Immunohistochemical studies on the progesterone receptor (PR) in the sow uterus during the oestrous cycle and in inseminated sows at oestrus and early pregnancy

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Abstract

Physiological changes in the sow uterus involve the regulation by progesterone and its receptor proteins (PR). Therefore, the aim of the present study was to investigate the localization of PR during different stages of the oestrous cycle and in inseminated sows during early pregnancy by use of immunohistochemistry. Uterine samples were collected from cyclic and inseminated sows at different stages of the oestrous cycle and early pregnancy. The samples were fixed in 10% formaldehyde and embedded in paraffin. Immunohistochemistry was done by use of a mouse monoclonal antibody to PR. The highest PR immunostaining in the surface epithelium was observed at oestrus/5–6 h after artificial insemination (AI) and early dioestrus/70 h after AI. In the glandular epithelium, the highest level of PR was found at oestrus with the lowest at late dioestrus/d 19. Higher levels of PR were observed in inseminated groups compared with cyclic sows. In the myometrium, a high level of PR was found at oestrus, while stromal PR cells were constantly present throughout the oestrous cycle and at different stages of early pregnancy. In conclusion, this study shows that the immunopresence of PR in the sow uterus differed between uterine compartments at the same reproductive stage. Differences were also found for some uterine compartments between cyclic and inseminated/early pregnant sows. The relatively consistent immunostaining of PR in the stroma strengthens a stromal role in the regulation of physiological activities in the sow uterus during the oestrous cycle as well as early pregnancy.

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Introduction

The uterus is influenced by the ovarian steroid hormone progesterone, which induces glandular and stromal differentiation as well as cell proliferation (Graham & Clarke 1997). A stimulatory effect by progesterone on uterine protein secretion has been shown in pigs in several studies (Adams *et al.* 1981, Roberts *et al.* 1987, Vallet *et al.* 1998). During pregnancy progesterone has an important role in preparations for uterine reception of the early embryos and for the maintenance of pregnancy. The effects of progesterone are mediated by interactions of the hormone with specific intracellular progesterone receptors (PR). The importance of PR was studied in knockout mice and it was shown that the adult female PR mutant displayed significant defects in all reproductive tissues including the uterus (Lydon *et al.* 1996, Conneely *et al.* 2001). A recent review described that the absence of PR was associated with uterine release of the luteolytic

prostaglandin F_{2α} (PGF_{2α}) in cyclic ewes and with the onset of uterine gland differentiation in pregnant ewes (Spencer *et al.* 2004). In gilts, Geisert *et al.* (1994) showed by immunohistochemical staining that PR proteins were absent in both surface and glandular epithelia at d 12–18 of the oestrous cycle and of pregnancy. Moreover, Persson *et al.* (1997), by use of enzyme immunoassay, showed that the levels of PR protein were highest in the non-pregnant gilt endometrium at oestrus and metoestrus and that it was low at d 14–18 as well as at d 25–30 of pregnancy. However, uterine physiology of gilts may differ from that of sows that have experienced a number of pregnancies. To our knowledge, no studies of PR using immunohistochemistry, have been done on the sow uterus.

Our previous studies on oestrogen receptor alpha (ERα) and proliferative activities in the sow uterus revealed that ERα may be related to proliferation in the epithelia (Sukjumlong *et al.* 2003, 2004b). However, physiological

changes in the uterus should involve regulation via PR as well. As PR can be induced by both oestrogen and progesterone (Ing & Tornesi 1997), it is implied that many of the reproductive physiological responses attributed to PR may be due to the combined effects of oestrogen and progesterone acting through their respective receptors.

Studies in different species have shown that oestrogen increased the presence of PR while progesterone itself decreased PR in many reproductive tissues (Geisert *et al.* 1994, Dhaliwal *et al.* 1997, Ing & Tornesi 1997, Bouchard 1999). However, these effects may not be similar for all cell types of the uterus. The localization of steroid receptors can differ among specific tissue compartments of the uterus, even at the same plasma steroid level. This has been shown for PR in different species (Lessey *et al.* 1988, Ohta *et al.* 1993, Wathes & Hamon 1993, Geisert *et al.* 1994, Dhaliwal *et al.* 1997) as well as for ER α in our previous studies on both cyclic and inseminated/early pregnant sow uteri (Sukjumlong *et al.* 2003, 2004b).

