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Oestrogen and progesterone receptor binding capacity and oestrogen receptor alpha expression (ERα mRNA) along the cervix of cycling ewes

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Abstract. The aim of the present work was to study the oestrogen receptor (ER) and progesterone receptor (PR) binding capacity and the oestrogen receptor alpha (ERα) mRNA concentration in cranial and caudal cervix during the ovine oestrous cycle. Cervical samples of synchronised Corriedale ewes were obtained on Day 1 ($n = 7$), 6 ($n = 6$) or 13 ($n = 7$) after oestrus detection (Day 0). The ER and PR binding capacity by ligand-binding assay and the ERα mRNA concentration by solution hybridisation in both cranial and caudal zones of the cervix were determined. The ER and PR binding capacity were higher ($P < 0.005$) on Day 1 than on Days 6 and 13 in both cranial and caudal zones. The ERα mRNA concentrations were higher ($P < 0.0001$) on Day 1 than on Days 6 and 13 only in the caudal zone. The PR binding capacity and ERα mRNA concentration were higher ($P < 0.005$) in the caudal than in the cranial zone on Day 1. The ER and PR expression in the ovine cervix varied during the oestrous cycle in agreement with the known upregulation exerted by oestrogen and downregulation exerted by progesterone. Differences in ER and PR expression along the longitudinal axis of the ovine cervix were found, reflecting histological and functional differences between the cranial and caudal zones.

Additional keyword: ewe cervical steroid receptors.

Introduction

The ewe cervix acts as a barrier against the passage of the transcervical instruments for artificial insemination (AI) (Lightfoot and Salamon 1970; Halbert *et al*. 1990*a*, 1990*b*; Campbell *et al*. 1996) and for embryo transfer (ET) procedures (Armstrong and Evans 1983; Kraemer 1989; Croy *et al*. 1999). Treatments with oestrogen combined with relaxin (Nemec *et al*. 1988), oxytocin (Khalifa *et al*. 1992; Flohr *et al*. 1999), or prostaglandin E2 (Barry *et al*. 1990; Mylne *et al*. 1992; McKelvey *et al*. 1997) have been performed to increase cervical dilatation and to facilitate the passage through the ovine cervix into the uterine lumen. Those studies suggest that cervical dilatation and penetrability varies along the oestrous cycle and are under the influence of the ovarian steroid hormones. In addition, the cervix of the ewe supports the passage of normal motile sperm by modifications of the physical and molecular composition of the cervical mucus (Lee *et al*. 1986), associated with cyclic changes in morphology and secretory capacity of the luminal epithelium (Moré 1984). These events are modulated by ovarian steroid hormones.

The genomic actions of oestrogen (E) and progesterone (P) depend on their circulating concentrations and on the target tissue's sensitivity in terms of their specific and high-affinity nuclear receptor concentrations (ER and PR, respectively) (Clark *et al*. 1992; Meikle *et al*. 2004). It is accepted that E upregulates ER and PR expression, whilst P downregulates both receptors (Ing *et al*. 1993; Couse *et al*. 2006). This regulation is consistent with the higher uterine ER and PR protein and ERα mRNA and PR mRNA concentrations at oestrus than in the luteal phase found in ruminants (Miller *et al*. 1977; Rexroad 1981; Vesanen *et al*. 1988; Ott *et al*. 1993; Meikle *et al*. 2001*a*; Tasende *et al*. 2005*b*). However, data on cervical ER and PR expression during the oestrous cycle are contradictory. Cyclic variations in ER and PR (Stanchev *et al*. 1984) and in ERα mRNA and ERα protein (Wang *et al*. 2000) concentrations were found in pig and rat, respectively. On the contrary, no variations in cervical ER concentrations were found in cow (Vesanen *et al*. 1991) and mare (Re *et al*. 1995), species in which PR concentration varies.

