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Collagen and matrix metalloproteinase-2 and -9 in the ewe cervix during the estrous cycle

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ABSTRACT

The cervical collagen remodeling during the estrous cycle of the ewe was examined. The collagen concentration determined by a hydroxyproline assay and the area occupied by collagen fibers (%C), determined by van Gieson staining, were assessed in the cranial and caudal cervix of Corriedale ewes on Days 1 ($n = 6$), 6 ($n = 5$), or 13 ($n = 6$) after estrous detection (defined as Day 0). In addition, the gelatinase activity by *in situ* and SDS-PAGE gelatin zymographies and matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9, respectively) expression by immunohistochemistry were determined. The collagen concentration and %C were lowest on Day 1 of the estrous cycle ($P < 0.04$), when MMP-2 activity was highest ($P < 0.006$) and the ratio of activated to latent MMP-2 trend to be highest ($P = 0.0819$). The MMP-2 activity was detected in 73% of the homogenized cervical samples, and its expression was mainly detected in active fibroblasts. By contrast, the MMP-9 activity was detected in 9% of the samples, and its scarce expression was associated with plasmocytes, macrophages, and lymphocytes. Matrix metalloproteinase-2 expression was maximal on Day 1 in the cranial cervix and on Day 13 in the caudal cervix and was lower in the cranial than in the caudal cervix ($P < 0.0001$). This time-dependent increase in MMP-2 expression that differed between the cranial and caudal cervix may reflect their different physiological roles. The decrease in the collagen content and increase in fibroblast MMP-2 activity in sheep cervix on Day 1 of the estrous cycle suggests that cervical dilation at estrus is due to the occurrence of collagen fiber degradation modulated by changes in periovulatory hormone levels.

1. Introduction

The tortuous nature of the ovine cervix restricts transcervical artificial insemination and embryo transfer procedures [1–3]. However, natural cervical dilatation occurs at estrus [4], and many studies have examined the physiological mechanism of cervical dilatation for transcervical cannulation improvement [5,6]. Fibrillar collagen and high-molecular-weight proteoglycan complexes are the main components of the extracellular matrix (ECM) of the cervical connective tissue [7–9]. The biochemical interactions between these structural elements are critical to the cervical remodeling process that results in cervical dilation [10,11].

In the ewe, the proposed model for cervical dilatation at estrus involves a central role of periestrous endocrine changes that drive ECM remodeling processes and, consequently, cervical dilatation [4–6]. These periestrous endocrine changes include the preovulatory increase of estradiol and gonadotropins [4–6,12] and the activation of the

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prostaglandin E2/oxytocin (PGE2/Ox) system [13–15]. Cervical production of PGE2 stimulates smooth muscle relaxation and hyaluronan-like glycosaminoglycan (GAG) synthesis *via* an autocrine or paracrine mechanism, causing disaggregation of collagen fibers and cervical dilatation [16,17]. In other species, some evidence indicates that degradation of collagen fibers may also be involved in cervical dilatation. For example, a decrease in collagen content was measured chemically [18] and histologically in pregnant women at term [19]. The degradation of collagen in the ECM depends on the activity of matrix metalloproteinases (MMPs), which are the only enzymes capable of degrading denatured fibrillar collagen [20]. In particular, the expression of MMP-2 and -9 (also called gelatinases A and B, respectively) increases in the human cervix at the end of pregnancy [21], indicating a likely role of MMP-2 and -9 in the cervical dilatation process.

We hypothesized the coexistence of increased collagen fiber disaggregation and increased enzymatic collagen degradation in the sheep cervix around the estrus. The changes in the collagen content and distribution and in the MMP-2 and -9 abundance and activity along the cervix of the ewe during the estrous cycle were examined, particularly at the expected time of artificial insemination and embryo transfer (Days 1 and 6 after estrus, respectively).

2. Materials and methods

2.1. Animals and treatments

The experiment was carried out at the experimental field of the Veterinary Faculty of the University of Uruguay, Canelones, Uruguay (35°S), during the breeding season of Corriedale ewes (February through March). Animal experimentation was performed in compliance with regulations set by the Veterinary Faculty of the University of Uruguay. The adult Corriedale ewes were kept under natural daylight conditions. They grazed on native pastures and were given water *ad libitum*. Vasectomized rams fitted with marking crayons were kept with the ewes for 2 months before the start of the study to confirm the normal cyclic conditions of the ewes.

