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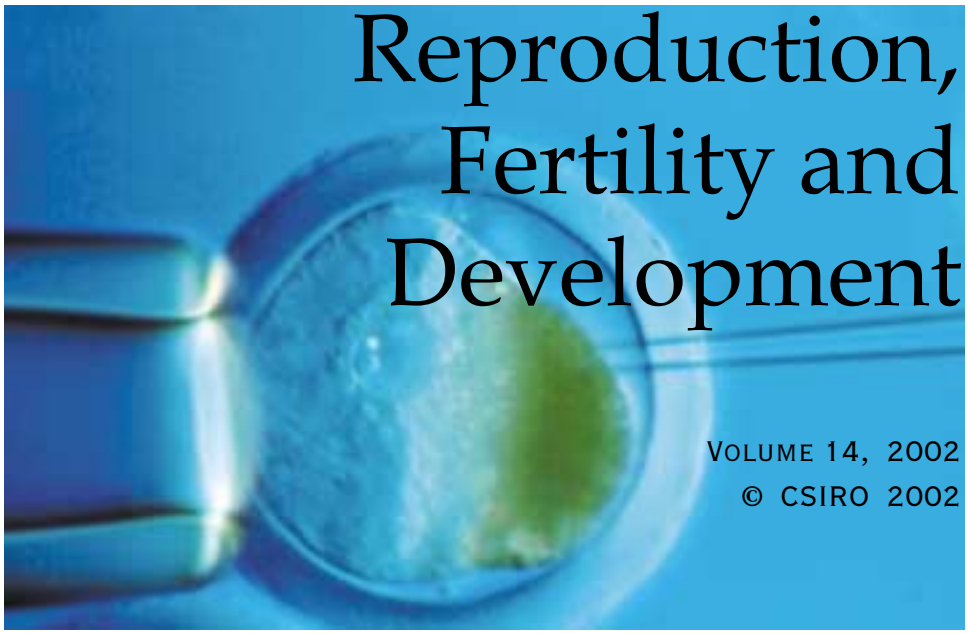

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Immunohistochemical distribution of oestrogen and progesterone receptors and tissue concentrations of oestrogens in the cervix of non-pregnant cows

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Abstract. An immunohistochemical study of the expression of oestrogen (ER) and progesterone receptors (PR) in different regions along the longitudinal and vertical axes of the cervix of non-pregnant cows was performed. Animals were separated into two groups depending on the presence or absence of a functional corpus luteum in their ovaries, as indicated by blood progesterone concentrations. The high progesterone group (HP4) had serum progesterone concentrations >2.0 ng mL^{-1} (n = 6) and the low progesterone group (LP4) had serum progesterone concentrations ≤0.5 ng mL^{-1} (n = 4). Significantly higher concentrations of oestrogen were found in the cervical tissue of animals in the LP4 group than those in the HP4 group (473 ± 53 *v.* 149 ± 46 pg g^{-1} wet weight; P<0.01). Furthermore, there was a significant effect of tissue layer (epithelium to deep stroma) on the number of ER (P<0.01) and PR (P<0.05) immunoreactive nuclei per 1000 cells. For both ER and PR the proportion of cells expressing the receptor increased from epithelium to subepithelial stroma (P<0.01) and from subepithelium to deep stroma (ER P<0.05; PR P=0.061). When the number of receptor-positive cells were expressed per mm^2 tissue, differences between the subepithelial stroma and the deep stroma became even more marked. In addition, the vaginal part of the cervix had significantly more (P<0.01) ER and PR immunoreactive nuclei per 1000 cells than the uterine part, but these differences were no longer apparent when a correction was made for cell density. There was no relationship between progesterone status of the animals, nor local tissue oestrogen concentrations and ER or PR immunoreactivity in the cervix of these non-pregnant cows. Instead, a strong relationship between both longitudinal and vertical positioning of tissue in the cervix and expression of both receptor types was shown. In addition, a strong correlation between ER and PR expression in the subepithelial stroma (R = 0.85, P<0.01) and the deep stroma (R = 0.83, P<0.01) was evident. In conclusion, these results demonstrate that in studies of steroid hormone receptor expression in the cervix, careful description of sampling site and depth are necessary if the results are to be interpreted meaningfully.

Introduction

The presence of oestrogen and progesterone receptors (ER and PR) in the cervix, and the characteristic changes that this organ undergoes during the oestrous cycle and pregnancy, have identified it as a target for both of the important reproductive steroid hormones. Moreover, the expression of ER and PR in the cervix has been reported to vary according to the stage of the oestrous cycle in sheep (Zhao *et al.* 1999), dogs (Vermeirsch *et al.* 1999; Vermeirsch *et al.* 2000) and rats (Wang *et al.* 2000). Similarly, in non-pregnant cows (Vesanen *et al.* 1991; Vesanen 1993) and horses (Re *et al.* 1995), concentrations of cervical cytosolic PR, but not ER, have been reported to be suppressed by high serum progesterone levels. In contrast, there appears to be no clear relationship between immunohistochemically detected ER or PR in the cervix and the stage of the menstrual cycle in women (Kupryańczyk and Möller 1988; Cano *et al.* 1990; Kupryańczyk 1991; Snijders *et al.* 1992).

