, C.P., Khalid, M., 2010. The effect of estradiol on COX-2, EP2, and EP4 mRNA expression and the extracellular matrix in the cervix of the hypogonadotrophic, ovariectomized ewe. Theriogenology 73, 620-628.

Kershaw, C.M., Scaramuzzi, R.J., McGowan, M.R., Wheeler-Jones, C.P., Khalid, M., 2007. 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. 76, 124-129.

Klewitz, J., Großheim, B., Schwenzer, K.,Pansegrau, U., Burger, D., Sieme, H. Effects of topical medications on the dimensions of the cervix in estrous mares. 2014 J. Equine Vet. Sci. 34 151

Kunkitti, P., Srisuwatanasagul, S., Chatdarong, K., 2011. Distribution of estrogen receptor alpha and progesterone receptor, and leukocyte infiltration in the cervix of cyclic bitches and those with pyometra. Theriogenology 75, 979-987.

LeBlanc, M., 2012. Antibiotic uses in equine reproduction: local versus systemic therapy, Proceedings of the 18th Annual Meeting of the Italian Society of Equine Veterinarians SIVE, Bologna, pp. 62-66. Available at https://www.ivis.org/proceedings/sive/2012/lectures/leblanc3.pdf (accessed 10th June 2019)

LeBlanc, M.M., Neuwirth, L., Asbury, A.C., Tran, T., Mauragis, D., Klapstein, E., 1994. Scintigraphic measurement of uterine clearance in normal mares and mares with recurrent endometritis. Equine Vet. J. 26, 109-113.

Leethongdee, S., Kershaw-Young, C.M., Scaramuzzi, R.J., Khalid, M., 2010. 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 73, 1257-1266.

Leppert, P.C., 1995. Anatomy and physiology of cervical ripening. Clin. Obstet. Gynecol. 38, 267-279.

Lieux, P., 1970. Relationship between the appearance of the cervix and the heat cycle in the mare. Vet. Med. Small. Anim. Clin. 65, 859-866.

Lunelli, D., Cirio, S.M., Leite, S.C., Camargo, C.E., Kozicki, L.E., 2013. Collagen types in relation to expression of estradiol and progesterone receptors in equine endometrial fibrosis. Adv. Biosci. Biotech. 4, 599-605.

Mizrachi, D., Shemesh, M., 1999. Follicle-stimulating hormone receptor and its messenger ribonucleic acid are present in the bovine cervix and can regulate cervical prostanoid synthesis. Biol. Reprod. 61, 776-784

Montoya, T.I., Maldonado, P.A., Acevedo, J.F., Word, R.A. 2015 Effect of vaginal or systemic estrogen on dynamics of collagen assembly in the rat vaginal wall. Biol Reprod. 92(2), 1-9

Nallasamy.,S., Yoshida, K., Akins, M., Myers, K., Iozzo, R., Mahendroo, M. 2017 Steroid Hormones Are Key Modulators of Tissue Mechanical Function via Regulation of Collagen and Elastic Fibers. Endocrinology 158(4), 950–962.

Noden, P.A., Oxender, W.D., Hafs, H.D., 1975. The cycle of oestrus, ovulation and plasma levels of hormones in the mare. J.Reprod. Fertil. Supp. 23 189-192.

Pinzone, J.J., Stevenson, H., Strobl, J.S., Berg, P.E., 2004. Molecular and cellular determinants of estrogen receptor alpha expression. Mol. Cell. Biol. 24, 4605-4612.

Pycock, J., 2004. Pre-breeding checks in mares. In Practice 26, 78-85.

Rambags, B.P., Van Tol, H.T., Van Den Eng, M.M., Colenbrander, B., Stout, T.A., 2008.

Expression of progesterone and oestrogen receptors by early intrauterine equine conceptuses. Theriogenology 69, 366-375.

Reilas, T., Rivera del Alamo, M.M., Liepina, E., Yeste, M., Katila, T., 2016. Effects on the equine endometrium of cervical occlusion after insemination. Theriogenology 85, 617-624.

Rodriguez-Pinon, M., Tasende, C., Casuriaga, D., Bielli, A.Genovese, P., Garofalo, E. G., 2015 Collagen and matrix metalloproteinase-2 and -9 in the ewe cervix during the estrous cycle. Theriogenology 84 (5), 818-826

Soh, Y.M., Tiwari, A., Mahendroo, M., Conrad, K.P., Parry, L.J., 2012. Relaxin regulates hyaluronan synthesis and aquaporins in the cervix of late pregnant mice. Endocrinology 153, 6054-6064.

