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

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Abstract

The complex anatomy of the ovine cervix limits the success of trans-cervical artificial insemination in sheep. However, there is a degree of natural relaxation of cervix at oestrus that is accompanied by an increase in the water content. As hyaluronan (HA) has a high affinity for water molecules, in this study, we tested the hypothesis that the HA content of the cervix, the proportion of different size fragments of HA and expression of its receptor CD44 vary with the stage of the oestrous cycle. Oestrous was synchronized in 25 Welsh mountain ewes, and their cervices were collected either during luteal phase (n = 8) or pre-LH (n = 8) or post-LH (n = 9) surge stage of the oestrous cycle. The pre-LH surge group had the highest HA content (2.96 ng/mg of cervical tissue), which was significantly (P < 0.05) higher than that observed for the post-LH surge (2.04 ng/mg) group. The luteal phase group had a mean HA content intermediate between the pre- and post-LH surge groups, and was significantly different from either. The frequency of cervical samples containing both sizes of HA fragments (small and large) was significantly higher (P < 0.05) in the pre-LH surge group compared with the luteal and the post-LH surge groups, whereas that in post-LH surge group was significantly (P < 0.05) higher than that in the luteal group. The number of cervical samples that contained only small HA fragments was significantly (P < 0.05) higher in the pre-LH surge group compared with the luteal or post-LH surge groups, whereas the number of samples containing only large HA fragments was significantly (P < 0.05) higher in the post-LH surge group compared with the luteal or pre-LH surge groups. Overall mean expression of CD44 in the vaginal and mid regions was significantly (P < 0.001) higher than that in the uterine region, with no difference between the vaginal and mid regions of the cervix. Pattern of CD44 expression depended on the stage of the oestrous cycle. At the luteal stage, CD44 expression did not vary among epithelial, sub-epithelial, circular and longitudinal smooth muscle layers, whereas at the pre- and post-LH surge stages, the expression in the epithelial layer was significantly (P < 0.001) higher than that in the other three layers. In general, CD44 expression in the transverse smooth muscle layer was significantly (P < 0.05) lower than the expression in all the other layers at all the stages of the oestrous cycle. The results indicated that the HA varied with the steroid status. Higher HA values at a time when cervical relaxation is naturally higher may indicate its involvement in remodelling of the cervix at oestrus.

Introduction

Schemes for genetic improvement of sheep can make a significant economic impact on the UK sheep industry. However, maximum benefit depends on the use of artificial insemination (AI) with frozen–thawed (FT) semen because fresh semen is viable for only 8 h and sheep farms are geographically widely distributed. Fertility following cervical AI with FT semen is poor and not commercially acceptable. The use of FT semen with laparoscopic intrauterine AI gives acceptable results but the procedure itself is not welfare friendly. A practical solution is trans-cervical intrauterine insemination. However, the cervical lumen in sheep is convoluted, and the passage of an inseminating pipette into the uterus is not currently practicable. Lambing rates are considerably increased with an increase in the depth of cervical insemination (Salamon & Maxwell 1987), but the inseminating pipette can rarely be inserted more than 1 cm into the sheep cervix (Alvarez et al. 1998). This is mainly due to the second and third cervical rings being frequently out of alignment with the first.

The sheep cervix consists mostly of connective tissue including collagen, elastin and the macromolecular components that make up the extracellular matrix.
The degree of relaxation of cervix varies with the stage of the oestrous cycle, and is highest at oestrus. Taken together, this may mean that HA fragments of a particular molecular weight may be more active and/or available to carry out their function at oestrus. However, it remains to be determined whether the proportion of different size fragments of HA in the cervix varies with the stage of the oestrous cycle in a manner that parallels the degree of relaxation of the cervix.