The aim of the present study was to investigate the localization of the PR, by immunohistochemistry, in the sow uterus during different stages of the oestrous cycle and in inseminated sows at oestrus and during early pregnancy.

Material and Methods

Experimental animals and management

The experimental plan was approved by the Ethical Committee for Experimentation with Animals. Thirty-three crossbred sows (Swedish Landrace $\times$ Swedish Yorkshire) were used and the study was performed in two experimental groups of sows: fifteen non-pregnant cyclic sows (experiment A) and eighteen inseminated/early pregnant sows (experiment B). The mean parity numbers of these experimental sow groups were 3.4 ± 0.7 and 3.4 ± 0.6 , respectively. The sows were purchased from a commercial herd and brought to the Department of Obstetrics and Gynaecology directly after weaning. They were kept in individual pens and were fed according to the Swedish breeding stock standard for dry sows (Simonsson 1994). The sows had shown normal reproductive performance before selection to the experiment. After weaning (5 weeks of lactation) careful oestrous detection was done by inspection of vulva reddening and swelling as well as control of standing reflex by help of a boar. The ovulation time was checked by ultrasonography. Before slaughter, blood samples were collected from the external jugular vein for analysis of oestradiol-17 β (Mwanza *et al.* 2000) and progesterone (Rojkittikhun *et al.* 1993).

In the inseminated/early pregnant groups of sows (experiment B), all sows were inseminated once at 20–15 h before expected ovulation in their second oestrus after weaning (estimated from results of ultrasound examinations at the first oestrus). At insemination, a semen dose containing 10×10^9 spermatozoa in Beltsville Thawing Solution (BTS; Pursel & Johnson 1976) from two boars of proven fertility was used (Kaeoket *et al.* 2002).

Tissue collection

The cyclic sows (experiment A) were slaughtered at five different stages of the oestrous cycle: at oestrus (d 1, 11.5–17.5 h after the start of the second standing oestrus after weaning; $n = 3$), early dioestrus (d 4, 70.5–71 h after ovulation; $n = 3$), dioestrus (d 11–12; $n = 3$), late dioestrus (d 17; $n = 3$) and pro-oestrus (d 19; $n = 3$). In experiment B, the inseminated sows were divided into 5 different groups and slaughtered at oestrus, 5–6 h after artificial insemination (AI) (group 1; $n = 4$), at 20–25 h after ovulation (group 2, 36–43 h after AI; $n = 4$), at 70 h after ovulation (group 3, 83–93 h after AI; $n = 4$), on day 11 (group 4, the first day of standing oestrus = day 1; $n = 3$) and on day 19 (group 5; $n = 3$).

Immediately after slaughter, the reproductive organs were examined for normality and tissues were sampled. The uterine samples were collected at the mesometrial side, 20–30 cm from the tip of the uterine horn and fixed in 10% formaldehyde for up to 4 days. The samples were embedded in paraffin blocks, cut in 4 μ m thick sections and placed on Superfrost Plus glass slides (Menzel-Glaser, Freiburg, Germany) before immunohistochemistry was carried out.

Immunohistochemistry

After the sections were deparaffinized in xylene and graded alcohol, antigen retrieval technique was used in order to enhance the reaction between antigen and antibody by boiling in 0.01 M citric buffer pH 6.0, 2 $\times$ 5 min in a microwave at 750 watt. All the immunohistochemical procedures were carried out as described earlier (see Sukjumlong *et al.* 2003). Briefly, a standard avidin-biotin immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories Inc., USA) was applied to detect the PR proteins. The primary antibody used was a mouse monoclonal antibody to both PR-A and PR-B (PR-2C5, Zymed Laboratories, Inc., San Francisco, USA) at the dilution of 1:200. The incubation time for the primary antibody was 1 h at room temperature. A negative control was obtained by replacing the primary antibody with non-immune serum (IgG_{2a}) of the same concentration as the primary antibody. In the final step, 3,3'-diaminobenzidine (DAB) (Dakopatts AB, Älvsjö, Sweden), a chromogen, was added to visualize the bound enzyme (brown colour). All sections were counterstained with Mayer's hematoxylin followed by mounting in glycerine-gelatin before investigation. Selected sections were photographed with a Nikon microphot-FXA photomicroscope (Nikon Corporation, Tokyo, Japan).