Tamada, H., Kawata, N., Kawate, N., Inaba, T., Kida, K., Hatoya, S., Akune, A., Nakama, K., Kohsaka, T., Takahashi, M., Sawada, T., 2012. Factors associated with patency of the uterine cervix in bitches with pyometra. Res. Vet. Sci. 93, 1203-1210.

Troedsson, M.H., 2006. Breeding-induced endometritis in mares. Vet. Clin. North Am.: Equine. Pract. 22, 705-712.

Wathes, D.C., Guldenaar, S.E.F., Swann, R.W., Webb, R., Porter, D.G., Pickering, B.T., 1986. A combined radioimmunoassay and immunocytochemical study of ovarian oxytocin production during the periovulatory period in the ewe. J. Reprod. Fertil. 78, 167-183.

Zhang, Q., Collins, V., Chakrabarty, K., Rose, J.C., Wu, W.X., 2007. Regulation of the prostaglandin enzymatic system by estradiol and progesterone in nonpregnant sheep cervix. Reproduction 133, 1027-1034.

Zhao, Y., Williams, L.M., Hannah, L.T., Ross, A.W., McKelvey, W.A., Robinson, J.J., 1999. Oestrogen and progesterone receptor immunoreactivity and c-fos expression in the

ovine cervix. J. Reprod. Fertil. 115, 287-292.

Campbell et al., cervix
Figure legends

Figure 1

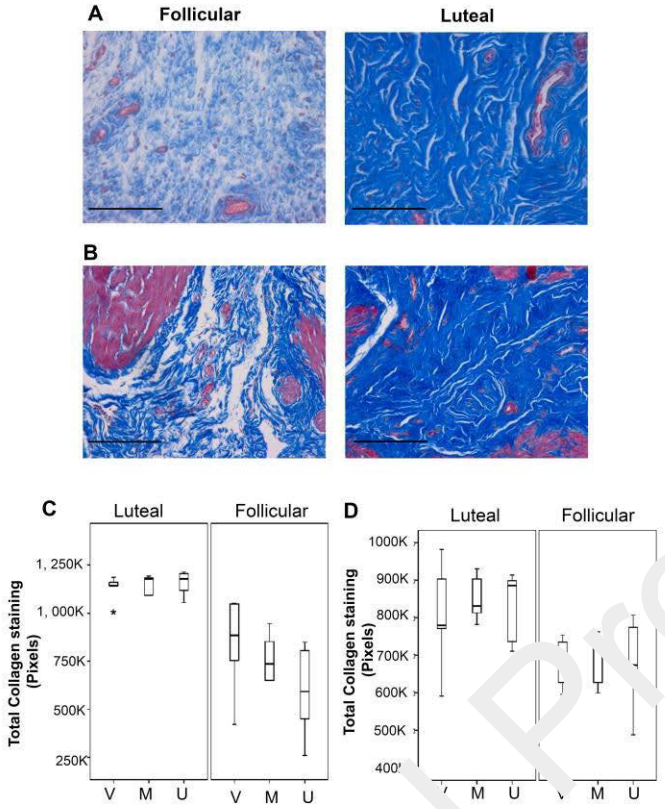

Fig. 1. A. Collagen content in the mare cervix. A. Massons Trichrome staining of sections of the cervix showing representative images of the sub-epithelial stroma (A) and deep stroma (B) from the follicular (left panel) and luteal phases (right panel); Quantification of total collagen staining in the sub-epithelial stroma (C) and deep stroma (D) in the caudal- (V), mid- (M) and cranial- (U) cervical tissue during the follicular (n = 5 mares) and luteal (n = 6 mares) phase of the oestrous cycle; Total collagen staining is described in pixels as calculated in Volocity as described in the methods using five independent measurements per mare; Box and Whisker plots show the 5 to 95 percentile and median value' Outliers are indicated by an asterisk