In the ewe, information about the cervical ER and PR binding capacity is scarce. The presence of the cervical ER binding protein in ovariectomised ewes (Tang and Adams 1981, 1986), as well as the cervical ER, PR, ERα mRNA, and PR mRNA in prepubertal lambs (Meikle *et al*. 2001*b*; Rodríguez-Piñón *et al*. 2005) have been previously demonstrated. On the other hand, during the postpartum period in the breeding season, the cervical ER and PR concentrations were low after parturition and increased in late postpartum, associated with the presence of the E-active large follicles in the ovarian surface (Rodríguez-Piñón *et al*. 2000). This suggests that changes in the cervical steroid receptors are related to the levels of ovarian steroid hormones. We are unaware of any reports of the cervical ER and PR binding capacity during the oestrous cycle in ewes. However, by immunohistochemical technique, both ER and PR were detected

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in the epithelium around the time of oestrus, but not in the luteal phase (Zhao *et al*. 1999). In addition, in bovine cervix, the ER and PR immunoreactivity increased from the cranial to the caudal region (Breeveld-Dwarkasing *et al*. 2000, 2002), suggesting that the distribution of these steroid receptors could be different along the longitudinal axis of the ruminant cervix.

The aim of the present work was to study the ER and PR binding capacity and the ERα mRNA concentration of the cranial and caudal ovine cervix in relation to levels of circulating E and P during the oestrous cycle, including the days when AI and ET would be performed (Days 1 and 6 after oestrus, respectively).

Materials and methods

Experimental design

The experiment was performed at the experimental field of Veterinary Faculty, Canelones, Uruguay (35°S), during the breeding season of Corriedale ewes (February to March). Animal experimentation was performed in compliance with regulations set by the Veterinary Faculty, University of Uruguay.

Twenty adult Corriedale ewes (bodyweight, mean ± pooled s.e.m., 39.1 ± 0.85 kg) were synchronised with two doses, 6 days apart, of a prostaglandin F2α analogue intra-muscularly (i.m.) (150 μg, Glandinex, Laboratorio Universal, Montevideo, Uruguay). From Day 10 of the first oestrous cycle, ewes remained with two vasectomised rams with marking crayons and were checked twice a day (at 600 hours and 1800 hours) for service marks indicative of oestrus (day of oestrus = Day 0). All animals were located under natural daylength, grazed on native pastures and given water *ad libitum*. The ewes were killed on Day 1 ($n = 7$), 6 ($n = 6$) or 13 ($n = 7$) after oestrus detection. The whole cervices were dissected into three equal length segments named cranial, middle and caudal zones and including all the histological layers. The cranial (next to the uterus) and caudal (next to the vagina) zones were selected for the present study. The tissues were frozen in liquid nitrogen and stored at −80°C until receptor and transcript determinations were carried out. The number of ruptured follicles and corpus luteum (CL) present in the ovarian surface were recorded.

For circulating P determination, blood samples were collected daily from Day −1 of expected oestrus until the killing time in the second oestrous cycle. For oestradiol-17β (E_2) determinations, the samples were collected every 8 h during the 48 h period before the killing time. The samples were centrifuged (900*g* for 15 min at 4°C) within the first hour after collection and serum was stored at −20°C until hormone assays were performed.

Hormone determinations by radioimmunoassay (RIA)

Progesterone concentrations were assayed by direct solid-phase ^{125}I radioimmunoassay (RIA) method (Count-A-Count TKPG; Diagnostic Products Corporation, Los Angeles, CA, USA), according to the manufacturer's instructions and as previously described for sheep (Garófalo and Tasende 1996). All samples were performed in duplicate in the same assay. The sensitivity of the assay was 0.1 nM, and the intra-assay coefficient of variation was less than 10%.