The biological actions of HA may be mediated by binding to its cell surface receptor, CD44 (Aruffo et al. 1990), or receptors like RHAMM (Slevin et al. 2007), versican (Ruscheinski et al. 2008) and HA-binding proteins such as heavy chain subunit of the inter- 

α-trypsin inhibitor protein family (ITI; Zhuo et al. 2003). The CD44 family belongs to a larger group of HA-binding proteins, termed the hyaladherins (Toole 1990). CD44 mediates HA-dependent cell adhesion, which has been observed in a number of different cell types, including epithelial cells (Culty et al. 1992), leukocytes and fibroblasts (Underhill 1992). The interaction of HA to the well-characterized receptor, CD44, activates a signalling cascade involving small GTP-binding proteins and MAP kinase signalling pathways (Itano & Kimata 2002). While the amount of HA bound at the cell surface is influenced by several factors, e.g. type or isoform of CD44 (Stamenkovic et al. 1991) or the presence of the carbohydrate side chains of CD44 (Lokeshwar & Bourguignon 1991), it remains to be determined whether CD44 expression in the cervix is regulated by different hormonal milieus during different stages of the oestrous cycle. Thus, our hypothesis in the present study was HA content, the ratio of different sizes of HA and the expression of its receptor CD44, change in the cervical stroma depending on the stage of the oestrous cycle in sheep.

**Results**

**Plasma progesterone and LH concentrations**

All the ewes exhibited plasma progesterone (P₄) profiles characteristic with the stage of the oestrous cycle. As expected, higher P₄ concentrations (4.02 ± 0.32 ng/ml) were observed in the luteal ewes and basal (0.22 ± 0.02 ng/ml) in the pre- and post-LH surge ewes (Scott et al. 2001). The LH profiles showed basal LH concentrations during the luteal and early follicular phase with concentrations increasing 31–34 h after PGF₂α injection. The LH surge (23.2 ± 4.3 ng/ml) coincided with the GNRH injection and lasted 4–5 h, at these levels it was similar to that reported by (Scott et al. 2001). At the culling time, the ewes in the luteal and pre-LH surge groups had basal/lower LH concentrations (0.6–1.2 ng/ml), whereas those in the post-LH surge group had been exposed to the LH surge at least 3 h before the collection of cervices.
HA content

The data on the HA content are reported on the basis of dry weight of the cervix. The HA content of the cervix did not differ among the regions of the cervix. However, there was a significant ($P \leq 0.05$) difference among different stages of the oestrous cycle. The pre-LH surge group showed the highest HA content (2.96 ± 0.39 ng/mg of cervical tissue), which was significantly ($P \leq 0.05$) higher than that observed for the post-LH surge group (2.04 ± 0.19 ng/mg). The luteal phase group had a mean HA content (2.28 ± 0.22 ng/mg) intermediate between the pre- and post-LH surge groups, and was not significantly different from either (Fig. 1).

Changes in tissue hydration were observed in relation to stage of the cycle. The cervical tissue collected from the pre-LH animals had the highest water content, which contained significantly ($P \leq 0.05$) more (75.7%) fluid than the tissue collected from the luteal group. However, the moisture content of the cervix did not differ between the post-LH surge group and the luteal or the pre-LH surge groups.

HA fragment size

The number of cervical samples containing both small (200–2000 kDa) and large (≥2000 kDa) HA fragments was highest (33/75; 44%) followed by those containing only large HA fragments (27/75; 33%) or only small fragments (15/75; 20%). The frequency of cervical samples containing different sizes of HA fragments did not vary among the three regions of the cervix, but was significantly ($P \leq 0.05$) different among the three stages of the oestrous cycle (Fig. 2). The frequency of samples containing both sizes of HA fragments (small and large) was significantly higher ($P \leq 0.05$) in the pre-LH surge group compared with the post-LH surge group, which was significantly ($P \leq 0.05$) higher than that in the luteal group. The number of cervical samples that contained only small HA fragments was significantly ($P \leq 0.05$) higher in the luteal group compared with both the pre- and post-LH surge groups, whereas the number of samples containing only large HA fragments was significantly ($P \leq 0.05$) higher in the post-LH group compared with the luteal or pre-LH surge groups (Fig. 2).