It has also been reported that expression of both ER and PR in the cervix differs in different tissue layers and cell types (Kupryańczyk and Möller 1988; Cano *et al.* 1990; Snijders *et al.* 1992; Zhao *et al.* 1999) and even in different regions along the longitudinal axis (Kupryańczyk and Möller 1988; Cano *et al.* 1990), although these reported differences were based on semiquantitative or qualitative descriptions. Interestingly, regional differences in the

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expression of the oxytocin receptor within the bovine cervix also vary with the changing steroid environment (Fuchs *et al.* 1996). Finally, our recent observations that the mucosa of the bovine cervix is divided into two stromal layers that are clearly different in collagen biochemistry (V. N. A. Breeveld-Dwarkasing, M. de Boer-Brouwer, J. M. te Koppele, R. A. Bank, G. C. van der Weijden, M. A. M. Taverne and F. M. F. van Dissel-Emiliani, unpublished data) and cell composition (Breeveld-Dwarkasing *et al.* 2000), suggest that regional differences in the expression of steroid hormone receptors are likely. These findings, and the reports of cyclical and regional differences in receptor expression in other species, led us to investigate whether there is a relationship between serum progesterone concentrations and the expression of ER and PR in different regions of the bovine cervix. Immunohistochemistry was used to assess receptor expression in the epithelium, subepithelial stromal and deep stromal layers of the vaginal, middle and uterine segments of the cervix obtained at a slaughterhouse, of cows with either high or low serum progesterone concentrations. Because it is difficult to identify cows that are in oestrous at the time of slaughter under such conditions, local tissue oestrogen levels were also determined in the different sections of cervical tissue to find additional confirmation of the endocrinological status of the animals and to examine whether it affected ER or PR expression.

Materials and methods

Sample collection

Cervices were recovered from non-pregnant Holstein Friesian cows after slaughter. Immediately after stunning, jugular vein blood samples were collected from each cow for determination of the progesterone concentrations by means of a validated, direct solid-phase ^{125}I RIA (Dieleman and Bevers 1987) with a sensitivity of 47 pg mL^{-1}. The inter-assay coefficient of variation (CV) was 11% (n = 16) and the intra-assay CV was 7.5% (n = 20). Based on the serum progesterone (P4) concentrations, the cows were divided into two groups: (*i*) the low progesterone group (LP4) with serum P4 concentrations ≤0.5 ng mL^{-1} (n = 4); and (*ii*) the high progesterone group (HP4) with serum P4 concentrations >2.0 ng mL^{-1} (n = 6). Care was taken to ensure that the progesterone concentrations correlated with ovarian morphology (presence, size and texture of the corpus luteum and follicles) and the macroscopic features of the cervix (such as abundant clear mucoid discharge). Circular slices were cut from the vaginal (V), middle (M) and uterine (U) segments of each cervix (Fig. 1*a*) and, from these slices, smaller wedge-shaped pieces were cut, fixed in 4.5% formaldehyde for 48 h and then embedded in paraffin. Furthermore, tissue samples from each region were snap-frozen in liquid nitrogen and stored at –80°C until assessed for their concentrations of oestrogens (discussed later).

Local tissue oestrogen concentrations

The frozen samples of the superficial layer (S, epithelium and subepithelial stroma) and the deeper stromal layer (D) (Fig. 1*b*) from the three cervical segments (V, M, U) were used for the analysis of tissue oestrogen concentrations. As the cervical tissue was quite hard and could not be homogenized using a potter, the samples were digested using enzymes before extraction. After chopping 0.2 g of the tissue in an Eppendorf cup using a pair of scissors, 100 mL of water was added and the mixture was heated at 95°C for 10 min to destroy steroid-converting enzymes. Next, 1 mL of Hanks buffer containing 4 mg mL^{-1} collagenase, 1.6 mg mL^{-1} dispase and 0.24 mg mL^{-1} DNAse, which was filtered through Sep pak R C18 cartridges (Waters®, Millford, MA, USA) to remove any interfering substances, was added and the samples were incubated for 22 h at 37°C. After incubation, the samples were centrifuged (5 min, 9000*g*) and 1 mL of the supernatant was transferred to a fresh tube. The pellet was resuspended in 100 mL of methanol and then vortexed, centrifuged and resuspended once more in 100 mL of methanol, with the second supernatant having been added to that from the first centrifugation. Next, an additional 2 mL of water was added and the aqueous phase was re-extracted using Sep pak R C18 cartridges. After priming and extraction, the steroids were removed from the cartridge using 80% methanol. After evaporation and redissolving in assay buffer, the oestrogen concentration was measured using a biotin-streptavidin EIA kit that used a sheep anti-17β oestradiol-17-HS antibody, as described by Palme and Moestl (1993). These investigators reported cross-reactivity for this antibody of 100% with oestrone, 70% with oestradiol 17-β, 19% with oestradiol 17-α and <0.01% with oestradiol-sulfate. The sensitivity of the assay was 0.7 fmol/well and the inter- and intra-assay CVs were 14.3% (n = 50) and 12.4% (n = 20) respectively.

