Acta Histochemica 120 (2018) 438–445

Contents lists available at ScienceDirect

Acta Histochemica

journal homepage: www.elsevier.com/locate/acthis

Changes in mucins and matrix metalloproteases in the endometrium of early pregnant alpacas (*Vicugna pacos*)

Daniela E. Barraza[a], Renato Zampini[a,b], Silvana A. Apichela[a,c], Joel I. Pacheco[d], Martin E. Argañaraz[a,b,*]

[a] *Instituto Superior de Investigaciones Biológicas (INSIBIO), CONICET-UNT, and Instituto de Biología "Dr. Francisco D. Barbieri", Facultad de Bioquímica, Química y Farmacia, UNT, Chacabuco 461, T4000ILI, San Miguel de Tucumán, Argentina*

[b] *Cátedra de Biología Celular y Molecular, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco 461, San Miguel de Tucumán, T4000ILI, Tucumán, Argentina*

[c] *Cátedra de Zootecnia General I, Facultad de Agronomía y Zootecnia, Universidad Nacional de Tucumán, Florentino Ameghino s/n, El Manantial, 4105, Tucumán, Argentina*

[d] *Instituto Veterinario de Investigaciones Tropicales y de Altura, Universidad Nacional Mayor de San Marcos – UNMSM, Sede Marangani, Cuzco, Peru*

ARTICLE INFO

Keywords:
Mucin
Metalloproteases
Early pregnancy
Alpacas

ABSTRACT

South American Camelids (SAC) have unique reproductive features, one of which is that 98% of the pregnancies develop in the left uterine horn. Furthermore, early pregnancy is an uncharacterized process in these species, especially in regard to the ultrastructural, biochemical and genetic changes that the uterine epithelial surface undergoes to allow embryo implantation. The present study describes the uterine horn luminal surface and the characteristics of the mucinous glycocalyx in non-pregnant and early pregnant (15 days) female alpacas. In addition, the relative abundance of Mucin 1 and 16 genes (MUC1 and MUC16) was determined, as well as the relative mRNA abundance of matrix metalloproteinases (MMPs) that could be involved in MUC shedding during early pregnancy. Noticeable changes were detected in the uterine luminal epithelium and glycocalyx of pregnant alpacas in comparison to non-pregnant ones, as well as presence of MUCs and MMPs in the endometrial environment. The decrease in glycocalyx staining and in the relative abundance of MUC 1 and MUC 16 transcripts in pregnant females would allow embryo attachment to the luminal epithelium and its subsequent implantation, as has been described in other mammals. These results suggest a crucial role of MUC1 and MUC16 and a possible role of MMPs in successful embryo implantation and survival in alpacas.

1. Introduction

The process of embryo implantation is actually a multistep event that involves several changes in the expression pattern of embryonic and uterine cell surface components. Although implantation aspects vary among species, interaction between the external surface of the embryonic trophectoderm and the apical surface of the uterine luminal epithelium (LE) is a general occurrence. The mechanism leading to embryo implantation in South American Camelids (SAC),whose process of embryo attachment and implantation is unique, has not been elucidated yet. Although camelids have two functional uterine horns and ovaries that contribute almost equally to ovulation, 98% of the pregnancies occur in the left uterine horn (LUH). This means that the embryo has to migrate to the LUH for implantation and that the right uterine horn (RUH) would be unsuitable to sustain pregnancy (Fernandez-Baca et al., 1979; Vaughan et al., 2013). In addition, the pregnancy rate 30 days post-mating in SACs is < 50% (Sumar et al., 1988), indicating that the embryo lossesare much higher in SACs than in other small ruminants (Diskin and Morris, 2008).

Similar to other epithelial surfaces, the mucosa of the female reproductive tract is lined with a glycocalyx, which allows diffusion of small molecules but inhibits cell adhesion and protects the upper tract from infectious agents (Aplin, 2010). The glycocalyx is formed by large, transmembrane glycoproteins known as mucins. Three major mucins have been identified in the uterine epithelia of multiple species: MUC1, MUC4, and MUC16 (Constantinou et al., 2015). MUC1 has been described in the uterine mucosa of dromedaries (Al-Ramadan et al., 2013), sheep (Wang et al., 2017), cows (Kasimanickam et al., 2014), mares (Wilsher et al., 2013), pigs (Ren et al., 2010) and humans (Shen et al., 2015) among others. Expression of MUC4 and MUC16 has been

* Corresponding author at: Cátedra de Biología Celular y Molecular, Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Chacabuco 461, San Miguel de Tucumán, T4000ILI, Tucumán, Argentina.
E-mail address: mearganaraz@fbqf.unt.edu.ar (M.E. Argañaraz).

https://doi.org/10.1016/j.acthis.2018.05.009
Received 9 March 2018; Received in revised form 18 April 2018; Accepted 10 May 2018

studied less and may be species-specific (Constantinou et al., 2015). The endometrium epithelial glycocalyx could offer an initial attachment site for embryos, but it also constitutes a barrier for implantation progression. This barrier function must be overcome in the context of embryo implantation to permit blastocyst attachment (Aplin, 2010). The mechanisms for removing mucins are species-specific and include hormonal suppression of gene expression or membrane clearance via proteases. For example, in rodents, sheep and pigs, MUC1 is downregulated through the loss of the progesterone receptor (PR) from the uterine epithelium (Bowen et al., 1997; Johnson et al., 2001). Nonetheless, mucin expression in rabbits and humans persists during the proposed receptive phase, although MUC1 is locally reduced at implantation sites (Hoffman et al., 1998; Spencer et al., 2004). Shedding of MUC1 at the implantation sites is accompanied by high levels of metalloproteases (Thathiah and Carson, 2004).

In an effort to better understand the molecular basis of embryo implantation in SAC, the uterine horns of non-pregnant and pre-implantation pregnant female alpacas were compared (LUH vs. RUH). Firstly, the endometrial surface was examinedby scanning electron microscopy and glycocalyx staining (PAS and Alcian Blue).Second, the relative abundance of MUC1 and MUC16 mRNA wasdetermined. In addition, presence of certain matrix metalloproteases (MMP14, MMP2 and MMP9) was examined in the endometrial fluid and tissue in relation to the embryo implantation process.

2. Materials and methods

2.1. Animals and sampling

A total of twelve two-year-old virgin females from the species*Vicugnapacos* (HuacayaAlpaca breed) were used in the study. The animals belonged to the veterinary research center (IVITA) at the Universidad Nacional Mayor de San Marcos in Marangani in the province of Canchis in the Cuzco region, Peru (14 °S, 71 °W; 3698 m altitude). The animals were maintained in outdoor paddocks and fed grass hay and water *ad libitum*. For the experiment, all females were allowed to mate once with a fertile adult male. Fifteen days after mating, the reproductive tracts were collected by necropsy according to the protocols approved by the local institutional animal care and ethics committee. The reproductive tracts were divided into two groups according to the ovary status: non-pregnant (NP) and pregnant (P). Females presenting a corpus luteum and embryos were considered pregnant. Progesterone was also assayed to confirm early pregnancy.