Classification of positively stained cells

The evaluations of positively stained cells were carried out as blinded preparation by the same person (S Sukjumlong). The results are presented for the different uterine compartments as follows: surface epithelium, glandular epithelium, subepithelial layer of the connective tissue

stroma and myometrium. In addition, superficial and deep-laying endometrial glands were evaluated separately since the staining patterns differed between these compartments. In the myometrium, only smooth muscle cells were studied. Blood vessels in both endometrium and myometrium were observed but not evaluated. The results of the immunostaining were evaluated in two ways, by a manual scoring method and by image analysis.

Manual scoring

The manual scoring of PR positive cells was done by classification into three different levels of intensity: weak, +; moderate, ++; and strong, +++. Since not all cells stained positively in some compartments of the uterus, i.e. the surface epithelium and the myometrium, the proportion of positive to negative cells was also included for these tissues. The proportions were estimated into four different levels (marked A–D): low proportion (<30% of positive cells, A); moderate proportion (30–60% of positive cells, B); high proportion (60–90% of positive cells, C) and almost all cells positive (more than 90%, D).

Image analysis

In order to assess the PR immunostaining more quantitatively in each tissue compartment, a Leica microscope (Cambridge, UK) and Sony video camera (Park Ridge, NJ, USA) connected to a computer using an image analysis system (Leica Imaging System Ltd) was applied. Quantification of the immunostaining was performed on five randomly selected fields in each compartment. In the endometrial stroma, evaluations were done in the subepithelial connective tissue layer in which non-stromal elements (superficial endometrial glands, blood vessels) were interactively removed. Also in other compartments, proper areas were selected and blood vessels were

excluded interactively. By using the colour discrimination software, the nuclear staining intensity was divided into three different levels: weak, moderate and strong. However, it is difficult for the system to distinguish a faint background from weak staining in the cell nuclei and therefore, to avoid false positive staining, only moderate to strong intensity was included for the image analysis measurement. The results are presented as mean ratio of total area of positive nuclear staining per total area of cell nuclei.

Statistical analyses

The statistical analyses were performed only on the image analysis results by using the SAS statistical package (SAS for Windows, Version 6.12, Cary, NC, USA). The stage of oestrous cycle in experiment A and groups of inseminated/early pregnant animals in experiment B were regarded as independent variables. Mean values for each class of the independent variables were compared by using NPAR1WAY (Wilcoxon rank sum test). Spearman rank correlations between plasma levels of oestradiol-17 β , progesterone, and the ratio of PR-positive cells were performed in each experiment by using CORR procedure. *P* values <0.05 were regarded as statistically significant.

Results

Plasma hormone levels and pregnancy confirmation

The results of plasma hormone levels (Fig 1a and 1b) were shown previously by Kaeoket *et al.* (2001, 2002) for cyclic and inseminated sows, respectively. The cyclic sows (experiment A) showed high plasma levels of oestradiol-17 β at prooestrus and oestrus and a high level of progesterone during dioestrus (Fig. 1a). In the inseminated/pregnant sows (experiment B) (Fig. 1b), the highest level of plasma oestradiol-17 β was found at oestrus (group 1)

Figure 1 Plasma levels of oestradiol-17 β and progesterone (mean $\pm$ S.D.) in sows at different oestrous cycle stages (1a) and in inseminated sows (1b) at oestrous, 5–6 h after AI (group 1); 20–25 h after ovulation (group 2); 70 h after ovulation (group 3); d 11 (day 1 = first day of standing oestrous) (group 4) and d 19 (group 5).

while the plasma progesterone levels were high at d 11 and d 19 of early pregnancy (groups 4 and 5) (Fig. 1b).

In experiment B, the sows slaughtered before ovulation (group 1) had spermatozoa in the uterus, utero-tubal junction, isthmus and ampulla. All sows slaughtered after ovulation in experiment B were pregnant (groups 2–5) as embryos were observed (Kaeoket *et al.* 2002).

Immunohistochemistry of PR

Positive immunohistochemical staining was exclusively found in the nuclei of all uterine cell types and no cytoplasmic staining was observed (Figs 2 and 3). Negative controls showed no staining (Figs 2f and 2k). The manual scoring and image analysis results are shown according to different tissue compartments in Table 1 for cyclic sows (experiment A) and in Table 2 for inseminated/early pregnant sows (experiment B). For the blood vessels in both endometrium and myometrium, positive cells were rarely observed and therefore not quantified.

