Influence of Hyaluronan on endometrial receptivity and embryo attachment in sheep

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Running title: Role of HA during Implantation

Abstract (200 words for RFD)

An increasing number of reports suggests a role of hyaluronan (HA) in female reproduction and interest in its application in assisted reproduction is rising. However, there are contrasting data about the effectiveness of adding HA to the embryo-transfer medium on improving pregnancy rates. Using sheep as an experimental model, the studies reported here analysed the impact of HA infusion into the uterus on embryo attachment to uterine luminal epithelium (LE) and expression of selected markers of uterine receptivity. On Day 14 after natural mating (pre-attachment), uterine horns were infused with either (n = 4 each): PBS (control), HA (1 mg mL–1), HA + hyaluronidase 2 (Hyal2; 300 IU mL–1) or 4-methyl-umbelliferone (HA-synthesis inhibitor; 4MU, 1 mM). HA immunostaining on uterine sections collected on Day 17 was negative in the 4MU group and weak in the HA+Hyal2 group. In contrast to 4MU, which resulted in 100% attachment, HA infusion blocked embryo attachment in all treated animals. This was accompanied by the disappearance of mucin 1 and increased expression of osteopontin and CD44v6 in the LE of uteri with attached embryos. In conclusion, the presence of HA at the embryo–maternal interface during embryo implantation resulted in reduced endometrial receptivity and inhibited the interaction of trophoblasts with the LE, whereas clearance of HA favoured embryo attachment.

Introduction

Failure of implantation and early embryonic loss is a major cause of infertility in human (Macklon *et al.* 2002) and animals (Diskin and Morris 2008) which is estimated to be 25-40% or more, 70-80% of which occur between 8 to 16 days post-fertilization in cattle. Implantation failure is also an impediment to efficient assisted reproduction (Aplin 2006). Embryo implantation requires a highly coordinated set of sequential interactions between embryonal trophectoderm and endometrial luminal epithelium. Beside the importance of embryo quality and viability during implantation, the endometrial receptivity is indispensable (Sharkey and Smith 2003) and is the main determinant of successful pregnancy (Simon *et al.* 1998; Ledee-Bataille *et al.* 2002). In farm animals, uterine insufficiency for pregnancy is a major cause of early embryonic losses especially in high producing dairy cows, and in animals where oestrous synchronization and timed AI was used (Bridges *et al.* 2013). Thus, understanding the mechanisms regulating uterine receptivity and implantation can be useful to diagnose and treat recurrent pregnancy losses and improve pregnancy rates following embryo transfers.

The mechanism and timing of embryo attachment and implantation are highly variable across different animal species and humans. In contrast to humans and rodents, where immediate post-hatching "invasive" implantation takes place upon arrival of the blastocyst to the uterus, embryos in most farm animals including sheep exhibit protracted phase of preimplantation (Spencer *et al.* 2004). This extended window of superficial embryo-maternal interaction has been efficiently used in many studies as a model to understand the molecular and cellular mechanisms underlying implantation (Johnson *et al.* 2001; Spencer *et al.* 2004) however extrapolation of these mechanisms to human implantation should be handled with care. In sheep, once a blastocyst expands and hatches by day 8, it starts the orientation phase (from day 9 to 14) where it is immobilized but not attached. This is associated with accelerated increase in the number of cells and elongation of extraembryonic trophoblast (to reach a filamentous

form; 10 cm by day 14). The apposition phase then starts on day 14, when the filamentous conceptus is immobilised, but firm adhesion (which is hereafter sometimes referred to as implantation) does not occur until day 16-17 (Guillomot *et al.* 1981; Guillomot 1995).

For the adhesion process to be established, the interaction between the embryonal trophectoderm and uterine luminal epithelium (LE) is regulated by complex molecular and biochemical changes at the embryo-maternal interface; this involves expression or unmasking of several cellular adhesion molecules such as CD44,integrins (ITG) and osteopontin (OPN; also called secreted phosphoprotein 1, SPP-1; Aplin and Kimber 2004). Osteopontin is regulated by progesterone in humans (Omigbodun et al. 1997) and sheep (Johnson et al. 2000) and is known to bind several ITGs: α4β1, αvβ3, αvβ5, αvβ6 and specific splice variants of CD44 (v3, v6; Aplin 2006). In sheep, the prolonged exposure of endometrium to progesterone results in downregulation of progesterone receptors in LE by Day 11 and in glandular epithelium (GE) by Day 13 (Spencer and Bazer 2002). This is associated with reduced expression of the anti-adhesion protein mucin 1 (MUC1) in LE (Spencer et al. 2007) and increased secretion of some adhesion molecules like OPN (Johnson et al. 2001). OPN via binding to ITG is an acknowledged mediator of attachment between the trophoblast and the LE (Johnson 2003). Therefore, downregulation of MUC1 and upregulation of OPN, ITG and CD44 are considered as markers of uterine receptivity (Spencer et al. 2004).

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on embryo attachment to uterine luminal epithelium (LE) and expression of selected markers of uterine receptivity. On Day 14 after natural mating (pre-attachment), uterine horns were infused with either (n = 4 each): PBS (control), HA (1 mg mL–1), HA + hyaluronidase 2 (Hyal2; 300 IU mL–1) or 4-methyl-umbelliferone (HA-synthesis inhibitor; 4MU, 1 mM). HA immunostaining on uterine sections collected on Day 17 was negative in the 4MU group and weak in the HA+Hyal2 group. In contrast to 4MU, which resulted in 100% attachment, HA infusion blocked embryo attachment in all treated animals. This was accompanied by the disappearance of mucin 1 and increased expression of osteopontin and CD44v6 in the LE of uteri with attached embryos. In conclusion, the presence of HA at the embryo–maternal interface during embryo implantation resulted in reduced endometrial receptivity and inhibited the interaction of trophoblasts with the LE, whereas clearance of HA favoured embryo attachment.

Additional keywords: 4MU, CD44, hyaluronic acid, implantation, MUC1, osteopontin.

Introduction

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cause of early embryonic losses, especially in high-producing dairy cows and in animals where oestrous synchronisation and timed AI is used (Bridges et al. 2013). Thus, understanding the mechanisms regulating uterine receptivity and implantation can be useful to diagnose and treat recurrent pregnancy losses and improve pregnancy rates following embryo transfers.