To obtain uterine horn fluid (UHF), the embryo was carefully removed and then each uterine horn (UH) was clamped at both ends. A blunt needle attached to a syringe was inserted, and 4 ml of phosphate-buffered saline solution (PBS), pH 7.4, were flushed into the horn and then aspirated. Each flushing was centrifuged at 5000 × *g* (10 min, 4 °C) to pellet any cellular debris. After flushing, the endometria from the midsection of left and right UH (LUH and RUH) were dissected into 50 mm segments and subsequently placed in 4% formaldehyde-PBS solution pH 7.4 for histological assays, in Karnovsky solution for Scanning Electron Microscopy (SEM), or in RNAlater solution (Ambion, Austin, USA) for RT-PCR assays. RNAlater embedded samples and UHF were transported on dry ice and stored at −80 °C until further analysis.

2.2. Determination of progesterone levels

Blood samples for progesterone (P4) determinations were collected by jugular venipuncture at the time of tissue collection, before animal slaughter. Samples were centrifuged and plasma was stored at −20 °C until P4 assays were performed.

Hormonal analysis performed with electrochemiluminescence immunoassay (ECLIA) using a Roche Elecsys Cobas diagnostics kit (Roche Diagnostics, Indianapolis, USA), which is based on the sandwich principle. Assays were carried out in duplicate, according to the manufacturer's instructions, and samples were analyzed with a Roche Elecsys 2010 immunoassay analyzer (Roche Diagnostics). Results were compared with a calibration curve, which was specifically generated by a two-point calibration and a master curve provided via the reagent barcode. Values above 4 ng/ml were considered as normal for 14-day-pregnant animals (Bravo, 2002).

2.3. Scanning electron microscopy of the uterine mucosa

After Karnovsky fixation, NP (n = 3) and P (n = 3) alpaca tissue samples were treated according to Apichela et al. (2009); samples were thenmounted on aluminum stubs, coated with gold, and examined under a Carl ZeissVR Supra 55VP scanning electron microscope (Oberkochen, Germany) at Centro Integral de Microscopía Electronica (CIME),Tucumán, Argentina.

2.4. Histochemical methods: PAS and Alcian blue staining

LUH and RUH obtained from NP (n = 3) and P (n = 3) alpacas were previously fixed with formaldehyde-PBS and subsequently embedded in paraplast (Deltalab, Barcelona, Spain) for sectioning in 5 µm sections.Then they were subjected to standard histochemistry protocols for A) Periodic Acid-Schiff (PAS) staining, BIOPUR, Rosario, Argentina) and counterstained with Weigert's Hematoxylin (BIOPUR); this method is used for neutral or weakly acidic glycoconjugates; and B) Alcian blue (AB) staining with 1% Alcian Blue 8Gx solution (Biopack, Zarate, Argentina), pH 2.5, and counterstained with fast red (Sigma, St. Louis, USA); this method reveals presence of acid mucins with sulfate esters and carboxyl groups (Luna, 1968). Slides were observed under a Leica DM500 light microscope (Mannheim, Germany) and images were captured with a Leica ICC50 HD camera. Identical image acquisition settings and exposure times were applied.

ImageJ 1.42q software (NIH, Bethesda, USA) was used to measure the stained area of the LE according to Jensen (2013). For this analysis, we used two photographs (100X) of each sample with a total area analyzed of 16,518.1 pixels/mm^2. Three rectangular region of interest (ROI) with an area of 981.8 pixels/mm^2, were selected randomly. First, the images were converted to 8 bits, then a specific threshold wasdetermining for each staining and quantification was performed; data were expressed as pixels/µm^2. Other stained tissues were not quantified since they are not in direct contact with the embryos.

2.5. Semi-quantitative RT-PCR of MUC1, MUC16, MMP9, MMP2 and MMP14

Total RNA from LUH and RUH from NP (n = 5, one female was eliminated from the assays because of a cystic ovary) and P (n = 6)was isolated using the SV total RNA isolation system according to the manufacturer's instructions (Promega, Madison,USA). RNA was quantified spectrophotometrically at 260 nm, and RNA integrity was examined by electrophoresis on 1.5% agarose gels. One µg of RNA was reverse-transcribed with M-MLV reverse transcriptase (Promega) and oligo-dT primer (Promega) in a 25 µl reaction mixture according to the manufacturer's instructions.

Amplifications were conducted in a final volume of 10 µl containing 1 µl of LUH or RUH cDNA; 2 µl of 5X Green GoTaq Reaction Buffer, pH 8.5 (Promega,); 0.2 mM of each dNTP (Promega); 2.5 units of GoTaq DNA polymerase (Promega) and 1 µM of each primer pair (Table 1). Different amplification settings were assayed to determine optimal PCR conditions: 94 °C for 3 min, followed by 45 cycles at 94 °C for 10 s, 60 °C for 5 s, 72 °C for 5 s, and a final extension at 72 °C for 5 min. PCR products were analyzed with 1.5%agarose gel electrophoresis, and visualized with SYBR Safe DNA Gel Stain (Life technologies, Carlsbab, USA). For semi-quantitative measurement of the relative abundance, gel images were captured with an Optio M 90 Pentax digital camera (Tokyo, Japan), and the optical density of PCR products was quantified

Table 1
Sequences of primer pairs used, corresponding reference sequences as well as resulting fragment length.

GENE	ACC. NUMBER	FORWARD PRIMER	REVERSE PRIMER	SIZE (bp)
Mucin 1 (MUC1)	**XM_006214755.1**	**TGCTGCTATTCCCAGTGCTT**	**TGAGGTGTCATTGGTGGTCG**	**245**
Mucin 16 (MUC16)	**XM_006206471.1**	**TTGTTCCAGAGAAGCAGCCT**	**GGATGTCCACCCCTGTCTTG**	**97**
Metalloproteinase 2 (MMP2)	**GQ244429.1**	**CATGATGGAGAGGCTGACAT**	**GCTCATCGTCATCAAAGTGG**	**148**
Metalloproteinase 9 (MMP9)	**GU207475.1**	**GTTCGATGTGAAGACGCAGA**	**GTCCACCTGGTTCACCTCAT**	**175**
Metalloproteinase 14 (MMP14)	**XM_006217304.1**	**GAGGTTCTACGGTCTGCGAG**	**GGGGGTGTAGTTCTGGATGC**	**193**
HipoxantinFosforibosil Transferase (HPRT)	**XM_006215984.1**	**TGACACTGGCAAAACAATGCA**	**GGTCCTTTTCACCAGCAAGC**	**94**