CD44 expression

CD44 expression in the cervix was significantly ($P \leq 0.001$) affected by the region and the layer of the cervix as well as by the stage of the oestrous cycle. In the vaginal and mid regions, CD44 expression was significantly ($P \leq 0.001$) higher than in the uterine region, whereas no difference was observed between the vaginal and mid regions of the cervix (Fig. 3).

A significant ($P \leq 0.001$) interaction between the layer and the stage of the oestrous cycle indicated that the pattern of CD44 expression in the cervical layers was not the same for different stages of the oestrous cycle (Fig. 4b). CD44 expression in the transverse smooth muscle layer was significantly ($P \leq 0.05$) lower than the expression in all the other layers at all the stages of

![Figure 1](https://www.reproduction-online.org)

![Figure 2](https://www.reproduction-online.org)

![Figure 3](https://www.reproduction-online.org)

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the oestrous cycle, except in the sub-epithelial layer of the luteal and the pre-LH surge stages. At the luteal stage, CD44 expression did not vary among epithelial, sub-epithelial, circular and longitudinal smooth muscle layers, whereas at the pre- and post-LH surge stages, the expression in the epithelial layer was significantly \( (P \leq 0.001) \) higher than that in the other three layers. While there was no difference in the CD44 expression between the sub-epithelial, circular and longitudinal smooth muscle layers at the post-LH surge stage, at the pre-LH surge stage, the expression in the sub-epithelial layer was significantly \( (P \leq 0.001) \) lower than that in the circular and longitudinal smooth muscle layers of the cervix (Fig. 4b).

**Discussion**

The patency of the ovine cervix to semen is increased during the oestrous period to facilitate sperm transport and fertilisation (Hawk & Cooper 1977). However, the mechanism by which this is achieved and the role of HA in this process have not been thoroughly investigated. This study examined whether the HA, its varying fragments sizes and primary receptor CD44 vary in different regions of the ovine cervix at different stages of the oestrous cycle, which were confirmed by measuring plasma P4 and LH concentrations. At the culling time, the ewes in the luteal and pre-LH surge groups had basal/ lower LH concentrations that are comparable to the values reported earlier (Scott et al. 2001). The ewes in the post-LH surge group had been exposed to the LH surge at least 8 h before the cervices were collected. The significantly higher P4 concentrations observed in the luteal ewes and the basal values at the pre- and post-LH surge stages were also consistent with the results from earlier studies (Scott et al. 2001).

In our study, we observed that the HA content of the cervix was significantly higher in the pre-LH group compared with the luteal or post-LH surge groups, which may suggest that the cervical HA content varies with the steroid status. The higher HA content in the pre-LH surge group seems to be the result of accumulation during the luteal phase when these ewes were exposed to higher concentrations of P4 or alternatively rising oestrogen concentrations during the pre-LH surge stage might have played a role in this process. Evidence from a number of studies (Anderson et al. 1991, Rajabi et al. 1992, Ruscheinsky et al. 2008) suggest that higher P4 concentrations during pregnancy are implicated to increase the cervical HA content in a number of species. However, definite effects of oestrogens on the HA content are difficult to determine from the results of this study, and remain to be investigated. Nevertheless, HA content is reported to be the highest at term, at a time when P4 effect is withdrawn and there is relatively oestrogen dominance (Turnbull et al. 1974), a situation similar to that what we see in the pre-LH surge stage of the oestrous cycle.

In the present study, the HA content of the cervix was significantly higher at the pre-LH surge stage, which might explain a degree of natural relaxation of the cervix observed at oestrus (Granstrom et al. 1989) that is possibly achieved by loosening and dispersal of collagen fibres by water attracted by HA (Straach et al. 2004). It is noteworthy that in the present study, HA content is reported on the basis of dry weight of cervix and is, therefore, real and does not reflect any changes in the water content.