Figure 2

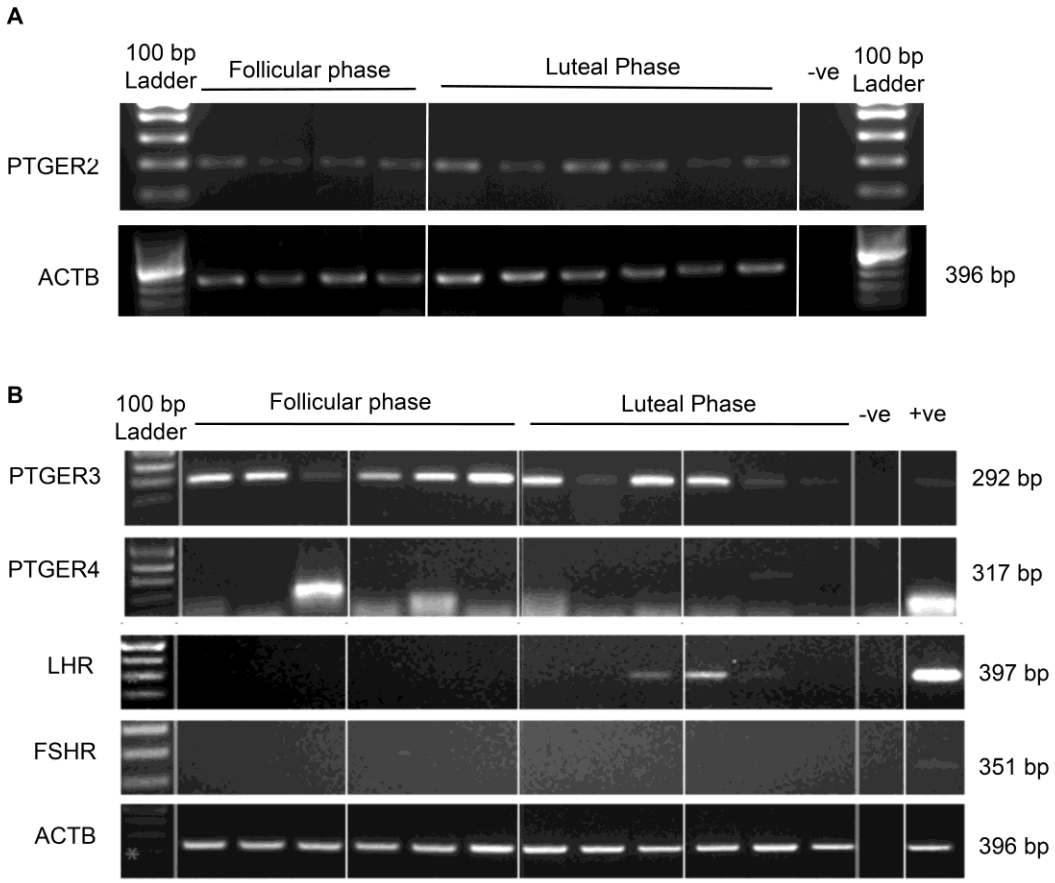

Fig. 2. Abundance of prostaglandin and gonadotrophin receptor mRNA in the mare cervix; A. Qualitative RT-PCR was used to determine the abundance of prostaglandin receptors *PTGER2 (A)* and reference gene (*ACTB*) in the mid-cervical tissue during the follicular phase (n = 4 mares) and luteal phase (n = 6 mares) of the oestrous cycle; B. *PTGER3, PTGER4, FSHR* and *LH* mRNA transcript abundance in the mid-cervical tissue during the follicular phase (n = 6) and luteal phase (n = 6) of the oestrous cycle; –ve indicates no template control and +ve indicates positive control tissue (LHR = endometrium; FSHR = follicle wall; PTGER3 and 4 = PBMC); Predicted size of bands stated in number of base pairs

Figure 3

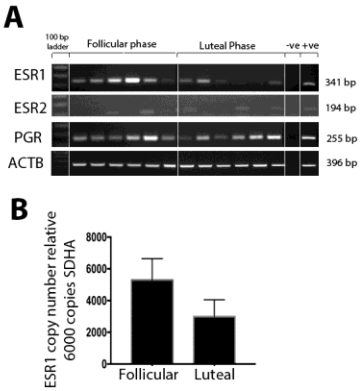

Fig. 3. Abundance of *ESR1, ESR2* and *PGR* mRNA transcript in the mare cervix; A. Qualitative RT-PCR was used to determine the abundance of *ESR1*, *ESR2* and *PGR* and a housekeeper (*ACTB*) in the mid–cervical tissue in the follicular phase (n = 6) and luteal phase (n = 6) of the oestrous cycle; –ve indicates no template control and +ve indicates positive control tissue (ESR1, PGR = endometrium; ESR2 = ovary); Predicted size of bands stated in number of base pairs; B. Absolute quantitative RT-PCR was used to determine abundance of *ESR1* mRNA transcript in the mid–cervical tissue in the follicular (n = 5) and luteal (n = 5) phase of the oestrous cycle; Data are the mean ± SEM copy number of *ESR1* normalised to 6,000 copies of reference gene *SDHA*