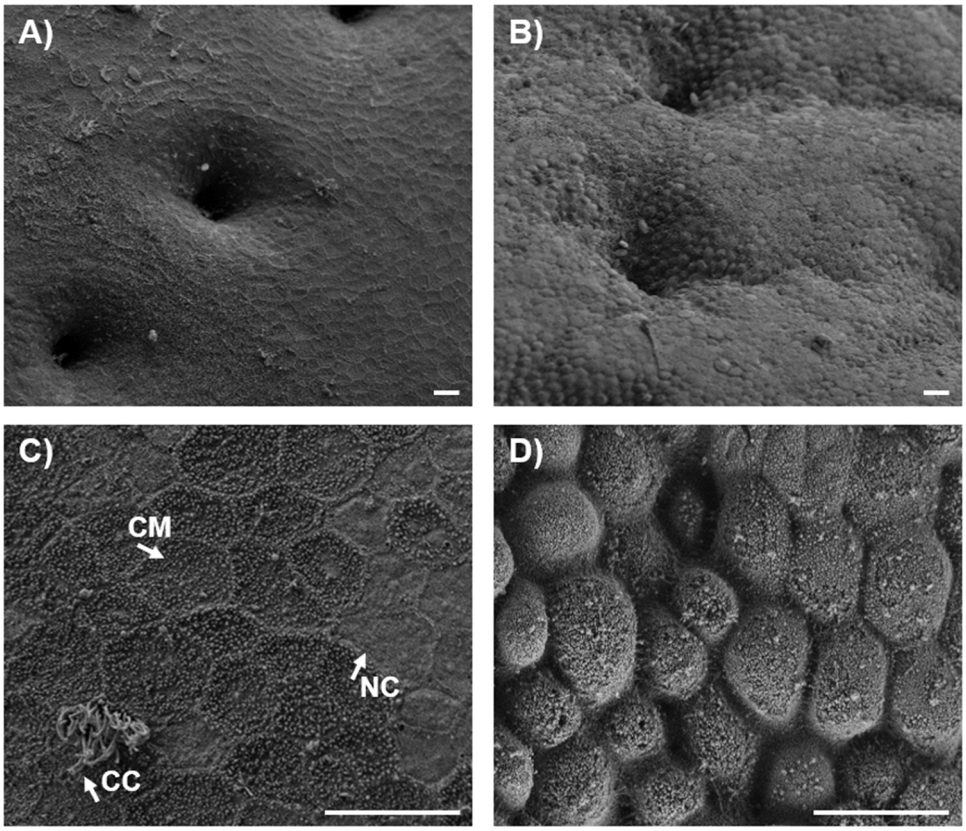

Fig. 1. Scanning electron microscope images of the endometrial surface of non pregnant (A, C) and pregnant alpaca (B y D) at different magnifications (bar = 10 μm). Arrows showed different cell types, CC: ciliated cell, NC: non ciliated cell, CM: non ciliated cell with microvilli.

using ImageJ 1.42q software (NIH). The relative abundance of MUC1, MUC16, MMP9, MMP2 and MMP14 transcripts was normalized against the HPRT reference gene (Table 1), and the transcript/HPRT ratio was calculated for each tissue analyzed (Diao et al., 2011). Molecular weight markers were used as calibration control between gels.

2.6. Gelatinzymography of left and right uterine horn fluid

Gelatinase activity in LUHF and RUHF (NP and P alpacas, n = 4 each) was assayed. Total protein was determined using a Micro BCA kit (Thermo Fisher Scientific, Rockford, USA). For zymography, 15 μg of total proteins were separated under non-reducing conditions on 8% polyacrylamide gels containing 1 mg/ml gelatin as described by Zampini et al. (2014). For semi-quantitative activity analysis, gel images were captured with an Optio M 90 Pentax digital camera, and the optical densitometry of gelatinolyticzymographicbands was quantified using ImageJ 1.42q software (NIH). One ng of Type I Collagenase (Clostridium peptidase A from Clostridium histolyticum, Sigma) was used as positive control and calibrator between gels. Additionally, UHFs were incubated in the presence of aminophenyl-mercuric acetate (APMA) to activate latent zymogen and induce transition of pro-MMP to MMP, and subsequently analyzed withzymography (Zampini et al., 2017). The molecular weight of the bands was calculated using GelAnalyzer freeware software (http://www.gelanalyzer.com, version 2010 by Istvan Lazar and Dr. Istvan Lazar, Hungary).

2.7. Statistical analysis

Statistical analysis was performed with InfoStat software (Di Rienzo et al., 2008). One-way Analysis of Variance (ANOVA) was used to analyze the following parameters: PAS and AB images, relative gene abundance and densitometry of gelatinolyticbands. When ANOVA showed significant differences ($p \leq 0.05$), Tukey test was used to determine the level of significance. Results were considered statistically significant at $p \leq 0.05$.

3. Results

3.1. Pregnancy rates and hormone profile

The percentage of pregnant alpacas obtained after a single copulation was 50% (6/12). Plasma progesterone of P females ranged between 4.5 and 10.2 ng/ml (7.4 ± 1.9 ng/ml), in correlation withthe development of a corpus luteum. All embryos collected had reached the