The results of the present study have shown that the luteal group had more cervical samples containing smaller HA fragments, and the post-LH surge group had more samples containing large HA fragments, whereas the pre-LH group had significantly more samples containing both small and large HA fragments. However, it is not known from the results of this study whether the enzymes resulting in synthesis or degradation are involved in generating and maintaining the observed HA polymer sizes. Nevertheless, HA polymers of different sizes have a vast array of functions (Kikuchi et al. 1996, Watanabe & Yamaguchi 1996, Ghosh & Guidolin 2002, Alaniz et al. 2009). Smaller HA fragments (200–2000 kDa) are involved in a variety of normal and pathological processes (Stern et al. 2006); they may come in sizes that overlap in the functions that they perform. Low-molecular weight HA administration has been shown to induce cervical ripening in rabbits (El Maradny et al. 1997), suggesting its role in cervical remodelling. HAase increases during cervical ripening (Obara et al. 2001) because of high concentrations of P4 and/or its receptor (Tanyildizi & Bozkurt 2002), and results in degradation of HA into smaller fragments. The increase in P4 seen in the luteal phase mirrors that seen during pregnancy, and this may help to explain why the luteal group has the highest frequency of smaller HA fragments in comparison with the other stages of the oestrous cycle.
In this study, the pre-LH surge group had significantly more samples that contained both small and large HA fragments. However, from the HPLC results, it is difficult to establish the ratio of HA fragments within the samples. While the presence of large HA fragments in the cervical samples may be explained on the basis of a decreased effect of HAase because of lower concentrations of P4 or its receptor (Obara et al. 2001, Uchiyama et al. 2004), the existence of smaller HA fragments within the same samples may be the result of an increased production of HAS1 and HAS3 (Itano & Kimata 2002), which may be stimulated by exposure to either the increased concentrations of PGs at luteolysis (Stuhlmeier 2007) or oestrogens (Uzuka et al. 1981) and/or LH (Schoenfelder & Einspanier 2003). However, to ascertain the definite effects of these hormones on HA synthase(s), it requires further studies.

The major HA receptor is CD44 that mediates HA-dependent cell adhesion in a number of different cell types, including epithelial cells (Culty et al. 1992) leukocytes and fibroblasts (Underhill 1992). Previously reported biochemical and cell biological properties of CD44 are consistent with its role as a cell surface glycoprotein (Brown et al. 1991). It is hypothesized that CD44 forms a direct association between HA and the cytoskeleton. However, the mechanism by which CD44 achieves this, including the signalling cascades that it regulates, is complex (Turley et al. 2002); nevertheless, the particular downstream signalling pathways lead to an onset of HA-dependent functions in various types of
tissues (Turley et al. 2002). Although in this study, we have focused only on CD44 that may alter or modulate the function of HA in the cervix; however, there are also other receptors like RHAMM (Slevin et al. 2007) and HA-binding proteins such as heavy chain subunit of the ITI (Izdi) or versican (Ruscheinsky et al. 2008), which may play a role in influencing HA functions in the cervix.

In the present study, we observed a significantly higher CD44 expression in the luminal epithelium of the cervix, a finding that is consistent with those of Fosang et al. (1984), El Maradny et al. (1997) and Ruscheinsky et al. (2008) who also reported the expression of CD44 being mainly around the basal layer of cervical epithelium. However, in addition to luminal epithelium, we also observed the expression in the sub-epithelial stroma layer, whereas studies such as those by Ruscheinsky et al. (2008) did not report any expression in this region. The differences between our results and those of Ruscheinsky et al. (2008) may be explained on the basis of either different species (ewes versus mice) and/or physiological status of the animals (non-pregnant versus pregnant). A higher expression of CD44 in the epithelium suggests a possible interaction between HA and its receptor CD44, and an increase in HA may upregulate CD44 function. This supports the fact that higher HA content and expression of CD44 were both observed during the same (pre-LH surge) stage of the oestrous cycle. The higher HA content during the pre-LH surge stage may be attributable to both luminal epithelial cells and fibroblasts, thus supporting the evidence that HA synthases are expressed in luminal epithelial cells of the mouse and human cervix (Straach et al. 2004), and is also consistent with unpublished data from our laboratory that HA synthases are expressed in both epithelium and sub-epithelial stroma. Therefore, the increased expression of the CD44 in the epithelium, as observed in the present study, is to ensure its availability to bind to its ligand. Significantly more mucus produced and/or accumulated naturally in the vaginal region of the ewe with increasing amounts towards oestrus (Rexroad & Barb 1977) explains the difference seen between regions of the cervix and stages of the oestrous cycle.