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endometrium to progesterone results in downregulation of progesterone receptors in LE by Day 11 and in glandular epithelium (GE) by Day 13 (Spencer and Bazer 2002). This is associated with reduced expression of the anti-adhesion protein mucin 1 (MUC1) in LE (Spencer et al. 2007) and increased secretion of some adhesion molecules like OPN (Johnson et al. 2001). OPN via binding to ITG is an acknowledged mediator of attachment between the trophoblast and the LE (Johnson 2003). Therefore, downregulation of MUC1 and upregulation of OPN, ITG and CD44 are considered as markers of uterine receptivity (Spencer et al. 2004).

Hyaluronan (HA) is another molecule involved in cell–cell adhesion in the extracellular matrix of many tissues. HA synthesis and distribution is evident in all cell types including the LE of the endometrium of several species including humans, mice and sheep (Brown and Papaioannou 1992; Salamonsen et al. 2001; Raheem et al. 2013). We have previously shown that the expression of HA synthases, HAS2 and HAS3, and HA receptor CD44 in the LE and GE of ovine endometrium is regulated by steroid hormones and varies according to the stage of the oestrous cycle (Raheem et al. 2013). It is thought that implantation failure could be reduced by providing a 'sticky' matrix for the embryos to attach. For this reason, HA (which is also called the magic glue (Girish and Kemparaju 2007) or EmbryoGlue (Hazlett et al. 2008)) was introduced to human IVF procedures as a supplement in embryo transfer medium to support implantation. However, the efficiency and controversy of HA remains inconsistent among different studies. In a few studies, HA was shown to be beneficial for embryo implantation (Svobodová et al. 2007; Urman et al. 2008; Nakagawa et al. 2012). Moreover, in attempts to develop human embryo culture media free from blood-derived additives (particularly albumin) HA was successfully used as a sole macromolecule in human embryo transfer medium, which resulted in high pregnancy and implantation rates (Simon 2003). A Cochrane meta-analysis of clinical trials concluded that HA inclusion in embryo transfer media significantly increases clinical pregnancy rates and live birth rates (Bontekoe et al. 2014). In addition, the use of HA in human frozen embryo transfer media significantly increased implantation rate without increasing the delivery rate (Hambiliki et al. 2010). In contrast, several studies revealed that the use of HA-enriched transfer medium does not improve implantation rates. In women who failed to conceive despite at least three previous embryo transfers, a 25% clinical

pregnancy and 14.2% delivered pregnancy rate were achieved using EmbryoGlue, when compared with women not using EmbryoGlue (39.2% and 39.2% respectively; P > 0.05; Dietterich et al. 2007). Among 120 cases, no statistical difference was found between clinical pregnancies in the control group and test group using EmbryoGlue (38% vs 42%) (Chao et al. 2008). Similar results were obtained by Marek et al. (2004). Another prospective, randomised study showed that in non-selected patients EmbryoGlue did not influence pregnancy rates in patients receiving either a Day-3 or Day-5 embryo transfer compared with standard culture media (Hazlett et al. 2008). This controversy leaves the role of HA during embryo implantation un-elucidated.The aim of this study was to further investigate the role HA at the time of embryo adhesion in sheep. We have examined the effect of uterine infusion of HA, HA fragments generated by Hyal2 or inhibition of HA by a specific HA synthesis inhibitor (4MU) on embryo attachment to the maternal endometrium. The infusion was performed just before implantation time in sheep. The number of embryos attached to uterine LE was investigated and the effects of different treatments on the expression of key markers of uterine receptivity were analysed.

Material and methods

Experimental design and animal treatments

All experimental procedures were done under a project licence approved by the Royal Veterinary College's Ethics and Welfare committee and complied with regulations in the UK Animal (Scientific Procedures) Act, 1986. A total of 16 proven fertile, non-pregnant Welsh-mountain ewes, 2–3 years old, were used for this study. Groups of four ewes (one ewe for each treatment) were synchronised to a common oestrus using vaginal sponges (Chronogest; Intervet) for 11 days. Ewes received 300 IU pregnant mare serum gonadotrophin (PMSG; Intervet) on the day of sponge removal. On Day 2 after sponge removal ewes were injected with Receptal (125 μg buserelin, gonadotrophin-releasing hormone (GnRH) agonist) to synchronise ovulation and each synchronisation group was hand mated by one of two proven

fertile rams. On Day 14 following mating each group of ewes was anaesthetised to expose the ovaries and uterus by laparotomy as described by Jackson (2004). The presence of mature corpora lutea (CL) on the ovaries was confirmed and 1 mL of one of the following four treatments was injected into each uterine horn: (1) sterile phosphate-buffered saline (PBS; control), (2) HA (400 kDa, 1 mg mL–1; H7630-Sigma), (3) HA (1 mg mL–1) treated with Hyal2 (HA+Hyal2; 300 IU mL–1) and (4) HA-synthesis inhibitor (4-methyl-umbelliferone (4MU); 1 mM). All treatments were dissolved in PBS (EGGTech) under sterile conditions and incubated for at least 2 h at 37°C before infusion. This period is enough to completely depolymerise HA by Hyal2 before infusion as tested by agarose gel electrophoresis and HA-binding protein staining described in our previous studies (Raheem et al. 2013). One ewe designated for the HA+Hyal2 group had to be killed due to illness. All ewes were sacrificed on Day 17. The presence of mature CL was confirmed on the ovaries and the reproductive tracts were harvested for further examination.

Histological examination using Haematoxylin and Eosin staining for detection of embryos and attachment to LE

Upon harvesting the uteri, right and left uterine horns were cut into 1 cm long pieces using sharp blade and immediately fixed in 4% buffered Formalin for 96 hours. After dehydration and embedding in paraffin, blocks were sectioned at 5 µm and mounted on glass slides. Endometrial sections of each animal were stained with H&E. Sections from the uterine horn ipsilateral to the ovary containing CL were examined first, while the other side was examined only if no embryo was found in the ipsilateral horn. The sections were dewaxed in Histoclear and rehydrated in a graded series of ethanol of 100%, 95%, 70% and 50% for 5 min each. The slides were incubated in 10% (v/v) Gill's Haematoxylin (Park Scientific Ltd., Northampton, UK) in distilled water (dH_2O) for 4 min. They were then dipped in alcohol acetic acid (1% v/v) for 30 sec. This was followed by incubation in 1% (w/v) Eosin (Sigma) in dH_2O for 1 min. The

slides were washed in running water for 5 min between each step. Finally, sections were dehydrated and mounted with Dibutylphthalate Polystyrene Xylene (DPX) mounting medium before applying coverslips and being visualised under a light microscope. Embryos were considered to be 'attached' if their trophectoderm was found to be in intimate contact with the luminal epithelium.