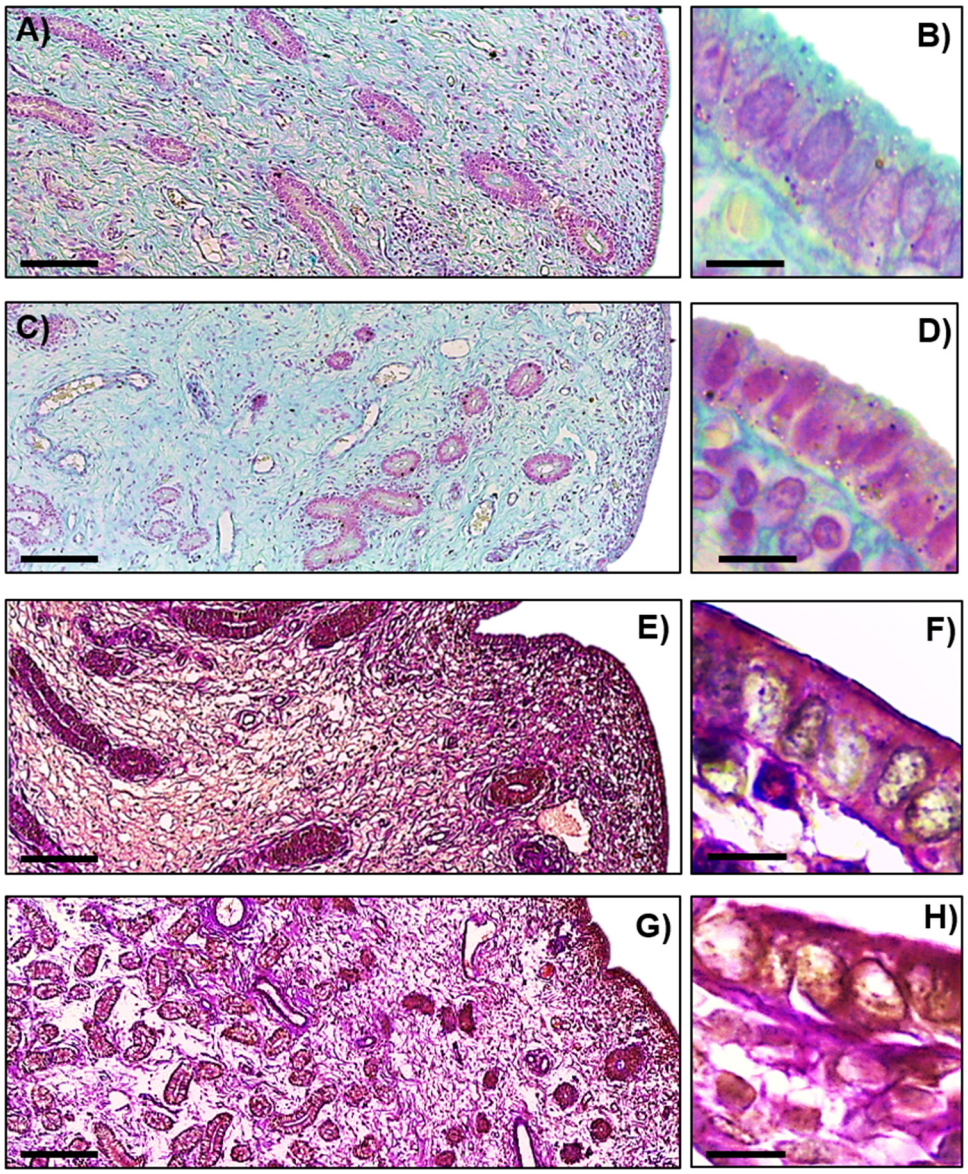

Fig. 2. Histological results of the endometrial mucosa of alpacas. A) Endometrial section of non-pregnant alpacas stained with Alcian Blue (pH 2.5), bar = 100 μm. B) Higher magnification of the luminal epithelium, bar = 10 μm. C) Endometrial section of pregnant alpacas stained with Alcian Blue (pH 2.5), bar = 100 um. D) Higher magnification of the epithelial mucosa, bar = 10 μm. E) Endometrial layer of non-pregnant alpacas stained with Periodic acid-Schiff (PAS), bar = 100 μm. F) Higher magnification of the luminal epithelium, bar = 10 μm. G) Uterus section of the mucosa of pregnant alpacas stained with PAS. H) Higher magnification of the respective epithelial mucosa, bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

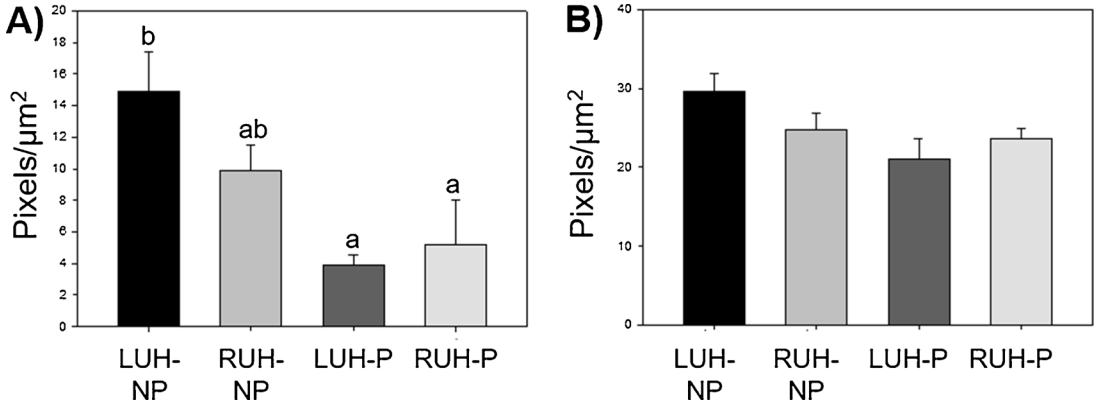

Fig. 3. Histograms of AlcianBlue and PAS stained area in the luminal epithelium of non-pregnant and pregnant endometrium, significant differences are indicated with different letters, data represent means ± SE (n = 3). A) Alcian Blue, ANOVA p = 0.024, Tukey p-value: LUH-NP vs. LUH-P p = 0.028, LUH-NP vs. RUH-P p = 0.049; B) PAS,ANOVA p = 0.085. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

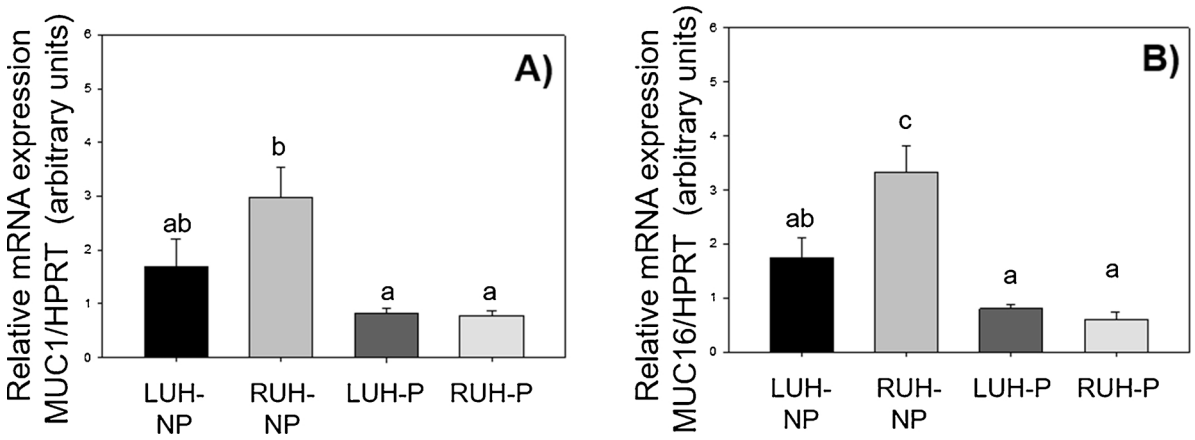

Fig. 4. MUC1 and MUC 16 mRNA relative abundance in left and right uterine horn of non pregnant alpacas (LUH-NP and RUH-NP) and pregnant alpacas (LUH-P and RUH-P). The MUC mRNA expression was normalized against HPRT gen. Data represent means ± SE (n = 5). Significant differences are indicated with different letters. MUC1: ANOVA p ≤ 0.001, Tukey p-value: RUH-NP vs. RUH-P p = 0.002, RUH-NP vs. LUH-Pp = 0.002; MUC16: ANOVA p ≤ 0.001, Tukey p-value: RUH-NP vs. RUH-P p = 0.001, RUH-NP vs. LUH-P p = 0.001, RUH-NP vs. LUH-NP p = 0.008.

elongated blastocyst stage. Five animals of the NP group were used for the different studies and one female was eliminated because ofa cystic ovary. Progesterone of NP animals ranged between 0.03–0.19 ng/ml (0.09 ± 0.07 ng/ml).