To determine which oestrogen reacted in the tissue extracts, we performed straight phase high performance liquid chromatography (Silica gel 60 Seibersdorf (10 m), 29 cm × 0.4 cm, starting solvent *n*-hexane/chloroform 50/50 for 25 min, followed by a linear increase of methanol to 10% methanol for 30 min; flow rate 2 mL min^{-1}). The elution time of oestrone was 9 min, of oestradiol 17-α was 18 min and of oestradiol 17-β 21 min.

After chromatography the individual fractions were collected, evaporated and redissolved in assay buffer. The EIA was used to measure steroid concentrations in the collected fractions.

Immunolocalization of oestrogen and progesterone receptors

The mouse anti-human ERα antibody (clone ID5) and the mouse anti-human PR antibody (clone 10A9) were both obtained from BioGenex® (San Ramon, CA, USA). The anti-ERα was a ready-to-use

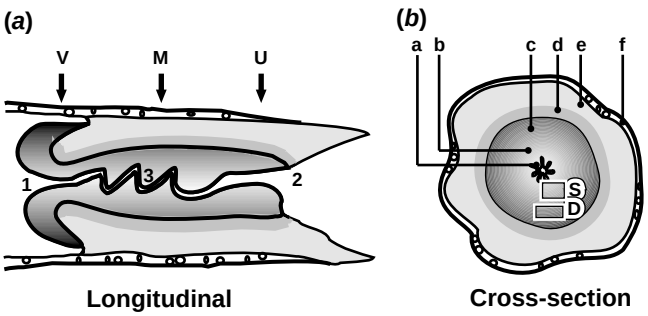

Fig. 1. Schematic diagrams of the bovine cervix. (*a*) Longitudinal section of the cervix. V, Vaginal; M, mid; U, uterine segment. 1, Portio vaginalis (external os); 2, uterine lumen; 3, cervical canal. (*b*) Cross-section of the cervix. Samples from the superficial stromal (S) and deep (D) stromal layer at each segment were used for the analysis of the concentration of total oestrogens. Oestrogen (ER) and progesterone receptor (PR) expression was assessed in: a, the epithelium; b, the superficial loose stromal band; and c, the deep dense collagenous layer of each segment. The other layers are the d, circular-oriented and e, longitudinally oriented muscle layer and f, the serosal lining with a thin layer of very loose connective tissue and blood vessels underneath.

solution and the PR was diluted 1:50 with BSA–C/TBS (50 µL of a 10% acetylated bovine serum albumin, diluted in 10 mL Tris-buffered saline) before use. Three 5-µm thin sections, 100 µm apart, were cut from wedges obtained at each cervical segment and mounted on a single, coated glass slide (Superfrost® plus, Erie Sc. Co. Portsmouth, NH, USA). Sections were deparaffinized with xylene and rehydrated with ethanol containing increasing concentrations of water, and finally in deionised water. Endogenous peroxidase activity was quenched with 3% peroxide in deionised water. To expose the antigens, sections were microwaved for three 5-min periods at 780 Watts in a household microwave and, thereafter, 10% normal goat serum was used as a blocking antibody. The sections were incubated with the anti-ER or -PR antibodies overnight at 4°C after which a biotinylated goat anti-mouse second antibody was applied to the sections, which were incubated for a further 1 h. Next, the slides were incubated for 1 h with streptavidin-biotin (ABC-elite kit) and, finally, 3,3′-diaminobenzidine tetrahydrochloride (DAB) was used to visualize the immunoreaction and the slides were counterstained with Mayers hematoxylin. For negative controls, sections of the cervix were incubated with normal mouse serum instead of the primary antibody.

Proportional scores of oestrogen and progesterone receptors (*number of positive nuclei per 1000 cells*)

The number of nuclei that stained positively for ER or PR in 1000 cells was counted at a magnification of 400× in three different layers along the cross-sectional axis. These three layers were: (*i*) the single-lined surface epithelium, not including the deeper epithelial crypts; (*ii*) the subepithelial stroma; and (*iii*) the deeper stroma. In each of the three tissue sections mounted on each glass slide, approximately 333 cells were counted to make a total of one thousand cells per layer. Any nucleus showing evidence of the DAB reaction product was considered to be positive irrespective of the intensity of DAB staining. In this way, a proportional score of the receptor-positive nuclei (ER or PR immunoreactive nuclei per 1000 cells) was obtained for the three different layers and the three different segments of the cervix for all of the animals in both groups.

Cell density and number of oestrogen and progesterone nuclei per mm²

The cell density of the subepithelial stromal layer was compared with that of the deep stromal layer by counting all fibroblast and muscle cell nuclei in an area equivalent to 2 mm² of the tissue section, using a 1 cm² grid at 400× magnification. The mean number of cells per mm² was calculated from triplicate countings. These numbers were then used to estimate the number of ER and PR immunoreactive nuclei per mm² (ER per mm² and PR per mm²) using the following formula:

$$\text{Number of ER or PR immunoreactive nuclei per mm}^2 = (A/1000 \times 100\%) \times B$$

where A is the number of immunoreactive nuclei per 1000 cells and B is the number of cells per mm².