Immunolocalization of HA and other markers of endometrial receptivity

Paraffin-embedded uterine sections (5 µm) were mounted on Superfrost slides (VWR International Co., Leicestershire, UK). For HA staining, sections were incubated with biotinylated-HABP (AMBS Biotechnology, Oxon, UK) at 2.5 µg/ml concentration overnight at 4^{o}C followed by Streptavidin-HRP (1:200 in PBS; GE Healthcare, Buckinghamshire, UK) for 1h at room temperature (RT).

Expression of CD44, MUC1, OPN and ITG αv and β3 were detected by immunohistochemistry (IHC) as described previously (Raheem et al. 2013) in different parts of the ipsilateral horn, particularly where an embryo was localised by H&E staining, using the following primary antibodies: rabbit polyclonal anti-MUC1 (1 : 100; ab15481; Abcam), mouse anti-human CD44 v6 (1 : 100; MCA1730; AbD Serotec), rabbit anti-OPN (1 : 200; ab8448, Abcam), rabbit anti-αv (1 : 200; Ab76609; Abcam) and rabbit anti-β3 (1 : 200; Ab75872; Abcam). Negative control sections were incubated with either normal rabbit or normal mouse IgG at the same concentration used for the primary antibody.

Semi-quantitative Analysis of Immunostaining

Immunostaining was semiquantitatively assessed using histochemical score (H-Score) as previously described by Ponglowhapan *et al.* (2008). This is calculated by multiplying the intensity (as a score of 0–3) of the brown diaminobenzidine (DAB) stain in each cell type (LE and GE) within the endometrium and the percentage of the area stained (0–100). The estimate

was based on five random fields per section from sections from at least three ewes. Images were scored by two independent experienced assessors blinded to the corresponding treatment. Numerical data generated by this scoring system were summarised as mean ± s.e.m.

Statistical analysis

The immunostaining H-Scores were analysed using ANOVA in SPSS 20.0 for Windows (IBM) while significant differences were further compared with Bonferroni post hoc tests. Differences of $P < 0.05$ were considered as significant.

Results

HA immunostaining in the endometrium after uterine infusion

To verify that infusion with Hyal2 and HA-synthesis inhibitor (4MU) affected endometrial HA as required in the experimental design, uterine sections collected on day 17 (3 days after uterine infusion) were immunostained using HA-binding protein (HABP). The HA-immunostaining in endometrium of the control group (infused with PBS) was moderate in the stroma (ST) and negative in the LE and glandular epithelium (GE). In the HA-infused group, HA was expressed in the ST similar to the control, but was also observed in the LE. In contrast, HA immunostaining was negative in the 4MU and HA+Hyal2 groups in all layers of the endometrium (Figure 1).

Effect of the uterine treatments on blastocyst attachment

The number of ewes in each treatment groups where an embryo and extra-embryonic membranes were detected by H&E staining is shown in Table 1. Ewes with no embryo in the uterus were excluded from further analyses. In the control group, an embryo was detected in three ewes and an attachment between the extra-embryonic membranes and the LE was evident in 2/3 of the embryos. Surprisingly, infusion with HA completely inhibited the process of attachment (0/3) where all embryos were found loose in the lumen of the uterine horn with no

attachment to the LE. However, interestingly, a distinctive effect was seen in the 4MU-infused group were all embryos (4/4) were strongly attached to the LE (Figure 2).

Effect of the uterine treatments on the expression and localisation of MUC1

In the PBS-infused group (control), MUC1 was restricted to the luminal surface of GE. LE was almost negative (Figure 3A). In other areas in the control uteri, weak MUC1 staining was detected but not at the sites of attachment with the embryo (Figure 3B). On the contrary, in the HA-infused group, MUC1 expression was not downregulated and strongly detected at the luminal surface of LE as well as the GE (Figure 3C), which was significantly higher than control when quantified by H-Score ($P<0.05$; Figure 3F). MUC1 expression in HA+Hyal2 at LE was lower than that in HA group (Figure 3D and 3F, $P<0.05$). In 4MU-group MUC1 expression was completely downregulated from LE and only weak staining was observed in GE (Figure 3E).

Effect of uterine infusions on OPN and integrins

In the PBS and 4MU infused groups, strong OPN immunostaining was seen at the luminal surface of LE. OPN expression at the luminal surface was decreased by HA infusion in the presence or absence of Hyal2 (Figure 4A). No OPN was detected in the stroma or trophectoderm cells. OPN was equally expressed in the GE in all treatment groups. OPN H-Score in the LE of the HA group was significantly lower than control ($P<0.05$, Figure 4B).

Integrin αv was equally expressed in LE layer of the endometrium and the embryonic TE layer in all treatment groups. Integrin β3 was only seen in the blood vessels but not in LE, GE, or TE (Figure 5).

CD44v6 immunostaining in the endometrium

CD44 was found to be expressed in all layers of the endometrium with highest staining intensity in embryonic trophoblasts. Staining was also intense at the luminal surface of LE. CD44 immunostaining in the LE was high in the control and 4MU groups (Figure 6A). The lowest CD44 staining was observed in HA+Hyal2 treatment group, which had a significantly ($P<0.05$) lower H-Score (Figure 6B).

Discussion

The aim of the studies presented here was to investigate the role of HA during embryo implantation (adhesion) to the luminal epithelium in sheep. The elongated blastocyst in sheep is known to adhere to the endometrial LE on Day 16 (Spencer et al. 2004). On this basis, uterine horns of pregnant ewes were infused on Day 14 with either PBS (control), HA or HA+Hyal2. Some ewes were infused with 4MU (a HA-synthesis inhibitor) to test the importance of endogenous HA. Animals were then sacrificed on Day 17 to check embryo adhesion. The size of HA used for infusion was ~400 kDa according to the manufacturer. This size is within the physiological range of the molecular weight of HA present in the ovine uterus during dioestrus as measured by agarose gel electrophoresis in a previous study (Raheem et al. 2013). The doses of HA, Hyal2 and 4MU were based on previous, related in vitro and in vivo studies (Marei et al. 2013, 2016).