3.2. Ultrastructure of luminal surface of alpaca uterine horns

The endometrial surface of NP alpacas was flattened and three types of epithelial cells could be distinguished: ciliated and non-ciliated cells, and non-ciliated cells with microvilli (Fig. 1C). Ciliated cells appeared solitary and they were disorderly interspersed among non-ciliated cells. In P alpacas, epithelial cells looked larger, with a globular appearance of the apical cytoplasm, and only cells with short microvilli were observed (Fig. 1D). Ciliated cells were absent in both uterine horns. No ultrastructural differences between uterine horns were observed.

3.3. Glycocalyx features in alpaca uterine endometrium

Presence of neutral and acid mucins was detected in the uterine endometrium. In general, the glycocalyx and the basal lamina of the luminal epithelium showed a positive mark. The uterine glands and the endothelium of vessels and capillaries also presented a distinguishing mark (Fig. 2). Neutral and acid stainingshowed similar positive marks in thetwo uterine horns (LUH and RUH) and during the physiological states (NP and P). Some differences were observed between NP and P endometrium for acid mucins. The overall trend showed that the colored area (pixels/μm^2) for acid mucins during pregnancy decreased, showing a reduction in LUH-P and RUH-P in the luminal epithelium with respect to LUH-NP; 10.9 fold (p = 0.028) and 9.7 (fold, p = 0.049) respectively (Fig. 3).

3.4. Relative abundance of MUC1 and MUC16 mRNA in alpaca uterine endometrium

MUC1 and MUC16 mRNA was detected in the two UHs both in NP and P females. MUC1 did not show any difference in mRNA relative abundance between UHs. However, differences between the reproductive stages were addressed. NP animals showed a greater relative abundance of MUC1 gene than P animals; the difference was significant between RUH-NP and RUH-P (p = 0.002), RUH-NP and LUH-P (p = 0.002) (Fig. 4A). Regarding MUC16, its transcripts showed a similar relative abundance profile than MUC1, and this was significantly higher for RUH than LUH of NP alpacas (1.89 fold, p < 0.008). P alpacas did not demonstrate any difference between the horns. In pregnant animals, MUC16 mRNA abundance in both UHs was significantly lower compared with RUH-NP, p < 0.001 (Fig. 4B).

3.5. Relative abundance of MMP9, MMP2 and MMP14 genes in alpaca uterine endometrium

Since MMP14 is involved in the shedding of MUCs and in the performance of other MMPs such as MMP2 and MMP9, their relative transcript abundance was analyzed in the endometrium. MMP14 was present at steady-state amounts in all samples assayed (p = 0.27). Regarding MMP9, no differences were found in NP nor in P UHs. In contrast, variations were observed between the physiological stages. Of all samples, the relative abundance of MMP9 mRNA was significantly greater in RUH of NP than RUH-P (p = 0.025) and LUH-P (p = 0.034) females.

The relative abundance of the MMP2 gene of both UHs in P females was higher than in NP females, p < 0.001(Fig. 5).

3.6. MMP presence in alpaca uterine fluid

Gelatin zymography was performed to detect metalloproteinases with gelatinolytic activity in alpaca UHF. Four gelatinolytic bands corresponding to MMPs were observed in all samples. Based on their molecular weights, the 62 and 92 kDabands corresponded to MMP2 and Pro-MMP9,respectively (Fig. 6a). These data were corroborated after treatment of UHF with APMA (a chemical activator of Pro-MMP); the Pro-MMP9 band disappeared andan 82 kDa corresponding to active MMP9 appeared (Fig. 6b).Densitometric analysis showed that MMP2 activity was similar among the UHFs assayed (p = 0.13), while theintensity of pro-MMP9 UHF from NP females was significantly higher than in P animals, ANOVA p-value = 0.003 (Fig. 6c).

4. Discussion

As knowledge about the reproductive physiology of SAC advances, itbecomes clearer that certain processes assumed in other domestic livestock cannot be extrapolated to SACs. As a consequence, the molecular and biochemical mechanisms that explain the differences promoting embryo implantation in the LUH, or even more, the basis of the embryo attachment in SACs, have not been elucidated yet.

In alpacas, implantation takes place around day 20 of pregnancy (20 days post-mating according to Olivera et al. (2003)). After 15 days of pregnancy the blastocysts are completely free within the uterine lumen,and luteal rescue have already occurred (Picha et al., 2013). In the current study, noticeable differences were observed 15 days post-mating in the epithelial surface of the endometrium between P and NP

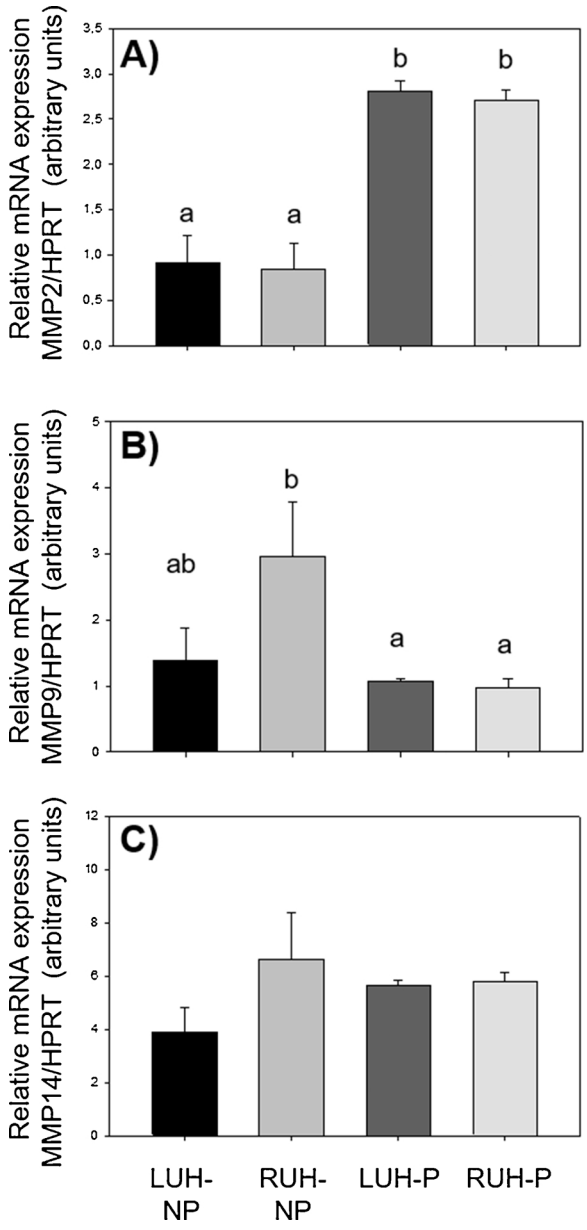

Fig. 5. Relative abundance of MMPs transcripts in the endometrium of non-pregnant and pregnant alpacas. Data represent means ± SE, n = 5. Different letters indicate significant differences. A) MMP2 mRNA expression, ANOVA p = 0.001, Tukey p-value: RUH-NP vs. RUH-P p = 0.001, RUH-P vs. LUH-P p = 0.001, LUH-NP vs. LUH-P p = 0.001, LUH-P vs. RUH-NP p = 0.001; B) MMP9 mRNA expression, ANOVA p = 0.020, Tukey p-value: RUH-NP vs. RUH-P p = 0.025, RUH-NP vs. LUH-P p = 0.034; C) MMP14 mRNA expression, ANOVA p = 0.27.

females, demonstrating a clear indication of preparation of the organ for embryo implantation and pregnancy support.