Statistical analysis

All the data are expressed as least squares means (LSM) ± SEM. The proportion of ER- and PR-positive nuclei in the epithelial, subepithelial stromal and deep stromal layers, and the number of ER- and PR-positive cells per mm² of the subepithelial stromal and deep stromal layers, were analysed using the General Linear Models procedure of SAS (1990). The same model was used to examine whether there was a relationship between cell density and location within the cervix or between cell density and progesterone status. The differences between HP4 and LP4 were tested against the variation within animals, nested within progesterone level. Non-significant interactions were removed from the model and significant interactions were studied in more detail. In addition, the correlation between the number of ER per mm² and PR per mm², and between receptor expression per mm² and the local oestrogen concentrations in the different sites were examined using Pearson's test. Differences were accepted to be statistically significant if $P<0.05$ and were described as a tendency if $0.05<P<0.10$.

Results

Serum P4 concentrations and local tissue concentrations of oestrogens

The serum P4 concentrations ranged from 2.1 to 5.7 ng mL^{-1} in the HP4 group and from 0.1 to 0.5 ng mL^{-1} in the LP4 group. High performance liquid chromatography analysis of cervical tissue demonstrated that oestrone and oestradiol 17-α were present in significant quantities, whereas oestradiol-sulfate was present only in trace amounts and oestradiol 17-β was not measurable at all. In some animals, not all of the six sites of the cervix were sampled because in some cases there was not enough tissue left to obtain samples from both deep and superficial layers within the same segment. However, because there were no significant differences in total oestrogen concentrations between samples of the subepithelial stromal and the deep stromal layer of the cervix, the data from these two layers within the same segment were pooled if both samples were present. By doing this, it was possible to calculate the mean total oestrogen concentrations for the three segment for all the animals of the LP4 group and for the five animals of the HP4 group, whereas only one HP4 animal had no samples to calculate the mean for the uterine segment (Fig. 2). The mean total oestrogen concentrations (pg g^{-1} wet weight) in the vaginal, mid and uterine segments of the cervices of the LP4 group (332 ± 47, 434 ± 51, 654 ± 51 respectively) were significantly higher than those in the equivalent segments of the HP4 group (149 ± 39, 159 ± 41, 138 ± 49 respectively).

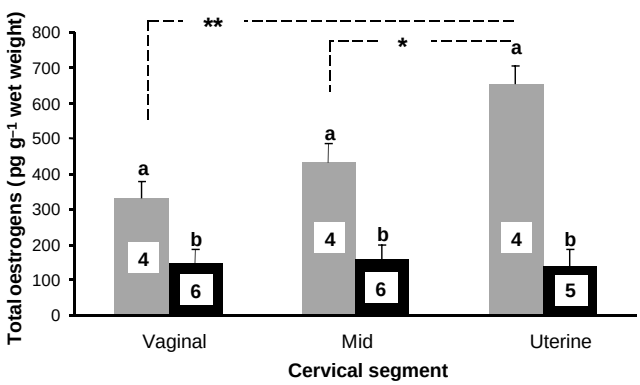

Fig. 2. Mean (LSM ± SEM) tissue oestrogen concentrations in the three longitudinal segments of the cervix in non-pregnant cows (pooled values of superficial and deep stromal layers). Within a segment, means with different superscripts differ significantly ($P<0.01$). Asterisks indicate significant differences ($^{*}P<0.05$ and $^{**}P<0.01$), between the cervical segments in LP4 cows. The numbers depicted in the bars represent the number of animals that provided samples for each mean. (■) LP4, Low progesterone group; (■) HP4, high progesterone group.

In addition, within the LP4 group, the mean total oestrogen concentration in the uterine segment of the cervix was significantly higher than that in the vaginal segment ($P<0.01$) or the mid segment ($P<0.05$; Fig. 1).

Immunolocalization of oestrogen and progesterone receptors

Immunohistochemistry demonstrated that ER as well as PR were present exclusively in the cell nuclei of the luminal epithelial cells, the epithelial crypts and of the fibroblasts and smooth muscle cells of all tissue layers of both the LP4 and the HP4 groups (Fig. 3). Conversely, vascular endothelium and smooth muscle cells in the walls of blood vessels did not stain positively for either receptor.

Proportional scores of oestrogen and progesterone receptors

There was a significant effect of cervical segment ($P<0.01$) and tissue layer ($P<0.01$) on the proportion of cells that contained ERs (the proportional score for ERs) in the P4