Our first concern was to check the effectiveness of the treatments in changing the HA profile in the endometrium. In the control group, immunostaining of HA using HABP revealed that on Day 17 of pregnancy HA is localised only in the ST of the ovine endometrium, while the LE and GE were devoid of HA staining. A similar pattern of HA localisation was observed in the endometrium of the ewe in our previous report during different stages of the oestrous cycle (Raheem et al. 2013) as well as in mouse (Teixeira Gomes et al. 2009) and human (Afify et al.

2006; Nykopp et al. 2010) endometrium. In the present study, uterine infusion with HA resulted in a positive staining of HA on the LE, suggesting that the infused HA can bind to the LE, possibly through hyaladherins like CD44, which was also found to be expressed. On the other hand, infusion with HA+Hyal2 resulted in very weak or no HA staining in the LE and stroma. Hyal2 is known to depolymerise HA into small fragments (~25 kDa), which can then be internalised for further degradation with lysosomal Hyals. This explains the weak HA staining seen in the Hyal2-treated group. Finally, as expected, no HA immunostaining could be seen in the 4MU-infused group, which is due to the potent inhibitory effect of 4MU on HA synthesis (Kultti et al. 2009). 4MU was shown to deplete intracellular pools of HA precursor and downregulate HAS2 and HAS3 mRNA expression in cancer cells (Kultti et al. 2009).

To evaluate the effect of different treatments on the adhesion of embryos to the LE, sections of uterine horns collected on Day 17 were examined by H&E staining. In the control group infused with PBS, two out of three had embryos attached to the LE. Based on previous studies mentioned in the introduction, our hypothesis was that HA can improve or at least have no influence on embryo implantation. Surprisingly, all embryos in the HA-infused group (3/3) failed to attach. When HA was infused together with Hyal2, 1/3 embryos was found attached. These results indicate that infusion of HA (especially of large molecular weight) is blocking the implantation process. However, and opposite to all expectations, infusion with 4MU resulted in strong embryo attachment in all treated ewes (4/4). These results are in line with previous reports that showed disappearance of HA at the maternal–embryo interface at the time of implantation in mice on Day 5–7 of pregnancy (Brown and Papaioannou 1992, 1993; San Martin et al. 2003). Moreover, increased decidual hyaluronan levels due to altered HA catabolism (by hyaluronidases) at the feto–maternal interface resulted in spontaneous abortion in mice on gestation Day 7.5 (Cordo-Russo et al. 2009). Blastocyst attachment to the endometrium involves initial apposition of the trophoblast to the LE surface. Several cell-

surface molecules are implicated in the initial attachment between trophectoderm and luminal epithelium. In order to explain the inhibition of embryo attachment by HA, we assessed the expression of some important components of the implantation cascade in sheep to examine how they are affected by HA and 4MU infusion.

Continuous exposure of the endometrium to progesterone in early pregnancy is known to downregulate progesterone receptors in LE and results in the loss of the cell-surface mucin MUC1 (Spencer et al. 2004). MUC1 was found to be expressed at the apical surfaces of uterine LE and GE but decreased significantly on the LE starting on Day 9 to become undetectable by Day 17 of pregnancy, when intimate contact between the LE and trophectoderm is established in sheep (Johnson et al. 2001). Endometrial receptivity in mouse is also associated with a decrease in MUC1 expression (Surveyor et al. 1995). MUC1 is a cell surface-associated glycoprotein with extensive O-linked glycosylation of its extracellular domain, which is normally protective and prevents pathogens from binding to epithelial cells (Moncada et al. 2003). In the uterus, downregulation of MUC1 at the time of implantation is critical to unmask cell-surface proteins such as adhesion molecules and their receptors (Brayman et al. 2004) and enable attachment between embryonal trophectoderm and uterine LE. In the present study, MUC1 immunostaining on Day 17 was found in the GE but not in the LE in the control group, which confirms the previously reported findings of Johnson et al. (2001). Our results showed that infusion with HA was associated with positive MUC1 staining on the apical surface of the LE. Since MUC1 is an effective inhibitor of cell–cell interactions, this explains the complete inhibition of embryo attachment in all ewes that received uterine HA infusion. It is also worth noting that the MUC1 expression on LE in the HA+Hyal2 group was less intense than with HA alone. This may suggest that the effect of HA on MUC1 expression is dependent on the size of HA. In marked contrast, 4MU resulted in the complete absence of MUC1 expression from the apical surface of the LE, which was associated with improved embryo attachment.

It was previously reported that overexpressed MUC1 at implantation sites was a major feature of non-obese diabetic (NOD) mice as opposed to normoglycaemic mice (Albaghdadi and Kan 2012). Hyperglycaemic conditions have been linked with upregulated HAS2 expression and increased HA synthesis in human vascular smooth-muscle cells (Sainio et al. 2010). It can be hypothesised that increased HA synthesis in the endometrium of diabetic patients may be a reason for the increased MUC1 and reduced uterine receptivity. However, this notion has not been investigated. In addition, MUC1 expression was significantly higher in patients with ovulatory polycystic ovaries than in fertile patients and it was suggested that expression of MUC1 in the infertile endometrium is significantly different from the fertile endometrium and appears to be a component of altered gene expression that potentially contributes to endometrial insufficiency (Margarit et al. 2010). Direct extrapolation of the present data from sheep to human is not possible due to significant differences in placentation mechanisms and timing. However, downregulation of MUC1 by 4MU could be a potential therapy for such cases. Local or systemic treatment with 4MU to reduce HA synthesis and MUC1 expression could be developed to treat patients suffering from miscarriage; however, further investigations are required on a wider scale using in vivo and in vitro models.