The modifications observed included ultrastructural and chemical variations that have previously been reported for other mammals. The striking reduction in acid mucin staining in the glycocalyx of P alpacas could be related to changes in the glycosylation profile as happens during early pregnancy and the menstrual cycle in women (Clark, 2015).

The decrease in mucin staining at an early stage in pregnant alpacas was accompanied by a reduction in the relative abundance of MUC1 and MUC16. It has been shown that expression of MUC1 and MUC16 in the uterus is negatively involved in implantation (Achache and Revel, 2006; Gipson et al., 2008). It could be expected that higher than normal concentrations of certain mucins could hamper the implantation process. Therefore, in all mammalian species studied to date conceptus attachment first requires loss of anti-adhesive molecules in the glycocalyx of luminal epithelium (Bazer and Johnson, 2014). Expression of MUC1 is controlled by progesterone and estrogen.For example, in the sheep, MUC1 in the luminal epithelium declines during conceptus elongation, between 9 and 17 days of pregnancy. This event is related to the rise of progesterone and the loss of PR in the endometrium, leading to the down-regulation of certain genes like MUC1 (Spencer et al., 2004). In pigs, who have epitheliochorial placentation like alpacas, progesterone also down-regulated MUC1 expression (Bowen et al., 1997). Ren et al. (2010) described that MUC1 expression in the luminal epithelium varied during implantation; it was lower at attachment sites than at interspatial areas. Similarly, progesterone levels could also inversely regulate MUC1 expression in the alpaca endometrium, as progesterone levels were higher at 15 days of pregnancy than in NP females. According to Bianchi et al. (2015), endometrial PR decrease until 10 days after GnRH induced ovulation in llamas, remaining low at 12 days. In addition, Al-Ramadan et al. (2013) reported that in 78–83 day pregnant dromedaries, expression of MUCl was spatially correlated toPR expressionin the endometrial stroma and they suggested a direct effect of the embryo on expression of PR and MUCl.

Regarding MUC16 mRNA, its expression is down-regulated during the luteal phase in bovine endometrium (Bauersachs et al., 2008) and during the receptive phase in human endometrium, and it is shed from the surface of the LE during uterodome formation (Gipson et al., 2008). In addition, MUC16 regulation is progesterone-dependent (Morgado et al., 2012), which suggests a common regulation of MUC16 during early pregnancy of alpacas.

Besides their down-regulation, a proteolytic clearance of MUCs from the LE glycocalyx has also been described in species such as humans, mice and rabbits during pregnancy (Aplin, 2006; Olson et al., 1998). In order to detect possible effectors of MUC shedding presence of MMPs in the UH environment was assayed, andchanges in MMP profile during pregnancycould be observed. Our resultshave shown that relative abundance of MMP14 transcripts remained unchanged for LUH and RUH, and P and NP females. On the other hand, relative MMP9 transcript abundance decreased in the endometrium of LUH and RUH during early pregnancy, in correlation with MMP9 presence in the UF. In contrast, relative abundance of MMP2 mRNA increased during early pregnancy, although MMP2 presence in the UF remained similar in both NP and P animals.

It has been reported that MMP14 (MT-MMP1) is capable of releasing MUC1 from the cell surface in humans, and overexpression or deficiency of the protease causes increased or inhibited MUC1 shedding, respectively (Thathiah and Carson, 2004). In addition, MMP14 activates pro-MMP2, concentrates its proteolytic activity on the cell surface and contributes to maintain high levels of MMP9, allowing for extensive degradation of the extracellular matrix (Cauwe et al., 2007). This means that MMP14 would be important for regulating MMP2 and MMP9 proteolytic activity in alpaca UH during early pregnancy.

In a previous study, we have addressed differences between the secretome of the RUH and LUH of 30-day pregnant alpacas, when the embryos are already implanted (Argañaraz et al., 2015). In addition, Abdoon et al. (2017) described differences between the LUH and RUH during different pregnancy stages in dromedaries. In the current study, remarkable differences between the UHs which could direct implantation in the LUH were not observed. A possible explanation could be that MUC shedding occurs in punctual sites. For example, in rodents and humansexpression of MUC1 in the LE is reduced only at the site of trophoblast invasion, suggesting that factors released by the blastocyst act either directly or in a paracrine fashion to trigger MUC1 loss by shedding and/or down-regulation (Aplin, 2006). The possible roll of MMP14, MMP2 and MMP9 in mucin luminal epithelial clearance of camelids should still be further studied.