For the E_2 assay, the serum samples were extracted with diethyl ether and assayed in duplicate with ^{125}I RIA (oestradiol double antibody, KE2D; Diagnostic Products Corporation) as previously described for sheep (Meikle *et al*. 1998; Tasende *et al*. 2002). All samples were determined in the same assay. The detection limit of the assay was 4.0 pM and the intra-assay coefficients of variation for three control samples were 25% (7 pM), 8% (44 pM), and 6% (122 pM).

Steroid receptors by binding assay

Ligand-binding assays for ER and PR were performed in soluble fraction of cervix as described previously (Garófalo and Tasende 1996; Rodríguez-Piñón *et al*. 2000). The term 'soluble fraction' refers to the supernatant fractions of tissue homogenates after a high-speed centrifugation, and does not imply cellular receptor localisation. Unless otherwise stated, the reagents were obtained from Sigma Chemicals (St Louis, MO, USA). The frozen cervical samples (300 to 500 mg) were sliced and homogenised in Tris buffer with a Polytron homogenizer (Polytron homogenizer PT-10, Kinematica AG, Littau Luzern, Switzerland). The soluble fractions were separated by a first centrifugation at 1000*g* for 15 min and then at 40 000*g* for 90 min. This and subsequent procedures were carried out at 0–4°C. The soluble fractions, in duplicate, were incubated with five to six increasing concentrations of ^{3}H-E_2 (86 Ci mmol^{-1}; 0.15–15 nM) or ^{3}H-ORG-2058 (40 Ci mmol^{-1}; 0.5–30 nM) (Amersham International, Buckhinghamshire, England), for determination of the total bound ^{3}H-labelled ligands. Identical duplicate samples were incubated with 200-fold molar excess of either unlabelled diethylstilbestrol or unlabelled ORG-2058, for determination of non-specifically-bound ^{3}H-labelled ligands. After 18 h incubation free hormones were removed and radioactivity was measured by liquid scintillation counting. Specific binding data were obtained by subtracting non-specific binding from total binding. A linear regression test of inverse Scatchard model analysis (Braunsberg 1984) was used to obtain the apparent dissociation constant (K_d, nM) and the concentration of receptor sites expressed as fmol mg^{-1} proteins. Protein concentrations were determined by the method of Lowry *et al*. (1951). The protein concentration was positively correlated with the amount of tissue used in the receptor assay, showing that the protein extraction procedure was similar in the different cervical samples.

ERα mRNA by solution hybridisation assay

The method used was previously validated for cervical ovine tissue (Meikle *et al*. 2001*b*). The hybridisation probe used was derived from plasmids containing 360 bp cDNAs from the ovine ERα, generously supplied by Dr N. Ing, Texas A&M University, TX, USA (Ing *et al*. 1996). Total nucleic acids (TNA) were obtained by digesting homogenised (Polytron homogenizer PT-10, Kinematica AG) cervical tissues (200–250 mg) with proteinase K in a buffer containing sodium dodecyl sulfate (SDS) followed by subsequent extraction with phenol-chloroform. The TNA content in the samples was determined spectrophotometrically at 260 nm and expressed as absorbance relative units (ARU). The ^{35}S-UTP-labelled cRNA was hybridised overnight at 70°C to TNA samples. The hybridisations were performed in

duplicates at two different concentrations in 40 μL of hybridisation formamide buffer under two drops of paraffin oil. After hybridisation, samples were treated with 1 mL of RNase buffer containing 40 μg RNase A, 118 U RNase T1 (Boeringer-Mannheim, Mannheim, Germany), and 100 μg calf thymus DNA for 45 min at 37°C to digest unhybridised RNA. Labelled hybrids protected from RNase digestion were precipitated with trichloroacetic acid and collected on filters (Whatman GF/C, Whatman Nederland B.V., Hertogenbosch, The Netherlands). Radioactivity was determined in a liquid scintillation counter. All samples from the experiment were determined in the same assay and the intra-assay CV was 16%. The ERα mRNA concentrations were expressed as amol ARU^{-1}.