Integrins are important elements required for cell–cell interaction; these are unmasked in the absence of MUC1 (Aplin 1997). Different α and β subunits of integrin heterodimers were found constitutively expressed on trophectoderm and the apical surface of LE and GE in both cyclic and early pregnant ewes (Johnson et al. 2001). In humans, unique mid-luteal phase expression of the integrin heterodimer αvβ3 coincides with the window of implantation and is thus considered as an endometrial biomarker of receptivity (Lessey 2011). Therefore, we examined αv and β3 expression in the uterine sections from this study. We found that the αv subunit is expressed in the LE and trophoblasts in all treatment groups with similar intensity. We also found that β3 was not detected at the feto–maternal interface on Day 17 in sheep. The β3

subunit was found in blood vessels and was detected using the same antibody on the LE during the oestrous cycle (data not shown), which excludes any technical issues. Both the αv and β3 subunit proteins were detected in early pregnant sheep endometrium (Johnson et al. 2001) although their expression was found to sharply decline on Day 17 (Wan et al. 2011). This is contrary to the observations of Kimmin et al. (2004), who showed that αv was not expressed at the LE during pregnancy in sheep. Nevertheless, based on our observations, at least the αv subunit is not affected by HA or 4MU treatments.

Osteopontin (OPN) is an important ligand for integrins. Since some integrins are located on both LE and trophectoderm, OPN is proposed as a bridging ligand at this interface (Johnson et al. 1999). OPN–integrin interaction was also suggested to mediate the interaction between gametes or embryos and epithelium in the oviduct (Gabler et al. 2003). In the endometrium, OPN was found to be produced by the GE as a component of histotroph but not by the LE (Johnson et al. 1999) and is involved in embryo adhesion (Johnson 2003). OPN can be detected on the luminal surface of the LE as it binds to its integrin receptors. Our results show that the expression of OPN varied between different treatment groups. OPN was strongly localised at the luminal surface of LE and GE in the control and 4MU groups, whereas its expression on the LE was reduced in HA-infused ewes and was nearly absent in the HA+Hyal2 group. The reduction of OPN at the LE may be due to the reduction of OPN production by the GE in these groups or to the masking of integrins by increased MUC1 expression. This can obviously contribute to the failure of embryos to attach. In ewes with uterine gland knockout (UGKO; no glandular epithelium development resulting from continuous administration of progestin to neonatal ewes), recurrent early pregnancy loss occurs due to the very low levels of OPN and other adhesion proteins like Glycam-1 (Gray et al. 2002).

CD44 is another cell-surface glycoprotein that can bind OPN. CD44 variants like CD44v6, which was examined in the present study, but not the standard form of CD44, can bind to both the amino- and C-terminal portions of OPN, which suggests that multiple domains on OPN can be bound to the CD44 variants (Katagiri et al. 1999). CD44 variants also cooperate with β1-containing integrins to enhance binding to OPN (Katagiri et al. 1999). At the same time, CD44 is the most common receptor for HA (Aruffo et al. 1990). Most biological processes of HA such as adhesion, migration, proliferation and angiogenesis involve HA–CD44-mediated signalling and cytoskeleton reorganisation, which are mediated by low molecular weight HA and CD44 interactions (Fujita et al. 2002). In the present study, we detected CD44v6 in the LE, GE and ST as well as the trophoblast of the control ewes, which supports the generally acknowledged concept of CD44 mediation of blastocyst attachment to the endometrium (Singh and Aplin 2009). We also observed a reduction in CD44v6 when HA was infused. This reduction was more distinct in the presence of Hyal2. Previous studies concomitant with our results have shown inhibitory effects of low molecular weight HA (average molecular weight of 2.5 kDa) on expression of CD44 (Yang et al. 2012). Similarly, injection of anti-CD44 into uterine horns was shown to cause a total inhibition of embryo implantation and embryo survival in rabbit (Illera et al. 2004). In contrast, complete inhibition of HA synthesis by 4MU shown in the present study is suggested to remove any competition by HA for CD44 receptors and enhance interaction between trophectoderm and the LE through OPN binding.

Since this study was performed in sheep using a limited number of ewes, further investigations are required on a wider scale. However, the study provides important evidence that clearance of HA is favourable for embryo implantation. In conclusion, the presence of HA at the feto–maternal interface during the process of embryo attachment results in reduced endometrial receptivity and inhibits interaction of trophoblasts with the LE.

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Table 1. Embryo adhesion on day 17 of gestation in sheep after uterine infusion on day 14 with 1 ml per uterine horn with phosphate buffered saline (Control); hyaluronan (HA; 1mg/ml); HA+Hyal2 (300 IU); and 4-methyubeliferone (4MU; 1mM). Assessment of attachment was determined in formalin-fixed uterine sections after H&E staining.

Treatment	Ewes in each group	Ewes containing embryos	Embryos attached to LE
Control	4	3/4	2/3
HA	4	3/4	0/3
HA+Hyal2	3	3/3	1/3
4MU	4	4/4	4/4

Figure legends

Figure 1. Immunolocalization of HA using biotinylated-HABP in uterine sections of ewes infused with PBS (Control), HA, HA+Hyal2 or 4MU. HA is shown as brown staining (DAB) while nuclei are counterstained with hematoxylin.