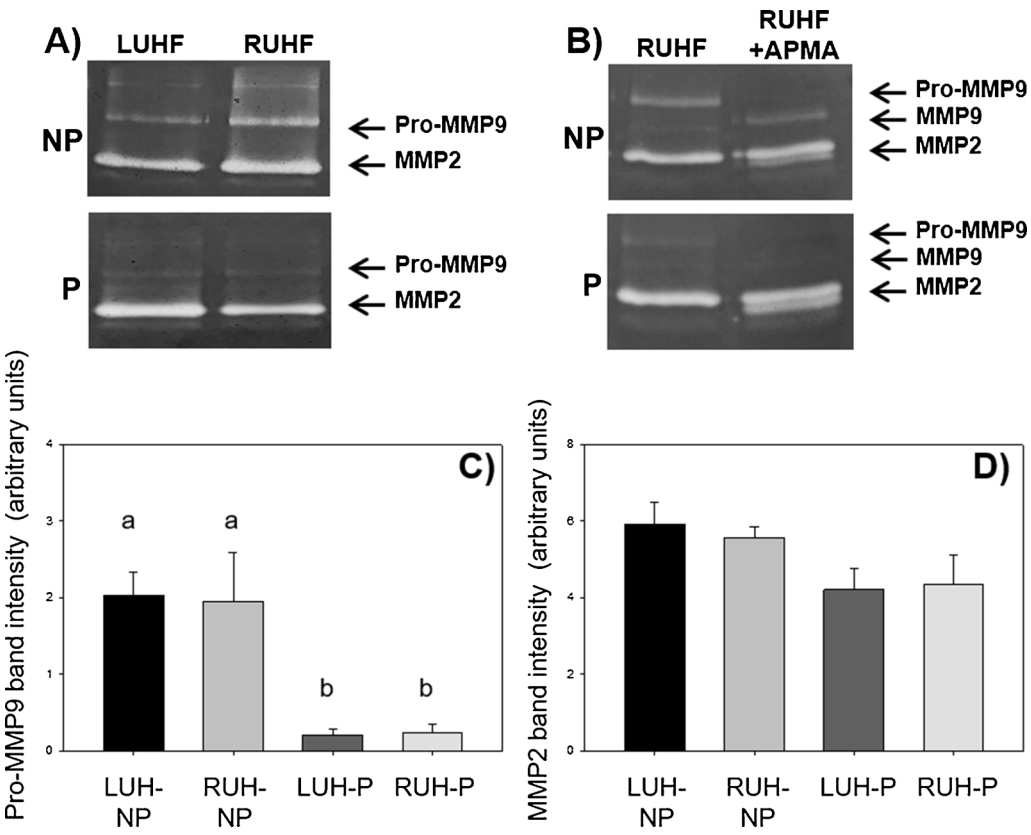

Fig. 6. Gelatinolytic activity of uterine horn fluid (UHF). Data represent means ± SE, n = 4. Different letters above bars indicate significant differences. A) UHF gel zymography from the right and left uterine horns of non-pregnant (NP) and pregnant animals (P). B) UHF treated with APMA, an activator of Pro-MMPs. C) Densitometric analysis of pro-MMP-9, ANOVA p = 0.003, Tukey p-value: LUHF-NP vs. LUHF-P p = 0.019, LUHF-NP vs. RUHF-P p = 0.020, RUHF-NP vs. LUHF-P p = 0.024, RUHF-NP vs. RUHF-P p = 0.026 and D) MMP-2, ANOVA p = 0.13.

5. Conclusion

Pregnancy affects MUC1 and MUC16 mRNA relative abundance and glycocalyx density, and relative abundance of MMPs is probably involved in mucin shedding. The timing of these changes coincides with that of uterus conditioning for the imminent embryo implantation. The specific molecular changes that promote preferential implantation in the LUH have to be elucidated yet. The current report describes an important step in the mechanism of embryo implantation in SAC,which helps to better understand the reproductive process of this species.

Author contributions

D. Barraza contributed with experimental assays and data analysis, R. Zampini contributed with zimography experimental design assays and data analysis, S. Apichela contributed with experimental design, analysis and interpretation of data and revising paper, J Pacheco contributed with the animal managing, sampling and revising paper. M. Argañaraz contributed with the experimental design, data analysis, interpretation and drafting the article.

Conflict of interest

The authors declare that there is no conflict of interest that prejudices the impartiality of this scientific work.

Acknowledgements

This research was partially supported by BID-PICT 2013-1495 and BID-PICT 2012-2701 (ANPCyT) and PIUNT D 525. The authors would like to thank Dr. F. Bonilla from Universidad Nacional de Tucumán, Argentina; Dr. D. Lombardo from Universidad de Buenos Aires, Argentina and Dr. G. Medina from CIP La Raya-UNA, Puno, Peru; for their collaboration.

References

Abdoon, A.S., Giraud-Delville, C., Kandil, O.M., Kerboeuf-Giraud, A., Eozénou, C., Carvalho, A.V., Julian, S., Sandra, O., 2017. Maternal recognition of pregnancy and implantation are not associated with an interferon response of the endometrium to the presence of the conceptus in dromedary camel. Theriogenology 1 (90), 301–308. http://dx.doi.org/10.1016/j.theriogenology.2016.11.033.

Achache, H., Revel, A., 2006. Endometrial receptivity markers, the journey to successful embryo implanta- tion. Hum. Reprod. Update 12, 731–746.

Al-Ramadan, S.Y., Ali Abdelhadi, M., AlthnianThnian, A., 2013. Analysis of the expression of progesterone and oestrogen receptors and their effect on Mucin-1 at both uterine horns of pregnant camel. J. Camel Pract. Res. 20 (1), 59–63.

Apichela, S., Jiménez-Díaz, M.A., Roldan-Olarte, M., Valz-Gianinet, J.N., Miceli, D.C., 2009. In vivo and in vitro sperm interaction with oviductal epithelial cells of llama. Reprod. Domest. Anim. 44 (6), 943–951. http://dx.doi.org/10.1111/j.1439-0531.2008.01125.x.

Aplin, J., 2010. Redefining endometrial receptivity: redefining endometrial receptivity. Indian J. Physiol. Pharmacol. 54 (5), 1–6.

Aplin, J.D., 2006. Embryo implantation: the molecular mechanism remains elusive. Reprod. Biomed. Online 13, 833–839.

Argañaraz, M., Apichela, S., Zampini, R., Vencato, J., Stelletta, C., 2015. Biochemical and protein profile of Alpaca (Vicugnapacos) Uterine Horn fluid during early pregnancy. Reprod. Domest. Anim. 50, 121–128. http://dx.doi.org/10.1111/rda.12460.

Bauersachs, S., Mitko, K., Ulbrich, S.E., Blum, H., Wolf, E., 2008. Transcriptome studies of bovine endometrium reveal molecular profiles characteristic for specific stages of estrous cycle and early pregnancy. Exp. Clin. Endocrinol. Diabetes 116 (7), 371–384. http://dx.doi.org/10.1055/s-2008-1076714.

Bazer, F.W., Johnson, G.A., 2014. Pig blastocyst–uterine interactions. Differentiation 87 (1–2), 52–65. http://dx.doi.org/10.1016/j.diff.2013.11.005.

Bianchi, C.P., Meikle, A., Benavente, M.A., Álvarez, M.A., Trasorras, V.L., Miragaya, M.H., Rodríguez, E., Aba, M.A., 2015. Oestrogen and progesterone receptors and COX-2 expression in endometrial biopsy samples during maternal recognition of pregnancy in llamas (Lama glama). Reprod. Domest. Anim. 50 (6), 980–988. http://dx.doi.org/10.1111/rda.12618.

Bowen, J.A., Bazer, F.W., Burghardt, R.C., 1997. Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biol. Reprod. 56, 409–415. http://dx.doi.org/10.1095/BIOLREPROD56.2.409.

Bravo, P.W., 2002. Female reproduction. The Reproductive Process of South American Camelids. Seagull Printing, Salt Lake City, pp. 1–29.

Cauwe, B., Van den Steen, P.E., Opdenakker, G., 2007. The biochemical, biological, and pathological kaleidoscope of cell surface substrates processed by matrix metalloproteinases. Crit. Rev. Biochem. Mol. Biol. 42 (3), 113–185.