Surface epithelium

Non-pregnant cyclic sows (Table 1) (Fig. 2)

The most prominent intensities (++/+++) as well as a high proportion of PR-positive cells was found at oestrus and early dioestrus while weak immunostaining and a low proportion was observed at the other stages. The results of image analysis showed that the mean ratio of PR-positive cells was significantly higher at oestrus and early dioestrus compared with other stages ($P < 0.001$).

Inseminated/early pregnant sows (Table 2) (Fig. 3)

The strongest intensity (++/+++) and a high proportion (D) of PR-positive cells was observed at 70 h after ovulation (group 3). At oestrus and 20–25 h after ovulation, the staining intensity was weak to moderate while weak staining as well as a low proportion was found at d 11 and d 19 (groups 4 and 5). The results from image analysis showed that the mean ratios of PR-positive cells were higher at oestrus and at 70 h after ovulation (groups 1 and 3) compared with the other groups (groups 2, 4 and 5) ($P < 0.001$).

Glandular epithelium

Non-pregnant cyclic sows (Table 1) (Fig. 2)

The manual scoring results showed that almost all glandular cells were PR-positive except for superficial glands at prooestrus and oestrus. The staining intensity in superficial glands was weak and/or moderate at all stages. The strongest intensity (++/+++) was observed in the deep glands at oestrus.

For the image analysis results, the highest mean ratio of PR-positive nuclei was found at oestrus in both

layers of glandular epithelium compared with the other stages ($P < 0.001$).

Inseminated/early pregnant sows (Table 2) (Fig. 3)

In the groups studied, almost all cells were PR-positive (D) but with different intensities. In the superficial glands, the strongest intensity (++) was observed at 70 h after ovulation (group 3) while weak staining was observed at 20–25 h after ovulation (group 2) and at d 19 (group 5). Strongest intensity (++/+++) in the deep laying glands was found at oestrus and 20–25 h after ovulation (groups 1 and 2). At d 19 (group 5) the staining intensity of most glandular epithelial cells was weak.

The image analysis showed that the highest mean ratio of PR-positive cells was found at oestrus (group 1) ($P < 0.001$) while the lowest mean ratio of positive cells was found at d 19 in both layers of glands compared with all other groups ($P < 0.001$).

Connective tissue (subepithelial layer)

Non-pregnant cyclic sows (Table 1) (Fig. 2)

Almost all cells were PR-positive at all stages of the oestrous cycle. However, the intensities differed, being strong at oestrus, strong/moderate at prooestrus and early dioestrus, and moderate at dioestrus and late dioestrus. For the image analysis results, the highest ratio of PR-positive cells was found at oestrus ($P < 0.001$) while the lowest ratio of positive cells was found at dioestrus which was significantly different to all other groups ($P < 0.001$).

Inseminated/early pregnant sows (Table 2) (Fig. 3)

The staining intensity of PR-positive cells was moderate/strong at oestrus and at 20–25 h after ovulation (groups 1 and 2) but moderate or moderate/weak in the other groups. The image analysis results showed higher ratios of PR-positive cells at oestrus (group 1) compared with other groups ($P < 0.001$).

Myometrium

Non-pregnant cyclic sows (Table 1) (Fig. 2)

The manual scoring method showed that almost all myometrial cells were PR-positive at all stages. The intensity of positive staining in the myometrium was moderate/strong at prooestrus and oestrus. At the other stages, the staining intensity was weak to moderate. The results from image analysis showed that the mean ratio of PR-positive cells was significantly higher at prooestrus and oestrus while low ratios of positive cells were found at the other stages ($P < 0.001$).

Inseminated/early pregnant sows (Table 2) (Fig. 3)

Almost all cells were PR-positive in all groups and moderate/strong intensity was found at oestrus and 20–25 h after

Figure 2 Immunohistochemical localization of PR in different tissue compartments of the sow uterus: a–f, surface epithelium (SE) and subepithelial layer of the connective tissue (STR); g–l, glandular epithelium (GE) and myometrium (M). Stages of the oestrous cycle: prooestrous, d 19 (a and g); oestrous, d 1 (b and h); early dioestrous, d 4 (c and i); dioestrous, d 11–12 (d and j); late dioestrous, d 17 (e and k); negative control, (f and l).