Statistical analysis

The ERα mRNA, ER and PR concentrations and K_d values were analysed by analysis of variance using a mixed model (Statistical Analysis Systems; SAS Institute, Cary, NC, USA) that included the fixed effects of day of oestrous cycle (Day 1, 6 or 13), cervical zone (cranial or caudal), and their interaction. Progesterone and E_2 serum concentrations were analysed by repeated-measures (Mixed Proc), and the statistical model included the effects of time of sampling, day of oestrous cycle, and their interaction. The correlation procedure available in SAS was used to study the relationship between ER, PR, ERα mRNA, and hormone concentrations. For the correlations between receptor and hormone concentrations, the mean of the E_2 or P concentrations pooled samples of 24, 16, 8, and 0 h (for E_2), and 24 and 0 h (for P) before the killing time, were considered. The results were expressed as the least square mean ± pooled s.e.m. The level of significance was $P < 0.05$, except where otherwise specified.

Results

Structures on the ovarian surface and hormone concentrations

All ewes killed on Day 1 had one ruptured follicle and those killed on Days 6 and 13 had one corpus luteum on the ovarian surface. There was a significant effect of time of sampling on P serum concentrations ($P < 0.0001$). Progesterone concentrations were low from Day −1 to Day 1 after oestrus, and then increased significantly starting from Day 3 to Day 13 (Fig. 1). There was a significant effect of time of sampling on E_2 serum concentrations ($P < 0.001$). Maximum E_2 concentrations were found between 24 and 8 h before oestrus detection. After this time E_2 concentrations decreased and remained at basal levels (Fig. 1).

Steroid receptor binding capacity

A single, saturable and high-affinity binding site for E and P were found in all samples. The K_d values were not affected by day of cycle or cervical zone. The K_d means (± pooled s.e.m., $n = 40$, nM) for ER and PR were 0.56 ± 0.06 and 1.04 ± 0.07, respectively.

There was a significant effect of day of oestrous cycle on ER concentrations ($P < 0.005$), but there was no effect of cervical zone. The ER concentrations were higher on Day 1 than on Days 6 and 13 in both cranial and caudal zones (Fig. 2*a*). There were no differences in ER concentrations between cranial and caudal zones on Days 1 and 6, while on Day 13 ER concentration tended to be lower ($P = 0.054$) in the cranial than in the caudal zone (Fig. 2*a*). The ER concentrations were positively correlated with E_2 concentrations only in the caudal zone ($r = 0.48$, $n = 20$, $P < 0.05$), and were negatively correlated with P concentrations in both cranial ($r = -0.43$, $n = 20$, $P < 0.05$) and caudal zones ($r = -0.69$, $n = 20$, $P < 0.001$).

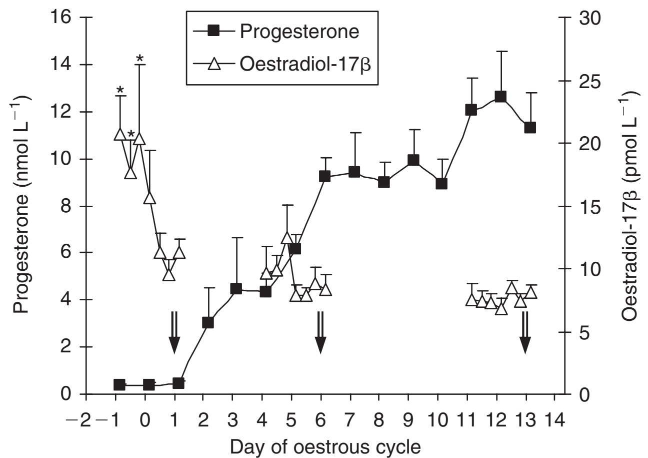

Fig. 1. Serum concentrations (mean ± pooled s.e.m.) of P (nM) and E_2 (pM) of ewes killed (arrows) on Days 1 ($n = 7$), 6 ($n = 6$) or 13 ($n = 7$) after oestrus (Day 0). Values marked with an asterisk differ significantly ($P < 0.001$).