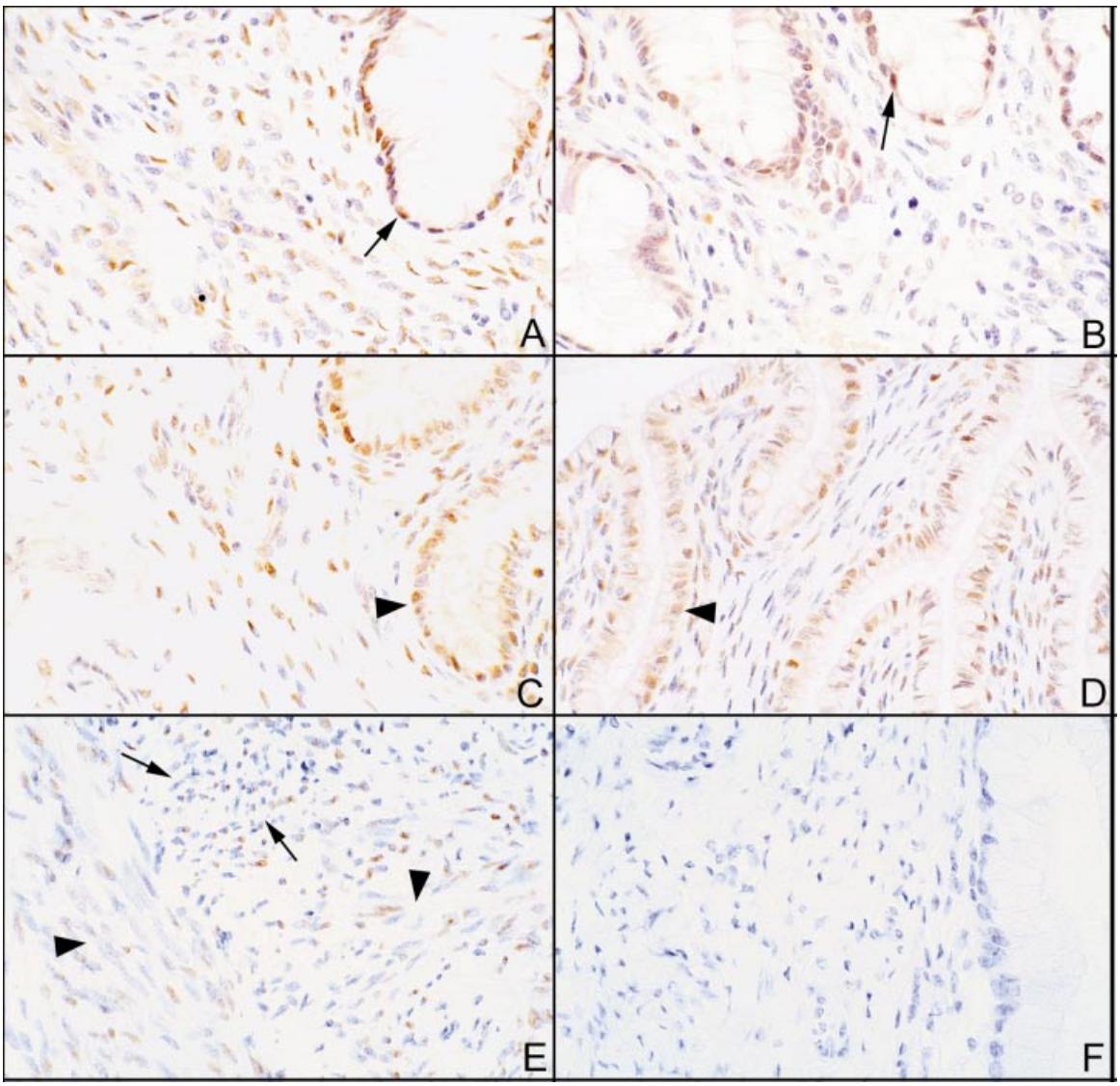

Fig. 3. Immunohistochemical staining pattern of (*A*,*C*,*E*) progesterone receptor (PR) and (*B*,*D*) oestrogen receptor (ER) in the epithelial and superficial stromal layer in cross-sections of the bovine cervix. Positive-reacting nuclei are stained brown. The animals of the low progesterone (LP4) group (*A*,*B*), show high secretory activity of the epithelial cells, indicated by the flattened nuclei and large mucus content (arrows), as opposed to the rounded nuclei and lower mucus content (arrowheads) of the epithelial cells in the high progesterone (HP4) group (*C*,*D*). (*E*) Progesterone receptor staining in the deep stromal layer, showing a higher cell density than in the superficial stromal layer. The fibrillar aspect of the deep stromal layer is illustrated by the mixed bundles of fibroblasts and by the smooth muscle cells with longitudinal orientation (arrows) and circular orientation (arrowheads). (*F*) Example of the negative control for PR (the control for ER is similar and, therefore, not shown). Magnification ×400.

groups, but not of the progesterone status of the cows (HP4 or LP4). Therefore, the values from both groups were pooled to further examine the effect of segment and tissue layer (n = 10, SEM = 16). In this respect, the mean proportional scores for ERs in the vaginal and mid segments were significantly higher than that in the uterine segment (P<0.01; Fig. 4*a*). Also, the mean proportional score for ERs in the deep stromal layer was significantly higher than those in the subepithelial stroma (P<0.05) and epithelium (P<0.01), and that in the subepithelial stroma was significantly higher than that in the epithelium (P<0.01; Fig. 4*b*).