The estrus was synchronized with two doses of a PGF2α analogue (intramuscularly, 150 mg, Glandinex; Laboratorio Universal, Montevideo, Uruguay) administered 6 days apart. From Day 10 after the second PGF2α treatment, ewes were checked twice daily (at 6 and 18 hours) for service marks of two vasectomized rams carrying marking crayons (day of estrus = Day 0). Seventeen ewes (bodyweight, mean ± pooled standard error of the mean, 39.0 ± 1.1 kg) showing spontaneous estrus were slaughtered on Days 1 (n = 6), 6 (n = 5), or 13 (n = 6) after the estrus detection. The day of the estrous cycle for each animal was confirmed by concentrations of circulating estradiol-17β (E2) and progesterone [12].

2.2. Cervical samples, wet weight, and water content

The cervices were weighed and dissected at a temperature of 0 °C to 4 °C into three transversal segments of equal length labeled cranial, middle, and caudal cervical zones (2–2.5 g per cervical zone). The cranial and caudal cervical zones were longitudinally cut into four equal segments (500–600 mg/segment). One longitudinal segment for each cervical zone was used to determine the water content by drying until a constant weight was reached at 80 °C for 3 hours; this water content was expressed as percentage humidity (%). Another longitudinal segment was weighed, sliced, and homogenized in PBS buffer (1/10, wt/vol) with a Polytron Homogenizer (Polytron Homogenizer PT-10; Kinematica AG, Littau Luzern, Switzerland). Aliquots of homogenates were stored at −80 °C until the spectrophotometric and SDS-PAGE zymography assays were performed. The third longitudinal segment was immediately fixed by immersion in buffered 4% formaldehyde (pH 7.4) for 24 hours and then stored in 70% ethanol for 10 days. Fixed cervices were then dehydrated and embedded in paraffin until histochemistry and immunohistochemistry assays were performed. The fourth longitudinal segment was embedded in tissue-freezing medium without fixation and stored at −80 °C until the *in situ* zymography was performed.

2.3. Collagen and total soluble protein content determined by spectrophotometry

The collagen content was measured indirectly by a hydroxyproline assay adapted from Bannister and Burns [22]. Aliquots of frozen homogenates were hydrolyzed in constantly boiling hydrochloric acid (3 N) at 90 °C for 24 hours. After a partial neutralization (pH = 2.3) with 3-N NaOH, the hydrolyzed samples were exposed to an oxidizing agent (chloramine-T 7%: water: PBS buffer, 7:100:500, v:v:v) for 15 minutes at room temperature (RT). Under these conditions, hydroxyproline was liberated by acid hydrolysis and oxidized to a pyrrole, which then reacted with the color reagent (30 g of 4-dimethylaminobenzaldehyde, 45 mL of 60% perchloric acid, and 250 mL of propan-2-ol) at 70 °C for 15 minutes. The absorbance of the resulting colored product was read at 550 nm. Readings were calibrated against standards prepared from L-4-hydroxyproline (Fluka, 56250) dissolved in 0.01-N HCl (0.5–30 μg/mL, $r = 0.9976$, $P < 0.0001$). All samples were analyzed in a single assay, with a sensitivity of 0.5 μg/mL and intra-assay coefficient of variation of 7%. The collagen concentration was calculated assuming that the hydroxyproline/collagen ratio is 14% [23] and was expressed relative to dry tissue mass (mg/g of dry tissue).

The total soluble protein concentration in the aliquots of frozen homogenates was determined by the method of Lowry et al. [24], using BSA (Fraction V, Sigma Chemical, St. Louis, MO, USA) as the standard (0.05–0.8 mg/mL, $r = 0.9960$, $P < 0.0001$). All samples were analyzed in a single assay, with a sensitivity of 0.05 mg/mL and intra-assay coefficient of variation of 4%. The total soluble protein concentrations (mg/g of dry tissue) were positively correlated with the amount of tissue used ($r = 0.7123$, $n = 86$, $P < 0.0003$), showing that the total proteins extracted were similar among cervical samples.

2.4. Collagen distribution determined by van Gieson staining

Van Gieson's picrofuchsin stain was used to observe the connective tissue fibers in deparaffinized and rehydrated cervical sections (5 μm). Sections were stained with iron hematoxylin for 10 minutes, washed in running water for