At all the stages of the oestrous cycle, the vaginal region of the cervix showed the highest CD44 expression in comparison with the mid or uterine regions. As significantly more mucus is produced and/or accumulated naturally in the vaginal region of the ewe (Rexroad & Barb 1977), higher CD44 in the vaginal region might be explained on the basis of the hypothesis that the HA contained in the mucus upregulates CD44 production and function. A general increase in mucus within the cervix close to oestrus (Rexroad & Barb 1977) may explain why the pre-LH group had high CD44 expression in both the vaginal and mid regions of the cervix. A build-up of mucus at the vaginal end may force it backward to the mid-cervical region. The mucus containing high levels of HA (Obara et al. 2001) may then upregulate CD44 expression.

It is worth noting that at present, there is very little information available about the role of HA in non-pregnant cervix. Exposure of the cervix to P4 during the luteal phase of the oestrous cycle is much shorter compared with that during pregnancy. Results of the present study show that HA content of the cervix varies during different stages of the cycle most probably due to different hormonal milieus. These hormones, particularly the steroids, seem to play the same role as they do during pregnancy and/or at the onset of labour but to a lesser extent, probably reflecting the length of exposure particularly to P4. However, the exact role of steroids and/or gonadotrophins in the function and/or regulation of HA in the ovine cervix still remain a subject for further investigation.

In conclusion, the results of the present study have shown that HA, fragment size and concentration vary considerably during the oestrous cycle. Higher HA values at a time when cervical relaxation is naturally higher may indicate its involvement in remodelling of the cervix at oestrus. It is therefore possible that HA can be utilized to assist in trans-cervical AI, it is commercially available (Lifecore Biomedical, Chaska, MN, USA) as high- or low-molecular weight powder and can be reconstituted to form a viscous solution for deposition in to the cervix of sheep via an inseminating pipette. We suggest that intra-cervical application of HA may promote remodelling of the cervix at oestrus to facilitate trans-cervical AI in sheep. As a matter of fact, low-molecular weight HA fragments have been reported to induce cytokines and chemokines that are implicated in inflammation and degradation of ECM (Noble et al. 1996). Moreover, low-molecular weight HA is angiogenic (West et al. 1985), and can induce cytokine production (Hiro et al. 1986), matrix metalloproteinase production (El Maradny et al. 1997) and nitric oxide synthesis (McKee et al. 1997), all of which are inducers of cervical ripening. Furthermore, it has been reported that the application of low-molecular weight HA to the cervix of rabbits actually starts cervical ripening (El Maradny et al. 1997).

**Materials and Methods**

**Animals and tissues**

The study was performed on 25 Welsh mountain ewes under Home Office authorisation in compliance with the Animal (Scientific Procedures) Act, 1986. The ewes were housed indoors on straw, and fed hay and a commercial concentrate diet with water always available.

Ewes were synchronized to a common day of the oestrous cycle by intravaginal Chronogest sponges (Intervet UK Ltd, Cambridge, UK) inserted for 12 days and 200 IU of pregnant mares serum gonadotrophin (Intervet UK Ltd, Buckinghamshire, UK) given i.m. at the time of sponge removal. All ewes
were in oestrus within 48 h of sponge withdrawal, which was termed day 0 of the oestrous cycle. On day 8 of the synchronized cycle (luteal phase), the reproductive tracts of eight animals were collected following death by captive bolt and exsanguination. On day 11, the remaining animals were administered i.m. with 125 μg of cloprostenol PGF2α analogue, Estrumate; Schering-Plough Animal Health, Welwyn Garden City, UK), and at 36 h after the PGF injection, the tracts were collected prior to the LH surge (n=8). At the same time, the remaining animals were administered 300 μg of GNRH (Sigma–Aldrich) to synchronize the LH surge. The tracts were collected 8 h post-GNRH following the LH surge (n=9). Jugal venous blood samples were taken for the duration of the study (every 48 h starting on day 0 of the synchronized oestrous cycle until PGF2α injection, then every 2 h thereafter) placed in heparinized tubes and centrifuged at 1700 g for 10 min at 4 °C. Plasma was separated and stored at −20 °C until analysed for LH and P4.