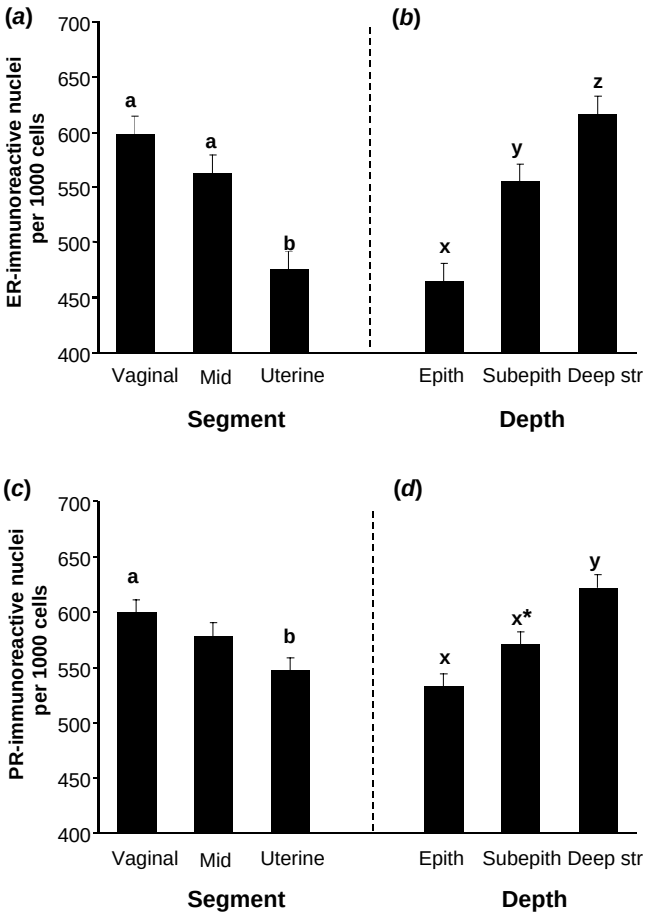

Fig. 4. Mean (LSM ± SEM) (*a*,*b*) oestrogen receptor (ER) and (*c*,*d*) progesterone receptor (PR) immunoreactive nuclei per 1000 cells in the cervix of non-pregnant cows; pooled values for low progesterone (LP4) group (n = 4) and high progesterone (HP4) (n = 6) group animals (LSM ± SEM, n = 10). (*a*) Relationship between the proportion of oestrogen receptor (ER)-immunoreactive nuclei and cervical segment. Means with different superscripts differ significantly (P<0.01). (*b*) Relationship between the proportion of ER-immunoreactive nuclei and the tissue layer. Differences between x and y and between x and z are significant at the P<0.01 level, whereas the difference between y and z is significant at the P<0.05 level. (*c*) Relationship between the proportion of progesterone receptor (PR)-immunoreactive nuclei and segment. Means with different superscripts differ significantly (P<0.01). (*d*) Relationship between proportion of PR-immunoreactive nuclei and tissue layer. Differences between x and y are significant at the P<0.01 level, whereby ∗ indicates that x^* tends (P=0.061) to be higher than x.

Similarly, there was a significant effect of cervical segment (P<0.05) and layer (P<0.01), but not of the progesterone status (HP4 or LP4) on the proportionional scores of PRs. To analyse the effect of layer and segment on the proportional scores of PRs, the values of the LP4 and HP4 groups were thus pooled (n = 10, SEM = 11). The mean proportional scores of PRs decreased successively from the vaginal segment to the mid segment to the uterine segment, with the difference between the vaginal and uterine segments reaching statistical significance (P<0.01; Fig. 4*c*). Furthermore, the mean proportional score for PRs in the deep stromal layer was significantly higher than that in the subepithelial stroma (P<0.01) or epithelium (P<0.01; Fig. 4*d*), whereas the subepithelial layer tended (P=0.061) to have a higher proportion of PR-containing cells than the epithelium.

Additionally, separate analysis of the different tissue layers in the cervix, to determine if progesterone status affected the proportion of cells containing ER or PR, revealed no significant effect. However, there was a significant effect of segment on the proportional scores for ERs, but not for PRs, in the deep stromal and subepithelial layers (P<0.05) and in the epithelial layer (P<0.01). In the vaginal segment of the epithelial layer, they were significantly higher than at the uterine side (528 *v.* 363, SEM = 31; P<0.05), and similarly in the subepithelial layer (593 *v.* 503, SEM = 19; P<0.05). Finally, the vaginal and mid segments of the deep stromal layer had significantly higher ER proportional scores than in the uterine segment (674 and 628 *v.* 557, SEM = 27; P<0.05) (Data not illustrated).

Cell density and number of oestrogen receptor- and progesterone receptor-positive nuclei per mm²

The ratio between the number of cells per mm² in the subepithelial layer and the deep layer varied between 0.41 and 0.55 in the different segments, but there was no relationship between these ratios and cervical segment or progesterone status. The mean cell density (cells per mm²) within each of the two layers was also unaffected by the progesterone status. As there was no effect of P4 status on the cell density, the values of LP4 and HP4 were pooled, revealing a significant effect of tissue layer (P<0.01) and segment (P<0.01). In all three segments, the cell density of the deep stromal layer was significantly higher than that of the subepithelial stroma (V, 207 *v.* 87; M, 223 *v.* 110; U, 283 *v.* 114; SEM = 14; P<0.01). In addition, the cell density of the uterine segment of the subepithelial layer was significantly higher than that of the vaginal segment (114 *v.* 87, SEM = 14; P<0.01), and similarly in the deep stromal layer (283 *v.* 207, SEM = 14; P<0.01) (Data not illustrated).