Figure 4

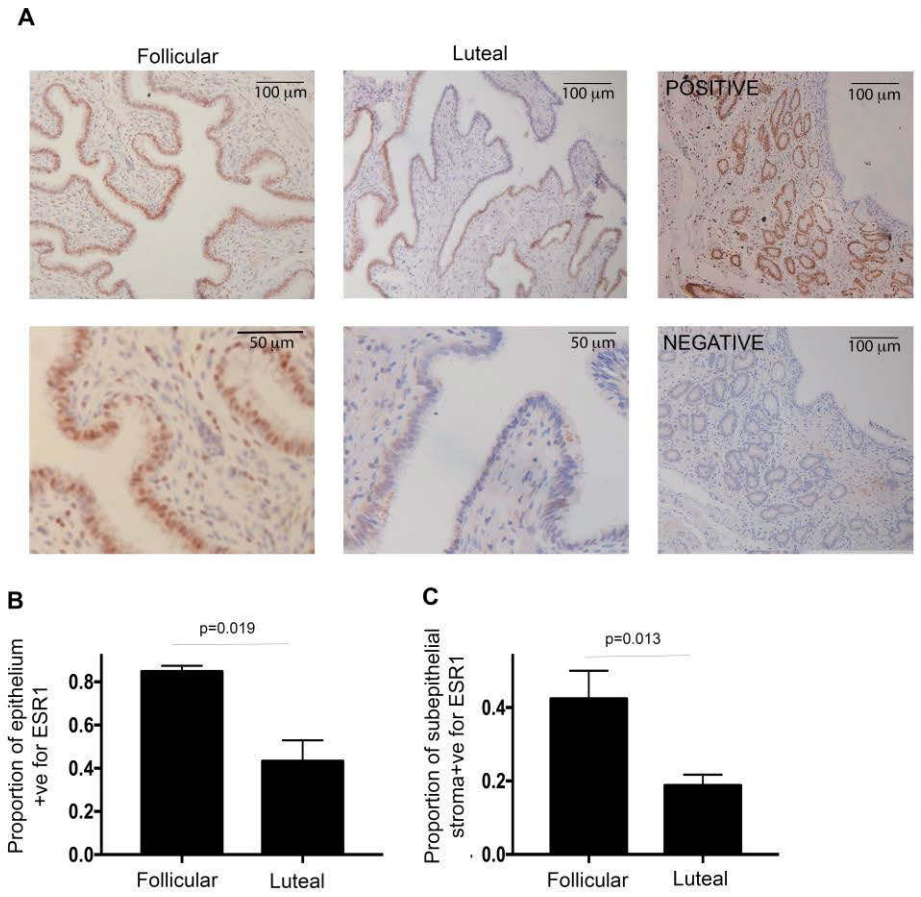

Fig. 4. ESR1 protein abundance is relatively greater the mare cervix in the follicular phase of the oestrous cycle; A. Representative brightfield images of sections of the mid–cervical tissue labelled with an anti-ESRα antibody during the follicular phase (left panel) and luteal phase (middle panel) at both low (upper panel) and high (lower panel) magnification; Sections of endometrium labelled with the antibody against anti-ESRα (positive control) or an isotype mouse IgG2a antibody (negative control) are shown in the right panel; ESRα positively labelled cells are represented by brown nuclear staining Proportion of surface epithelium (B) and sub-epithelial stroma (C) labelled with an anti-ESRα antibody during the follicular (n = 3) and luteal (n = 5) phase of the oestrous cycle; Data represent the mean ± SEM

