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

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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

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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