There was a significant effect of day of oestrous cycle ($P < 0.0001$) and cervical zone ($P < 0.0004$) on PR concentrations, but there was no interaction between them. The PR concentrations were higher on Day 1 than on Days 6 and 13 in both cranial and caudal zones (Fig. 2*b*). The PR concentrations were lower in the cranial than in the caudal zone on Day 1 (Fig. 2*b*). In both cervical zones, PR concentrations were positively correlated with E_2 concentrations (cranial zone, $r = 0.58$, $n = 20$, $P < 0.01$; caudal zone, $r = 0.44$, $n = 20$, $P < 0.05$), and negatively correlated with P concentrations (cranial zone, $r = -0.76$, $n = 20$, $P < 0.0001$; caudal zone, $r = -0.70$, $n = 20$, $P < 0.0005$).

Independently of the day of oestrous cycle, there was a positive correlation between ER and PR concentrations in both cranial ($r = 0.52$, $n = 20$, $P < 0.01$) and caudal zones ($r = 0.80$, $n = 20$, $P < 0.00001$), indicating a positive relationship between the expression of these two receptors.

ERα mRNA concentrations

There was a significant effect of day of oestrous cycle ($P < 0.0001$), cervical zone ($P < 0.005$), and interaction between them ($P < 0.05$) on ERα mRNA concentrations. In the caudal zone, ERα mRNA concentrations were higher on Day 1 than on Days 6 and 13 (Fig. 3). In contrast, no differences between days were found in the cranial zone (Fig. 3). The ERα mRNA concentrations on Day 1 were lower in the

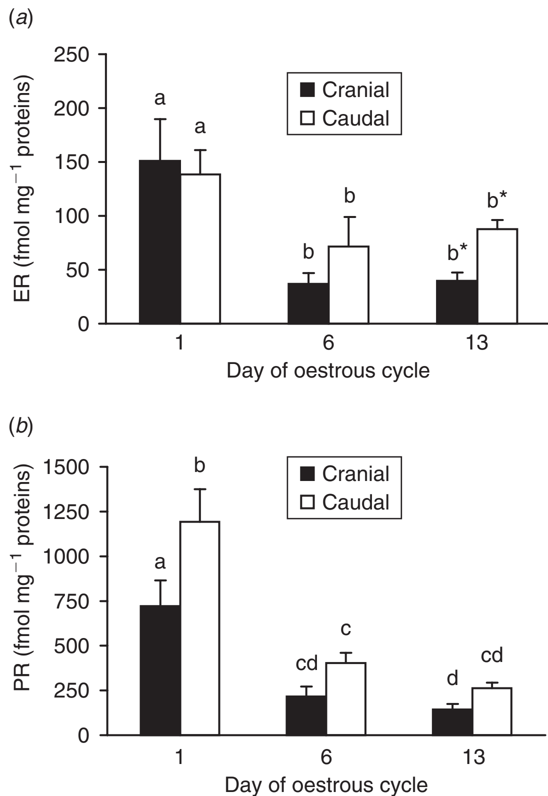

Fig. 2. Concentrations (fmol mg^{-1} proteins, mean ± pooled s.e.m.) of oestrogen receptor (ER, panel *a*) and progesterone receptor (PR, panel *b*) binding proteins in cranial and caudal cervical zones of ewes killed on Days 1 ($n = 7$), 6 ($n = 6$) or 13 ($n = 7$) after oestrus (Day 0). Bars marked with different letters differ significantly within days and zones ($P < 0.005$) and bars marked with asterisks tended to be different ($P = 0.054$).