Clark, G.F., 2015. Functional glycosylation in the human and mammalian uterus. Fertil. Res. Pract. Oct. 23 (1), 17. http://dx.doi.org/10.1186/s40738-015-0007-0.

Constantinou, P.E., Morgado, M., Carson, D.D., 2015. Transmembrane mucin expression and function in embryo implantation and placentation. Adv. Anat. Embryol. Cell. Biol. 216, 51–68. http://dx.doi.org/10.1007/978-3-319-15856-3_4.

Di Rienzo, J.A., Casanoves, F., Balzarini, M.G., Gonzalez, L., Tablada, M., Robledo, C.W., 2008. InfoStat, Versión 2008. grupo InfoStat, FCA.

Diao, H., Aplin, J.D., Xiao, S., Chun, J., Li, Z., Chen, S., Ye, X., 2011. Altered spatio-temporal expression of collagen types I, III, IV, and VI in Lpar3-deficient peri-implantation mouse uterus. Biol. Reprod. 84 (2), 255–265. http://dx.doi.org/10.1095/biolreprod.110.086942.

Diskin, M.G., Morris, D.G., 2008. Embryonic and early foetal losses in cattle and other ruminants. Reprod. Domest. Anim. 43, 260–267.

Fernandez-Baca, S., Hansel, W., Saatman, R., Sumar, J., Novoa, C., 1979. Differential luteolytic effects of right and left uterine horns in the alpaca. Biol. Reprod. 20, 586–595.

Gipson, I.K., Blalock, T., Tisdale, A., Spurr-Michaud, S., Allcorn, S., Stavreus-Evers, A., Gemzell, K., 2008. MUC16 is lost from the uterodome (pinopode) surface of the receptive human endometrium: in vitro evidence that MUC16 is a barrier to trophoblast adherence. Biol. Reprod. 78, 134–142.

Hoffman, L.H., Olson, G.E., Carson, D.D., Chilton, B.S., 1998. Progesterone and implanting blastocysts regulate Muc1 expression in rabbit uterine epithelium. Endocrinology 139, 266–271.

Jensen, E.C., 2013. Quantitative analysis of histological staining and fluorescence using ImageJ. Anat. Rec. 296 (3), 378–381.

Johnson, G.A., Bazer, F.W., Jaeger, L.A., Ka, H., Garlow, J.E., Pfarrer, C., Spencer, T.E., Burghardt, R.C., 2001. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol. Reprod. 65, 820–828.

Kasimanickam, R., Kasimanickam, V., Kastelic, J.P., 2014. Mucin 1 and cytokines mRNA in endometrium of dairy cows with postpartum uterine disease or repeat breeding. Theriogenology 81 (7), 952–958. http://dx.doi.org/10.1016/j.theriogenology.2014.01.018. e2.

Luna, L.G., 1968. Manual of Histological Staining Methods of the Armed Forces Institute of Pathology. McGraw-Hill Book Company, New York, Toronto, London, Sydney.

Morgado, M., Dharmaraj, N., Carson, D., 2012. SUN-12: MUC16 stimulation by proinflammatory cytokines in the Immortalized Human Endometrial Epithelial Cell Line, IHEEC, Houston, TX. The Endocrine Society's 94th Annual Meeting and Expo, June 23–26, 2012.

Olivera, L.V.M., Zago, D.A., Jones, C.J.P., Bevilacqua, E., 2003. Develpmental changes at the materno-embryonic interface in early pregnancy of the alpaca, Lamos pacos. Anat. Embryol. 207, 317–331. http://dx.doi.org/10.1007/s00429-003-0346-1.

Olson, G.E., Winfrey, V.P., Matrisian, P.E., NagDas, S.K., Hoffman, L.H., 1998. Blastocyst-dependent upregulation of metalloproteinase/ disintegrin MDC9 expression in the rabbit endometrium. Cell. Tissue Res. 293, 489–498. http://dx.doi.org/10.1007/S004410051141.

Picha, Y., Tibary, A., Memona, M., Kasimanickama, R., Sumar, J., 2013. Chronology of early embryonic development and embryo uterine migration in alpacas. Theriogenology 79, 702–708.

Ren, Q., Guan, S., Fu, J., Wang, A., 2010. Temporal and spatial expression of Muc1 during implantation in sows. Int. J. Mol. Sci. 11, 2322–2335. http://dx.doi.org/10.3390/ijms11062322.

Shen, F., Yan, C., Liu, M., Feng, Y., Chen, Y., 2015. Decreased expression of mucin-1 in endometriosis endometrium correlated with progesterone receptor B involved in infertility. Arch. Gynecol. Obstet. 291 (2), 439–445. http://dx.doi.org/10.1007/s00404-014-3419-x.

Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., 2004. Focus on implantation. Implantation mechanisms: insights from the sheep. Reproduction 128, 657–668.

Sumar, J., Fredriksson, G., Alarcon, V., Kindahl, H., Edqvist, L.E., 1988. Levels of 15-keto-13, 14-dihydro-PFG2 alpha, progesterone and oestradiol-17 beta after induced ovulations in llamas and alpacas. Acta Vet. Scand. 29, 339–346.

Thathiah, A., Carson, D.D., 2004. MT1-MMP mediates MUC1 shedding independent of TACE/ADAM17. Biochem. J. 382, 363–373.

Vaughan, J., Mihm, M., Wittek, T., 2013. Factors influencing embryo transfer success in alpacas: a retrospective study. Anim. Reprod. Sci. 136, 194–204.

Wang, X., Zhu, B., Xiong, S., Sheng, X., Qi, X., Huang, Q., Chen, C., Guo, Y., Ni, H., 2017. Expression and function of MUC1 in uterine tissues during early pregnancy in sheep after natural oestrous or artificially-induced oestrous. Theriogenology 108, 339–347. http://dx.doi.org/10.1016/j.theriogenology.2017.12.030.

Wilsher, S., Sue Gower, A.B., Allen, W.R., 2013. Persistence of an immunoreactive MUC1 protein at the feto-maternal interface throughout pregnancy in the mare. Reprod. Fertil. Dev. 25, 753–761. http://dx.doi.org/10.1071/RD12152.

Zampini, R., Apichela, S., Sari, L., Angiono, G., Lombardo, D., Miceli, D., Argañaraz, M., 2017. Segment specific expression of MMP/TIMP in the oviduct of llama (Lama glama) and gelatinolytic activity in the oviductal fluid. Int. J. Morphol. 35 (2), 615–623.

Zampini, R., Argañaraz, M.E., Miceli, D.C., Apichela, S.A., 2014. Detection of the matrix metalloproteinases MMP-2 and MMP-9 and tissue inhibitors of metalloproteinases TIMP-1 and TIMP-2 in llama (Lama glama) oviduct. Reprod. Domest. Anim. 49 (3), 492–498. http://dx.doi.org/10.1111/rda.12317.