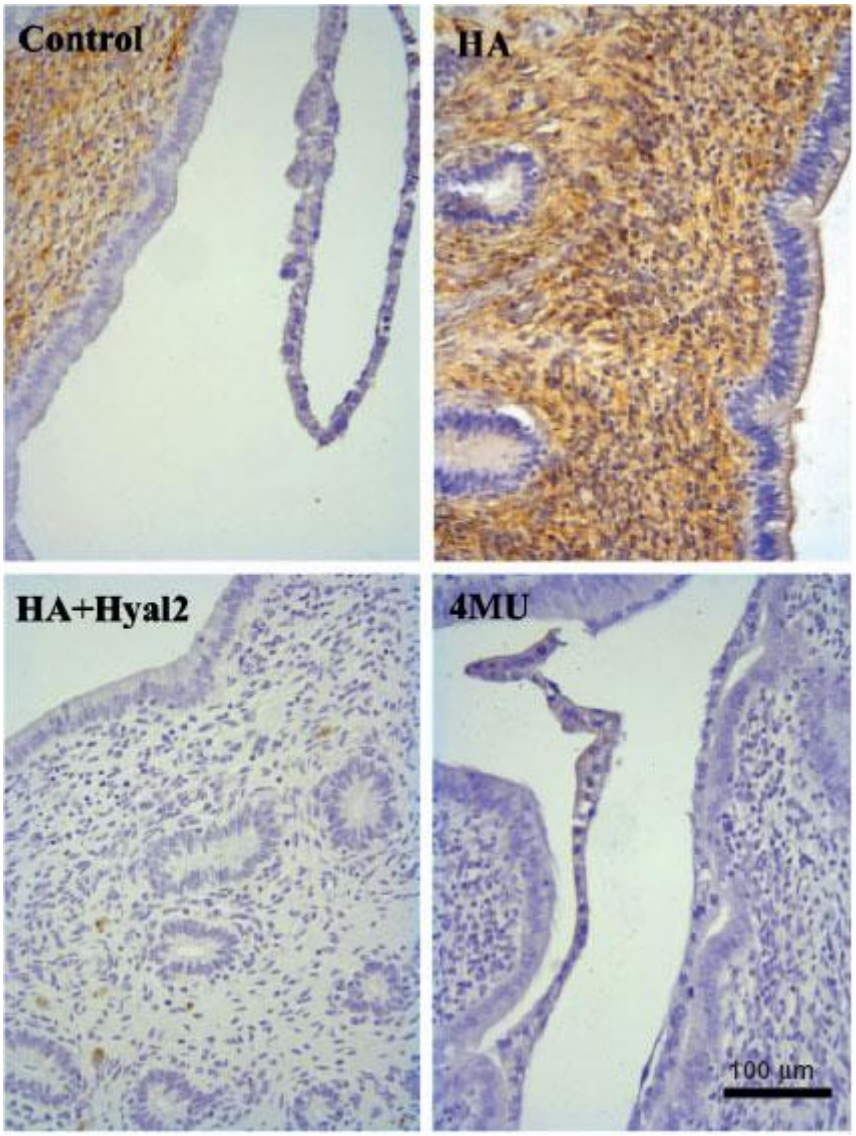

Figure 2. Representative images for uterine sections containing attached and not-attached embryos. Representative images are shown from **A,** HA-infused group (embryo not attached) and **B,** 4MU-infused groups (embryo attached) as shown by H&E staining on day 17 (3 days after infusion).

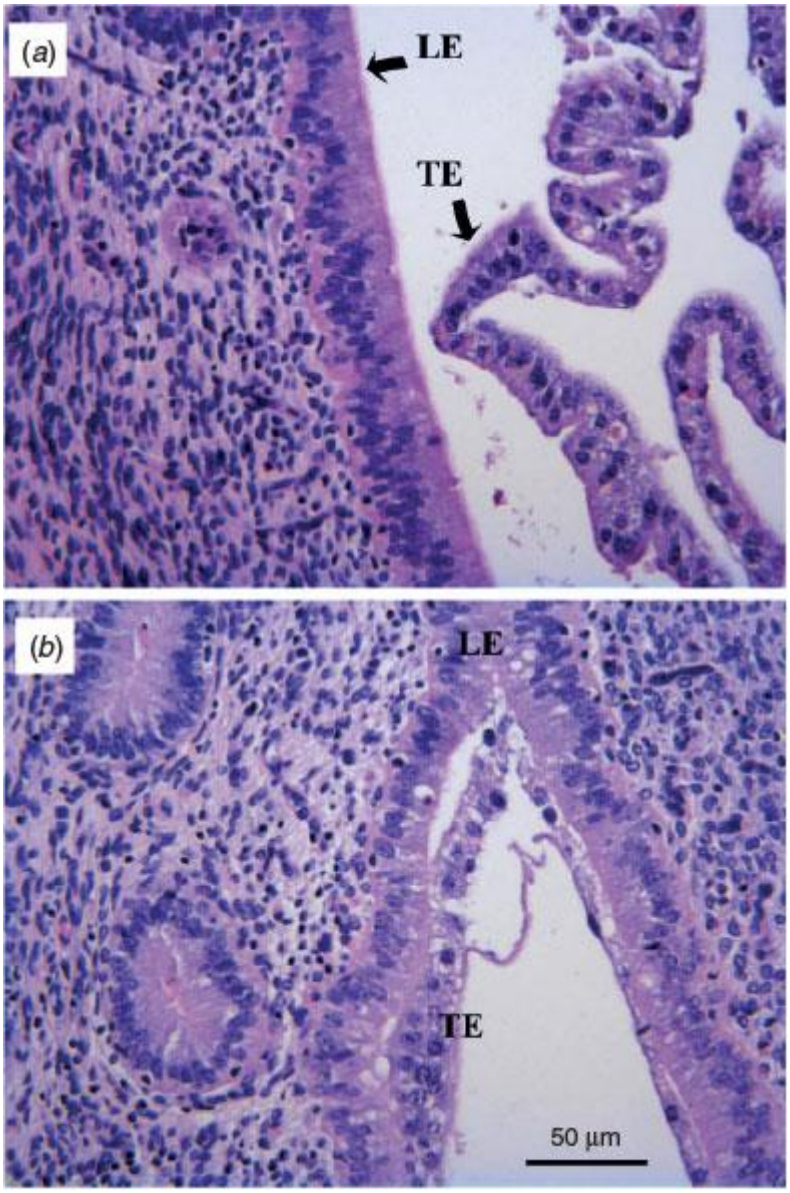

Figure 3A-E. Immunohistochemical localization of MUC1 in the uterine endometrium on day 17 of pregnancy in ewes infused with either PBS (Control), HA, HA+Hyal2 or 4MU on day 14. MUC1 is shown as brown staining (DAB) while nuclei are counterstained with hematoxylin. **Figure 3F.** Quantification of MUC1 immunostaining using HScore. Data is presented as mean ± SEM from at least 3 animals. Bars with different alphabets are significantly different at $P<0.05$.