When the number of ER- or PR-positive nuclei were expressed per mm² of tissue, differences between segments along the longitudinal axis disappeared (Fig. 5). In the LP4

group, the ER per mm^2 in the deep stromal layer were significantly higher (P<0.01) than they were in the subepithelial stromal layer at the uterine and mid segments (Fig. 5*a*), whereas in the HP4 group, this was the case in all three segments (P<0.01; Fig. 5*b*). The PR per mm^2 showed the same pattern as the ER per mm^2 with respect to the effects of tissue layer (P<0.01), cervical segment (P<0.01) and progesterone status (Fig. 5*c*,*d*).

No significant correlations were found between the local oestrogen concentrations in the different tissue sites and either ER per mm^2 or PR per mm^2. Similarly, there was no correlation between receptor expression (ER per mm^2 and PR per mm^2) and the serum P4 concentrations. The ER per mm^2 showed a strong positive correlation with the PR per mm^2 in both layers when the LP4 and HP4 groups were pooled (subepithelial layer, R = 0.85, P<0.01; deep stromal layer, R = 0.83, P<0.01).

Discussion

Given the relative length of dioestrus and oestrus in cows, the HP4 group was almost certainly more heterogeneous than the LP4 group with regard to the stage of the oestrous cycle. Nevertheless, the significant differences that were found with respect to local tissue concentrations of oestrogens are evidence that the LP4 (≤0.5 ng mL^{-1}) and the HP4 (>2.0 ng mL^{-1}) groups of animals differed markedly from each other in other endocrinological aspects. Interestingly, the present study demonstrated a change in the immunoreactive expression of ER and PR that depended on the depth of the cervical tissue (from epithelium to deep stroma) and thus closely resembled the gradient reported for ER and PR expression in the non-pregnant bovine uterus (Boos *et al.* 1996). Although previous investigators have also reported differences in the expression of ER and PR between different tissue layers within the cervix (Kupryańczyk and Möller 1988; Cano *et al.* 1990; Snijders *et al.* 1992; Zhao *et al.* 1999), none have demonstrated this apparent gradient. In addition, we demonstrated an increase in the proportion of nuclei per 1000 cells expressing both ER and PR from the uterine to the vaginal end of the cervix. At least in the case of ER, similar differences in expression along the longitudinal axis of the cervix have been reported for women (Kupryańczyk and Möller 1988; Cano *et al.* 1990). Furthermore, the current study demonstrated that the subepithelial stromal layer of the bovine cervix contains significantly fewer cells per mm^2 than the deep stroma, a difference that magnifies the differences in receptor expression between the two layers. The earlier observation that the relative proportions of smooth muscle and fibroblast cells in the subepithelial and deep stromal layers are different (Breeveld-Dwarkasing *et al.* 2000) may help to explain differing ER and PR concentrations in the two layers and further suggests that the effect of hormonal stimulation is likely to be different between the two layers. Another interesting finding of the current study was that when receptor expression in the tissue was corrected for cell density, longitudinal differences (uterine to vaginal segment) in receptor expression were no longer apparent because the uterine segment of the cervix is more cell dense than the vaginal segment.

Against expectations, the current study revealed no apparent effects of systemic progesterone or local tissue oestrogen concentrations on the expression of either ER or PR in the bovine cervix. This finding agrees with reports that immunohistochemical ER expression in the cervix of women does not vary during the menstrual cycle (Kupryańczyk and Möller 1988; Cano *et al.* 1990; Snijders *et al.* 1992), and that cyclical variations in the expression of PR are minor (Kupryańczyk 1991; Snijders *et al.* 1992) or non-existent (Cano *et al.* 1990). On the other hand, rather different results have been reported previously when cytosolic ER and PR concentrations were measured biochemically, using a dextran-coated charcoal extraction method, in the cervix of cows (Vesanen *et al.* 1991) and mares (Re *et al.* 1995). In both cases, medium to high serum progesterone levels reduced cytosolic PR concentrations but