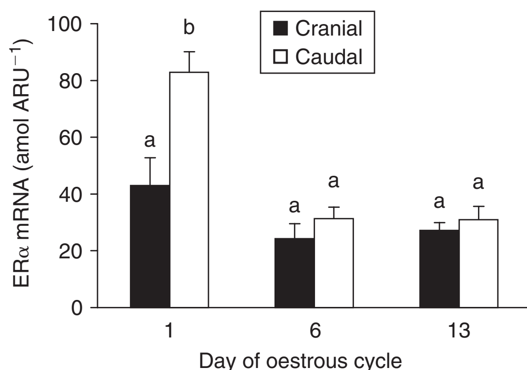

Fig. 3. Oestrogen receptor α messenger RNA (ERα mRNA) concentrations (amol ARU^{-1}, mean ± pooled s.e.m.) in cranial and caudal cervical zones of ewes killed on Days 1 ($n = 7$), 6 ($n = 6$) or 13 ($n = 7$) after oestrus (Day 0). Bars with different letters are significantly different within days and zones ($P < 0.05$).

cranial than in the caudal zone (Fig. 3). The ERα mRNA concentrations in the caudal zone were correlated positively with E_2 ($r = 0.59, n = 20, P < 0.01$) and negatively with P concentrations ($r = -0.74$, $n = 20$, $P < 0.0005$). There were no correlations between ERα mRNA and E_2 and P concentrations in the cranial zone. There was a positive correlation between ERα mRNA and ER concentrations only in the caudal zone ($r = 0.52$, $n = 20$, $P < 0.05$).

Discussion

In the present work we demonstrated that the expression of ER and PR in the ovine cervix varied during the oestrous cycle as well as along the longitudinal cervical axis.

High-affinity receptor binding proteins for oestrogen and progesterone were found in all cervical samples studied. The K_d values for ER and PR in all days of the oestrous cycle and all cervical zones studied were similar, suggesting that variations in the sensitivity of the ovine cervix to E and P may not depend on changes of affinity of the receptors, but rather on their binding capacity (ER and PR cervical concentrations). The cervical ER and PR K_d values were similar to those found in adult (Rodríguez-Piñón *et al*. 2000) and prepubertal ewes (Meikle *et al*. 2001*b*; Rodríguez-Piñón *et al*. 2005), as well as in other E and P ovine target tissues (Garófalo and Tasende 1996; Meikle *et al*. 2000; Tasende *et al*. 2005*a*, 2005*b*).

The cervical ER and PR concentrations were higher on Day 1 than on Days 6 and 13 of the oestrous cycle. This steroid receptor pattern is consistent with the stimulatory effect of the high E_2 levels found previous to oestrus and the inhibitory effect of the high P levels found during the luteal phase. The present findings in ovine cervix are in agreement with the E upregulation and P downregulation of the ER and PR expression found in ovine uterus during the oestrous cycle (Miller *et al*. 1977; Rexroad 1981; Ott *et al*. 1993; Tasende *et al*. 2005*b*). The existence of this ovarian steroid hormone regulation on the expression of ER and PR in ovine cervix during the oestrous cycle was confirmed by the positive correlation found between E_2 levels and ER (at least in caudal zone) and PR concentrations, and the negative correlation between P levels and ER and PR concentrations.

Similar cyclic variations in cervical steroid receptor expression during the oestrous cycle were also reported for ER and PR in the pig (Stanchev *et al*. 1984) and for ERα in the rat (Wang *et al*. 2000). On the contrary, cyclic variations in cervical ER concentration were not found in cows (Vesanen *et al*. 1991) or mares (Re *et al*. 1995), although the cervical PR concentrations in the same animals were higher in the non-luteal than in the luteal phase. In ovine cervix, the epithelial ER and PR immunoreactivity were detected in the first three days of the oestrous cycle, but were not detected during the luteal phase (Zhao *et al*. 1999). In a preliminary study we immunolocalised the ERα in the cervical epithelium of cycling ewes, and the percentage of ERα positive cells was higher around the oestrus than in the luteal phase (M. Rodríguez-Piñón, P. Genovese, R. González, P. Puime and A. Bielli, unpubl. data).