The cervix was cleared of all unwanted tissues, the anterior vagina was removed and the cervix was separated from the uterus with a transverse cut using a sterile scalpel. The cervix was divided transversely into six equal sections, alternate sections comprising the uterine region, mid region and vaginal region were fixed in neutral buffered formalin 30% (BDH, Poole, UK), and the other three sections were frozen on dry ice and stored at −80 °C. Fixed cervices were wax embedded, sectioned at 7 μm and mounted onto Superfrost Plus slides (BDH).

**Hormone assays**

Plasma P4 concentrations were determined following an extraction procedure, using a charcoal–dextran-coated RIA as described previously (Robinson et al. 2002). The limit of the sensitivity of the assay was 0.09 ng/ml, and the inter- and intra-assay coefficients of variation were 2.2 and 2.0% respectively. LH concentrations in the plasma were determined by ELISA using the commercially available kit (LH DETECT, CTK Biotech, Inc., San Diego, CA, USA) that detects the LH surge or whether LH concentrations are basal in the samples.

**Measurement of HA content**

Frozen cervical tissues at the uterine, mid and vaginal regions were digested in 0.1 M sodium acetate buffer, pH 5.8, containing 0.25 mg/ml papain (Roche Applied Science), 5 mM EDTA and 5 mM L-cysteine hydrochloride anhydrous at 60 °C for 16–18 h as described previously (Pitsillides et al. 1994). HA concentration in the digested tissue supernatant was assayed in duplicate by ELISA using the commercially available kit (LH DETECT, CTK Biotech, Inc., San Diego, CA, USA) that detects the LH surge or whether LH concentrations are basal in the samples.

**Determination of HA fragment size**

HPLC was used to determine the HA fragment size in uterine, mid and vaginal regions of the cervix from each animal. Cervical samples were digested overnight at 55 °C in lysis buffer that comprised 0.25 mg/ml papain (Roche Applied Science) in a 0.1 M sodium acetate buffer (pH 5.8) containing 5 mM EDTA (Sigma–Aldrich) and 5 mM L-cysteine hydrochloride anhydrous (Sigma–Aldrich). Previous studies have shown that HA content is not affected by this treatment (Pitsillides et al. 1994). The digested samples were analysed by size exclusion HPLC using a Waters 2695 separation module with a Tosohas TSK gel G6000 PWxl (pore size 13 μl), to separate sample components, and a Waters 2487 dual absorbance detector set at 206 nm wavelength, to measure magnitude and retention time of sample HA. The injected volume was 100 μl, the column flow was 1 ml/min Ringer's solution and the column temperature was 30 °C. A calibration curve for HA concentration was constructed for each batch of samples by running standards of known concentrations of rooster comb sodium hyaluronate (Sigma) in Ringer solution (0.0016, 0.003, 0.006, 0.0125, 0.025, 0.05, 0.1 and 0.2 mg/ml). The HA standards were run at the beginning and end of each HPLC session, to provide a calibration curve, which was highly linear over the range 0.025–0.400 mg/ml. The molecular weights of the standards were determined by laser light scattering. Curve relating retention time to HA molecular weights have been published previously (Coleman et al. 1997). The semi-logarithmic relationship was approximately linear from 0.21×10^6 to 3.9×10^6 kDa but the column did not appear to separate molecules larger than this. Retention times were essentially insensitive to pH outside the range 7.0–7.4.