Figure 3 Immunohistochemical localization of PR in different tissue compartments of the sow uterus: a–e, surface epithelium (SE) and subepithelial layer of connective tissue (STR); f–j, glandular epithelium (GE) and myometrium (M). Stages of pregnancy: group 1, 5–6 h after AI (a and f); group 2, 20–25 h after ovulation (b and g); group 3, 70 h after ovulation (c and h); group 4, d 11 after standing oestrous (d and i); group 5, d 19 after standing oestrous (e and j).

Table 1 Immunohistochemical staining of progesterone receptor (PR) presented as manual scoring (intensity and proportion) and as ratios of positive nuclei by image analysis unit results (mean $\pm$ s.d.) in different uterine tissue compartments of non-pregnant sows at different stages of the oestrous cycle.

Stage of the oestrous cycle	Surface epithelium	Glandular epithelium (superficial glands)	Glandular epithelium (deep glands)	Connective tissue stroma (subepithelial layers)	Myometrium
Pro-oestrus (d 19)*	-/+A (0.18 $\pm$ 0.2 ^b)	+A (7.16 $\pm$ 6.7 ^a)	+/++D (16.9 $\pm$ 14.4 ^a)	+/++++D (46.14 $\pm$ 11.3 ^a)	+/++++D (50.09 $\pm$ 23.1 ^a)
Oestrus (d 1)*	+/++++D (50.37 $\pm$ 28.7 ^a)	+/++B (12.30 $\pm$ 8.7 ^b)	+/++++D (62.59 $\pm$ 31.6 ^b)	++++D (66.54 $\pm$ 6.7 ^b)	+/++++D (59.16 $\pm$ 3.2 ^a)
Early dioestrus (d 4)*	+/++++D (67.85 $\pm$ 19.1 ^a)	++D (2.28 $\pm$ 3.1 ^{ad})	+/++D (0.67 $\pm$ 1.1 ^c)	+/++++D (50.03 $\pm$ 10.9 ^a)	+/++D (8.3 $\pm$ 3.6 ^b)
Dioestrus (d 10–11)*	-/+A (0.04 $\pm$ 0.1 ^b)	+D (0 $\pm$ 0.00 ^c)	++D (14.51 $\pm$ 24.6 ^c)	++D (22.81 $\pm$ 23.3 ^c)	+/++D (0.45 $\pm$ 0.7 ^c)
Late dioestrus (d 17)*	-/+A (0.02 $\pm$ 0.02 ^b)	+D (0.60 $\pm$ 0.8 ^d)	+/++D (0.27 $\pm$ 0.46 ^c)	++D (38.03 $\pm$ 11.54 ^d)	+/++D (1.60 $\pm$ 2.27 ^c)
Overall significant	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Staining intensity: -, negative; +, weak; ++, moderate; +++, strong.

A, low proportion (<30%); B, moderate proportion (30–60%); C, high proportion (>60–90%); D, almost all cells (>90%) are positive.

* day 1, first day of standing oestrus.

The different superscript letters are significantly different ($P < 0.05$)

Table 2 Immunohistochemical staining of progesterone receptor (PR) presented as manual scoring (intensity and proportion) and as ratios of positive nuclei by image analysis unit results (mean $\pm$ s.d.) in different uterine tissue compartments of inseminated sows at oestrus and early pregnancy.