The pattern of variation of the cervical ER and PR binding capacity during the oestrous cycle was similar to that found for the oxytocin receptor (OxR) binding capacity, with a maximum seen around the oestrus (Matthews and Ayad 1994). These results suggest that one of the E and P target genes in the cervix could be the OxR, since the sex ovarian hormones are important regulators of the OxR gene (Gimpl and Fahrenholz

2001). The ovarian steroid hormones could modulate several oxytocin effects on the cervix of cycling ewes by up or down-regulation of the OxR. Since oxytocin stimulates the release of prostaglandin E2 from the cervical mucosa of perioestrous cows (Fuchs *et al.* 2002), some of the oxytocin effects on the ruminant cervix could be mediated via paracrine release of prostaglandin. Additionally, E could also directly modulate the activity of prostaglandin E2, altering the ratio of content between their different receptor subtypes, as was demonstrated in cervix of ovariectomised ewes (Schmitz *et al.* 2006). The fact that the *post-mortem* depth of cervical penetration was greater in non-luteal than in luteal phase ewes (Kershaw *et al.* 2005) could suggest that a degree of natural cervical relaxation at oestrus may be a consequence of the previous high circulating E_2 levels acting via its nuclear receptor. The return of the cervical ER and PR binding capacity to the minimal values on Day 6 of the oestrous cycle, as well as the decrease in the cervical oxytocin receptor binding capacity early in the luteal phase (Matthews and Ayad 1994), suggests that the increment in cervical penetrability using these hormones could be less successful at the ET than at the AI time.

The caudal zone showed higher ERα mRNA concentration on Day 1 of the oestrous cycle than during the luteal phase, while no differences in the ERα mRNA concentration during the oestrous cycle was found in the cranial zone. The ERα mRNA concentrations in the caudal zone were positively correlated with the E_2 and negatively correlated with the P circulating levels, suggesting that the E_2 upregulation and P downregulation of the ERα are predominately at transcriptional level. The temporal association between cervical ERα mRNA and ER protein concentrations are in agreement with the results found for the ovine uterus (Ott *et al.* 1993), and support the hypothesis of a transcriptional mechanism to control the ERα expression in the caudal zone. The lack of relationship found between the ERα mRNA and ER protein concentrations in the cranial zone suggest that the expression of ERα could be regulated in a different manner, depending on the cervical zone. Recently, no differences in the ERα mRNA concentrations in the follicular and the luteal phases were found, but the cervical zone studied was not indicated (van Lier *et al.* 2006).

Interestingly, on Day 1 of the oestrous cycle, both ERα mRNA and PR concentrations were higher in the caudal than in the cranial zone, suggesting a differential level of ER and PR expression along the longitudinal axis of the cervix. This could reflect histological (Moré 1984) and functional (Hawk *et al.* 1978) differences between cranial and caudal regions of the ovine cervix. For example, the cranial cervix of the ewe appears to be more critical than the caudal cervix for sperm transport (Hawk *et al.* 1978). Similarly, the percentage of ER and PR immunopositive cells increases from the uterine to the vaginal ends of the bovine cervix (Breeveld-Dwarkasing *et al.* 2002). This gradient of positive ER and PR cells along the cervix was associated with regional differences in cell density (Breeveld-Dwarkasing *et al.* 2000) and collagen content (Breeveld-Dwarkasing *et al.* 2003). These findings are relevant for the interpretation of results obtained from *in vivo* sampling of the cervix, when only one region of the cervix can be reached or when different sampling regions are compared. The differential sensitivity to the ovarian steroid hormones in cranial and caudal zones of the ovine cervix could be relevant to the development of localised hormonal treatments for the increase of cervical penetrability.

In conclusion, the expression of ER and PR in the ovine cervix is higher at oestrus, decreases during the luteal phase, and is higher in the caudal than in the cranial cervix, reflecting their histological and functional differences.

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