**Immunohistochemistry**

The expression for CD44 was determined by immunohistochemistry (IHC) on wax-embedded cervix sections. The procedure for immunohistochemical localisation for CD44 was the same as previously described by Ponglowhapan et al. (2008) using a Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA). A mouse monoclonal anti-ovine CD44 antibody (MCA2219 AbD Serotec, Oxford, UK) was used as complex (Amersham Biosciences UK Ltd) diluted to 1:1000 in PBS–Tween were incubated for 30 min at 37 °C followed by 100 μl of 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) substrate at 37 °C for 20 min. The optical density of the wells was read at OD405, and the concentration of HA within the samples was determined against the optical density of the known standards. The concentration of cervical HA was expressed as μg of HA/mg of cervix (dry weight). Dry weight was determined using 200 mg of thawed tissue, which was put in an oven (37 °C) overnight to eliminate any moisture. The tissue was weighed and compared to the original (wet) weight. This value was used as a correction factor in the ELISA calculations. The limit of sensitivity of the HA ELISA was 1.6 ng/well of HA. The inter- and intra-coefficient of variations were 2.40 and 2.8% respectively.
primary antibody. Three sections per region of the cervix from each animal were examined for both positive antibody staining and negative controls. Negative controls were treated in the same way except that the primary antibody was substituted with normal mouse IgG (sc-2025 Santa Cruz Biotechnology, Inc., Heidelberg, Germany). The observed lack of staining in negative controls indicated high antigen specificity (Fig. 4a).

Quantification of IHC staining

Examples of positive and negative staining for CD44 are shown in Fig. 4a. The pattern and intensity of protein staining were determined semi-quantitatively using a histochemical score (HSCORE) that has been widely reported in a number of studies (Vermeirsch et al. 1999, Ponglowhan et al. 2007, 2008, Plante et al. 2009). In this study, all the analyses were carried out blind by one experienced assessor using HSCORE, incorporating both the distribution and the intensity of specific staining as described previously (Ponglowhan et al. 2008). For each animal, scoring was carried out separately on the five tissue layers (i.e. epithelium, sub-epithelial stroma, longitudinal muscle, circular muscle and transverse smooth muscle) for each of three regions of the cervix. Serosa was only present in some cervixes as it was lost during the fixation and embedding procedures and is, therefore, not included in the results. Briefly, the proportion of positively stained cells in five layers in each of three regions of the cervix was defined as the percentage area of the cell layer to the nearest 5% that had specific staining. The intensity of staining was graded as 0, 1, 2 or 3: an intensity score of 0 (Fig. 4a; B, D and F) indicated that there was no expression; 1 (Fig. 4a; A epithelial, sub-epithelial and muscle) light pink to pink, indicated weak staining; 2 (Fig. 4a; E epithelial and muscle) pink, indicated moderate staining; and 3 (Fig. 4a; epithelial and muscle) represented strong staining with a definite pink or red. Expression index was calculated for each sample by multiplying the percentage of positively stained cells with intensity of staining.

Statistical analysis

The data on HA content and CD44 were compared by factorial ANOVA using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK), among i) three regions of the cervix and ii) three stages of the oestrous cycle. The data on HA content and CD44 were compared by factorial ANOVA using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK), among i) three regions of the cervix and ii) three stages of the oestrous cycle. Data were compared by factorial ANOVA using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK), among i) three regions of the cervix and ii) three stages of the oestrous cycle. ANOVA was used to test for the effects of cervical layers.

Statistical analysis

The data on HA content and CD44 were compared by factorial ANOVA using GenStat 9th edition (Version 9.1.0.147, Lawes Agricultural Trust, VSN International Ltd, Oxford, UK), among i) three regions of the cervix and ii) three stages of the oestrous cycle, whereas data on CD44 expression were also compared for the additional factor of cervical layers.

HPLC analysis showed three different types of cervical samples containing either i) small (eluted after 8 min); ii) large (retention time between 6.3 and 6.7 min) or iii) both small and large (showing two peaks one between 6.3 and 6.7 min and other after 8 min) HA fragments. The frequency distribution of different sizes of HA fragments was compared among the three stages of the oestrous cycle and three cervical regions using $\chi^2$ analysis.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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