Figure 5

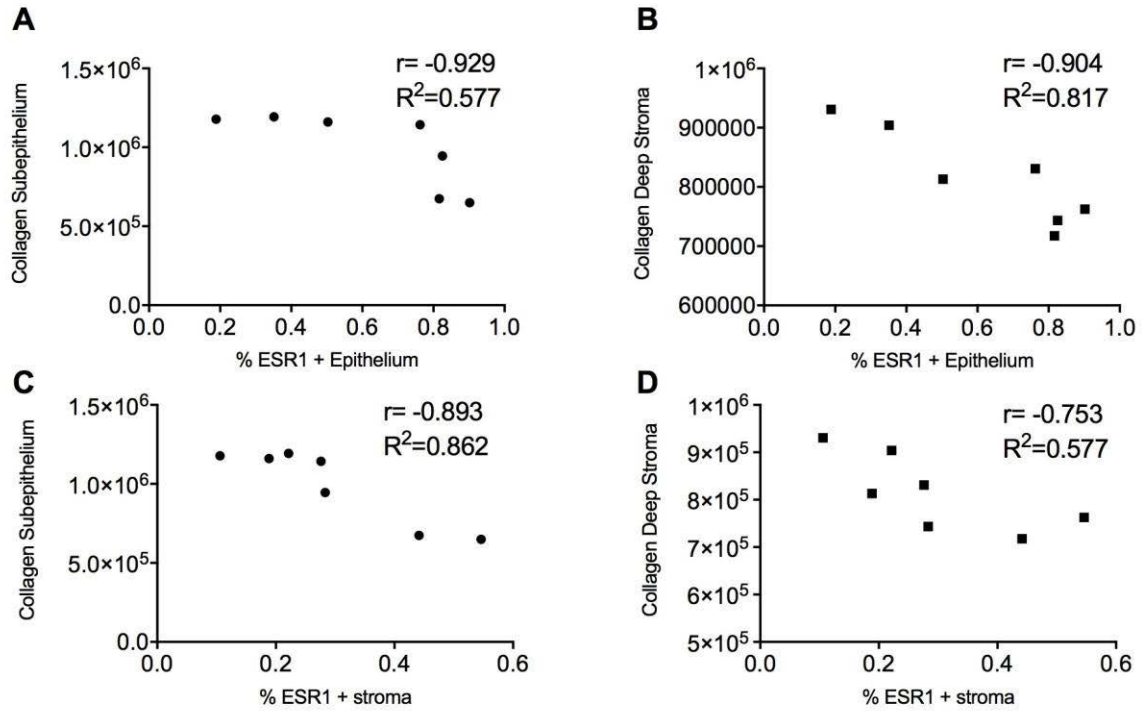

Fig. 5. ESR1 protein abundance and collagen content are highly correlated in the mare's cervix; Data shown in Figures 1 and 4 for a subset of mares (n = 7) where data were available from serial sections of the mid-cervical tissue that had been stained with Massons Trichrome and labelled with antibody against ESR1 was further analysed to assess the relationship between the collagen content and ESR1 protein abundance; r and R^2 values for Spearman (A, C) and Pearson tests (B, D) are shown

Table 1
Sequences of the primers used in RT-PCR and qRT-PCR and the optimised annealing

Gene	Primer Sequence 5′-3′	Predicted Product size (bp)	Optimal annealing temperature (°C)	Optimal primer concentration (μM)
ESR1	F TCCATGATCAGGTCCACCTTCT R GGTGTCTGTCATCTTGTCCA	341	55	0.25
ESR2	F TCAGCCTGTTCGACCAAGTG R CCTTGAAGTCGTTGCCAGGA	194	60	0.30
PR	F GTCAGTGGACAGATGCTGTA R CGCCTTGATGAGCTCTCTAA	255	55	0.5
LHR	F ATCCATTCCCTGCCTAACTATG R GGAGCAAGTCACATCAACGA	397	55	0.3
FSHR	F CTTTTGCATGGGGCTCTA R CCACATTGAGTATCAGGATGG	351	60	0.5
PTGER2	F CCTCCAAGCCCTTAGGTTTC R TATCCACAAGGGCCAGCTAC	191	60	0.5
PTGER3	F TTTCTTTGCCTCCACCTTTG R GCATTCTTTCTGCTTCTCTGTGT	292	60	0.3
PTGER4*	F ATCTTCGGGGTGGTGGGCAA R CGCTTGTCCACGTAGTGGCT	317	52 to 62 tested	0.5 tested
BETA ACTIN	F TCCCTGGAGAAGAGCTACGA R ACAATGAGGCCAGAATGGAG	496	60	0.5
ESR1 (qRT-PCR)	F CTCGGCTCCGTATGATGAAT R TCCAGAGACTTCAGGGTGCT	112	60	0.5
SDHA (qRT-PCR)	F CAGACGATTTATGGAGCAGAGG R CTGGATGGGCTTGGAGTAAT	105	60	0.5

Temperature and primer concentration; *Primers failed