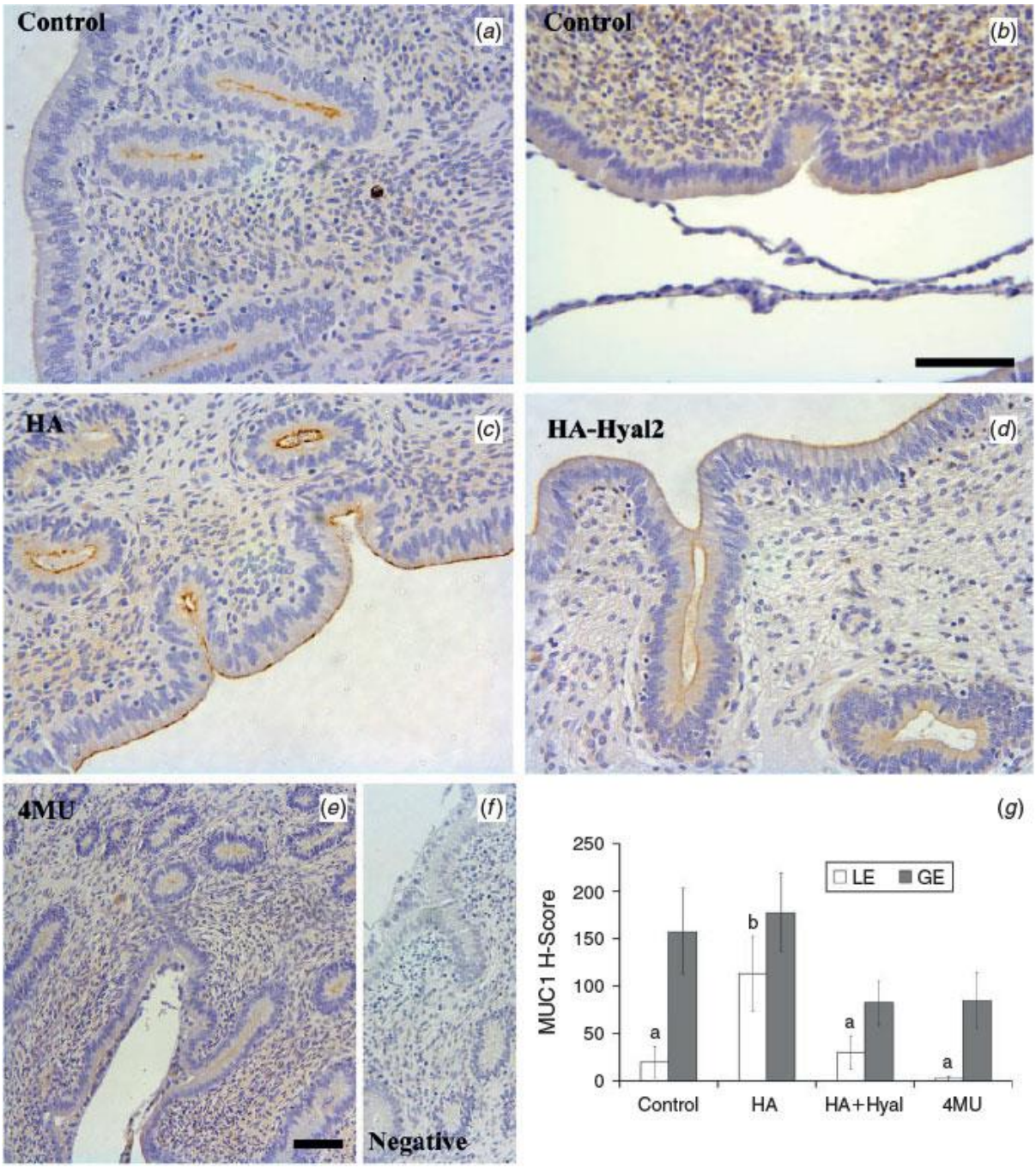

Figure 4A. Immunohistochemical localization of OPN in the uterine endometrium on day 17 of pregnancy in ewes infused with either PBS (Control), HA, HA+Hyal2 or 4MU on day 14. OPN is shown as brown staining (DAB) while nuclei are counterstained with hematoxylin. **TE**, Trophectoderm; **LE**, Luminal epithelium; **GE**, glandular epithelium. Figure 4B. Quantification of OPN immunostaining using H-Score. Data is presented as mean ± SEM from at least 3 ewes. Bars with asterisks are significantly different from control at $P<0.05$ within the same layer.