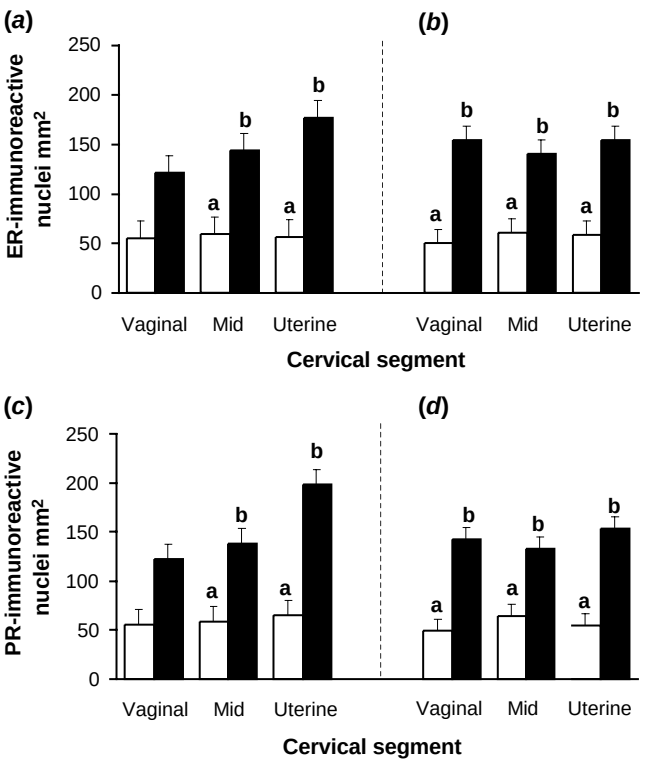

Fig. 5. (*a*–*d*) Mean (LSM ± SEM) receptor-positive nuclei per mm^2 in the (□) superficial and (■) deep stromal layers of three longitudinal segments of the cervix. Within a segment, means with different superscripts are significantly (P<0.01) different. Oestrogen receptor (ER)-immunoreactive nuclei per mm^2 in (*a*) the low progesterone (LP4) group (n = 4) and (*b*) the high progesterone (HP4) group (n = 6). Progesterone receptor (PR)-immunoreactive nuclei per mm^2 in the (*c*) LP4 (n = 4) and (*d*) HP4 (n = 6) groups.

did not affect cytosolic ER concentrations. However, the current study demonstrated clearly that cervical ER and PR expression differ both longitudinally and vertically, and this suggests that great care is needed when selecting tissue for analysis and may help to explain the differences in results between studies. Furthermore, the two techniques, immunohistochemistry and biochemistry, measure receptor expression in very different ways.

The results of the present study also differ markedly from reports of an almost complete absence of ER and PR immunoreactivity in the mucosal part of the cervix of sheep beyond days 0 to 3 of the oestrous cycle (Zhao *et al.* 1999) and the changes in receptor expression reported during the oestrous cycle of the dog (Vermeirsch *et al.* 1999, 2000) and rat (Wang *et al.* 2000).

Oestrogen stimulates an increased secretory activity in human cervical epithelium and it is suggested that this effect is mediated by ER (Gorodeski 1998). In the current study, all of the cervices from LP4 cows showed gross and histological signs of a highly secretory epithelium. Therefore, it was a surprise to find no significant differences in ER population between the cervical epithelium of the LP4 and HP4 groups. The absence of oestradiol-17β in the cervical tissue in the present study's experiment may explain the lack of effect on the epithelial ER population; however, it is in contrast to the findings of cyclical changes in ERα expression in the epithelium of the rat cervix (Wang *et al.* 2000). Conversely, Wang and colleagues performed their study on the vaginal part of the rat cervix, which has a stratified squamous epithelium that is markedly different to the columnar epithelium of the bovine cervix in the vaginal segment and thus may be subject to different hormonal regulation. It is also possible that epithelial functions might be controlled in a paracrine fashion via the action of steroid hormones on receptors located in stromal cells, as has been shown for the progesterone receptor in the mouse uterus (Kurita *et al.* 2000*a*, 2000*b*). The actions of oestrone, which is present in the tissue in substantial amounts, might be of interest, although, to date, we could find no indication in the literature about the affinity of oestrone for ER in the cervix.

It is also important to realize that qualitative descriptions of staining patterns or semiquantitative scoring methods based on a combination of the intensity of the staining reaction and the number of stained cells, as used in some of the previous studies, provide no indication of the amount of receptor protein present in the nucleus. Furthermore, because both steroid-occupied and steroid-unoccupied forms of the receptors are recognized by the antibodies (Press and Greene 1988), immunopositive staining of the nucleus does not itself necessarily reflect the level of hormone or receptor activity. Of course, it is possible that the effect of the steroid hormones on the cyclical changes in cervix physiology are not regulated simply and exclusively by receptor concentration. For example, oestrogens are thought to be able to induce non-genomic reactions in the absence of the ER (Lueng and Wathes 2000). Furthermore, endogenous suppressors of oestrogen activity exist, which, in competition with co-activators, decrease the transcriptional activity of the ER (Delage-Mourroux *et al.* 2000). Thus, the tissue-specific effects of oestrogen may be influenced by tissue- and cell-specific patterns of co-activators and co-suppressers of ER (reviewed by Klinge 2000). In addition, in the present study, the ER antibody used was directed against ERα and, although it was not always documented, this was probably the case in most of the other studies cited. Interestingly, the effects of oestradiol-17β binding to ERβ can oppose those of ERα binding (Paech *et al.* 1997) and this suggests that the balance of the different receptor isoforms may affect the biological effect of the ligand. However, to date, reports on the distribution of both ER isoforms in the cervix of non-pregnant species are limited. In rats, it has been reported that ERα and ERβ are distributed similarly within the cervix and that their expression changes in a parallel fashion during the oestrous cycle, although ERβ expression is much weaker (Wang *et al.* 2000). Wang *et al.* (2000) noted further that the immunohistochemically detected expression of both oestrogen receptor isoforms differed with respect to the region of the cervix.

In conclusion, the results of the current study demonstrate that oestrogen and progesterone receptor expression in the bovine cervix is not controlled simply by circulating steroid hormones or by tissue hormone concentrations. Furthermore, the regional differences in receptor expression described demonstrate clearly that great care is needed in the selection of tissue before conclusions can be made with regard to the effects of hormones on receptor expression.

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