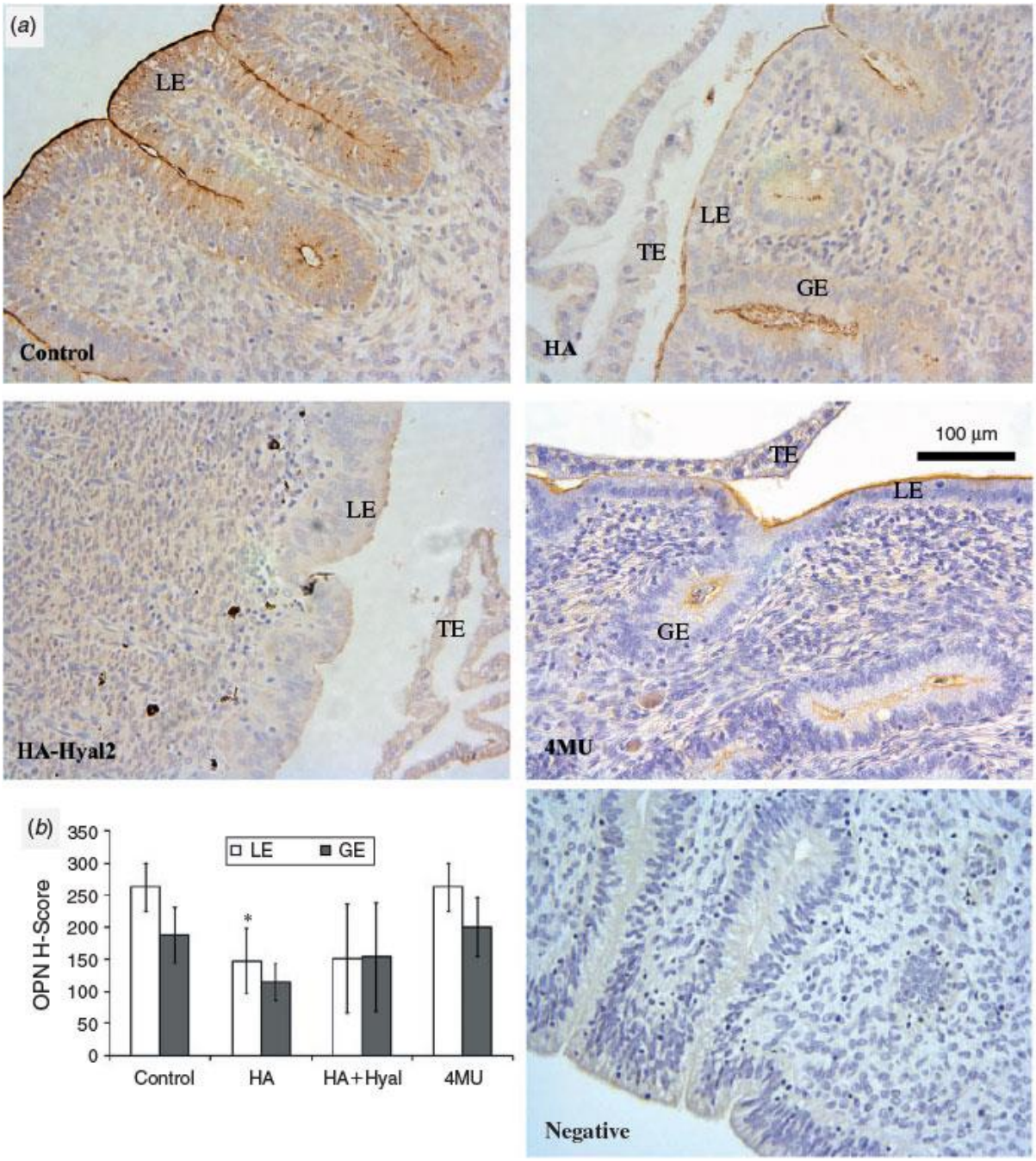

Figure 5. Immunohistochemical localization of integrins subunits αv and β3 in the uterine endometrium on day 17 of pregnancy in ewes. Representative images are shown from HA and 4MU groups. Integrins are shown as brown staining (DAB) while nuclei are counterstained with hematoxylin.

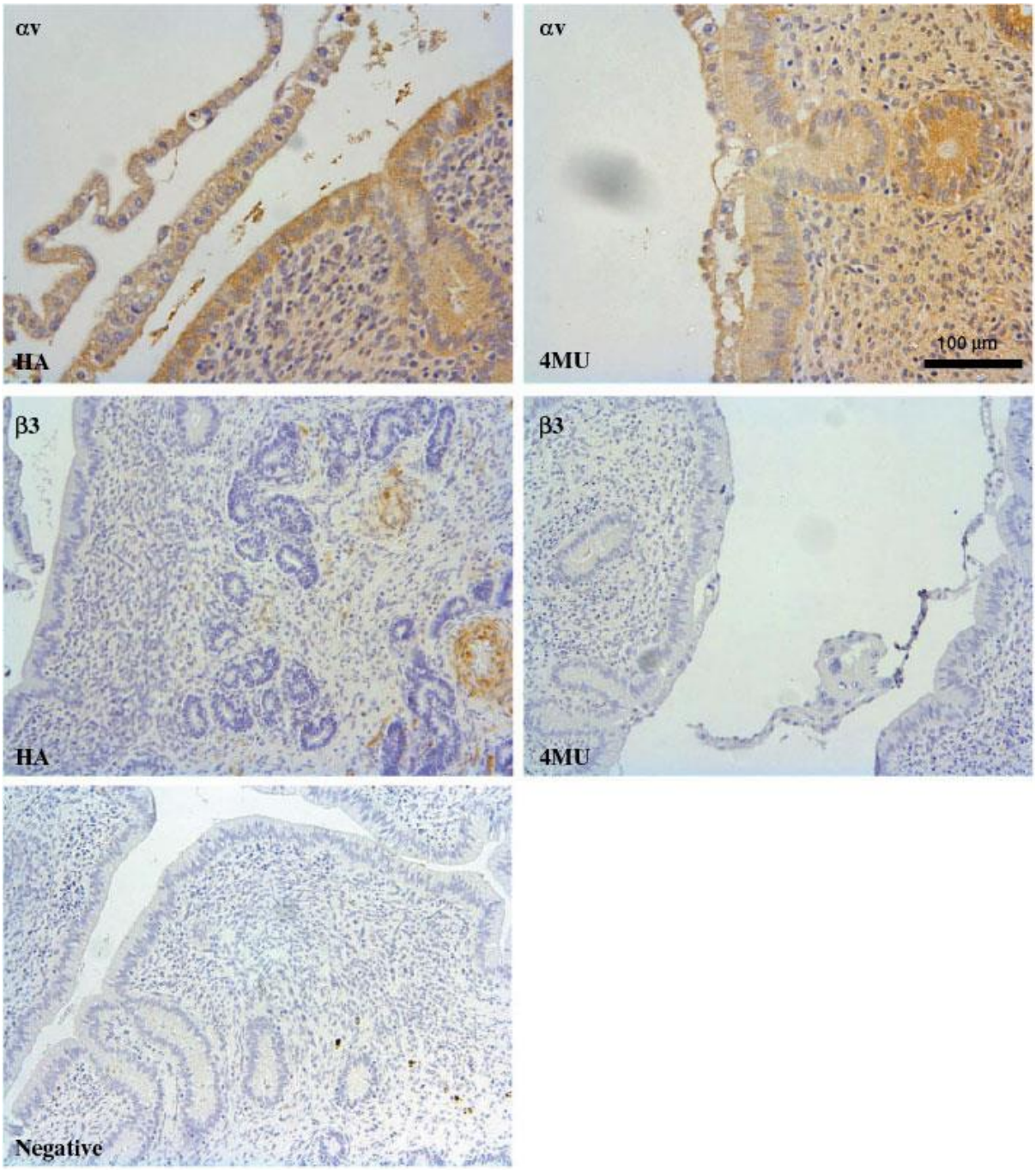

Figure 6A. Immunohistochemical localization of CD44v6 in the uterine endometrium on day 17 of pregnancy in ewes infused with either PBS (Control), HA, HA+Hyal2 or 4MU on

day 14. CD44v6 is shown as brown staining (DAB) while nuclei are counterstained with hematoxylin. **TE**, Trophectoderm; **LE**, Luminal epithelium; **GE**, glandular epithelium.
Figure 6B. Quantification of CD44v6 immunostaining using H-Score. Data is presented as mean ± SEM from at least 3 ewes. Bars with asterisks are significantly different from control at $P<0.05$ within the LE layer.