Group of pregnant sows	Surface epithelium	Glandular epithelium (superficial glands)	Glandular epithelium (deep glands)	Connective tissue (subepithelial layers)	Myometrium
Group 1 (oestrus, 5–6 h after AI)	+/++D (33.14 $\pm$ 8.14 ^a)	+/++D (19.07 $\pm$ 12.6 ^a)	+/++++D (87.75 $\pm$ 7.0 ^a)	+/++++D (60.24 $\pm$ 3.8 ^a)	+/++++D (46.7 $\pm$ 20.1 ^a)
Group 2 (20–25 h after ovulation)	+/++D (19.84 $\pm$ 18.01 ^b)	+D (6.22 $\pm$ 6.6 ^b)	+/++++D (44.9 $\pm$ 28.2 ^b)	+/++++D (34.97 $\pm$ 10.8 ^b)	+/++++D (36.11 $\pm$ 16.4 ^a)
Group 3 (70 h after ovulation)	+/++++D (49.71 $\pm$ 19.32 ^a)	++D (7.10 $\pm$ 12.1 ^b)	++D (10.7 $\pm$ 18.4 ^c)	++D (31.2 $\pm$ 13.1 ^b)	+/++D (7.59 $\pm$ 11.6 ^b)
Group 4 (d 11)*	-/+A (12.58 $\pm$ 24.75 ^c)	+/++D (4.24 $\pm$ 3.5 ^b)	+/++D (6.16 $\pm$ 5.04 ^c)	+/++D (32.8 $\pm$ 13.0 ^b)	+D (3.44 $\pm$ 3.43 ^b)
Group 5 (d 19)*	-/+A (3.27 $\pm$ 6.20 ^c)	+D (1.14 $\pm$ 2.07 ^c)	+D (1.88 $\pm$ 2.9 ^d)	++D (26.01 $\pm$ 8.4 ^b)	+D (1.9 $\pm$ 1.4 ^b)
Overall significant	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.01$

Staining intensity: -, negative; +, weak; ++, moderate; +++, strong.

A, low proportion (<30%); B, moderate proportion (30–60%); C, high proportion (>60–90%); D, almost all cells (>90%) are positive.

* day 1, first day of standing oestrus.

The different superscript letters are significantly different ($P < 0.05$)

ovulation (groups 1 and 2) whereas weak staining was found at d 11 and d 19 (groups 4 and 5). The mean ratios of PR-positive cells from image analysis at oestrus and 20–25 h after ovulation (groups 1 and 2) were significantly higher than at the other stages of early pregnancy (groups 3–5) ($P < 0.01$).

Results on correlation between image analysis results and plasma hormone levels

Non-pregnant cyclic sows (experiment A)

In cyclic sows, significantly positive correlations between PR-immunolabelling and plasma levels of oestradiol-17 β were found for the glandular epithelium (both superficial and deep glands) ($P < 0.001$), the connective tissue ($P < 0.001$) and the myometrium ($P < 0.001$). Negative correlations between PR-positive cells and the plasma levels of progesterone were observed in the same compartments ($P < 0.001$).

Inseminated/early pregnant sows (experiment B)

Significant positive correlations were found between plasma oestradiol-17 β levels and PR-positive cells in the uterus of inseminated/early pregnant sows in the superficial and deep glands ($P < 0.001$), connective tissue ($P < 0.001$) and myometrium ($P < 0.001$). The plasma progesterone levels were negatively correlated with PR-immunolabelling in all of the tissue compartments studied except the surface epithelium i.e. the superficial and the deep glands ($P < 0.001$), the connective tissue ($P < 0.001$) and the myometrium ($P < 0.001$).

Discussion

The localization of progesterone receptors (PR) was investigated in the uteri of cyclic and inseminated/early pregnant sows by using a mouse monoclonal antibody which recognizes an epitope present in both isoforms of the receptor (PR-A and PR-B). Both isoforms have been shown by western blot to be present in the porcine ovary (Slomczynska *et al.* 2000) and uterus (Geisert *et al.* 1994). The isoforms only differ with respect to an amino-terminal extension on PR-B (Gronemeyer *et al.* 1991). However, it has been suggested that the PR-A and PR-B proteins have different conformations (Mote *et al.* 2000) as there are differences in immunohistochemical binding patterns to cell clones specific to their respective isoform even if an antibody has been shown by western blot to detect both isoforms.

In the present study, immunostaining was evaluated by two different methods, a manual scoring and image analysis. The results from the manual scoring showed variations in patterns of PR-immunostaining with regard to both proportion and intensity while the image analysis quantified the total amount of clearly positive staining (moderate to strong) in randomly selected areas. When comparing the

two methods, the results for most compartments were in agreement with each other. However, differences were observed at some stages/groups and one explanation could be the difference regarding weak staining intensity which was estimated manually but excluded from the image analysis measurement as described above. Another explanation could be that the manual scoring method evaluated the whole tissue while the image analysis was limited to five randomly selected areas.

The present study showed that the level of PR-immunostaining was high in most compartments of the uterus at oestrus in cyclic sows (experiment A) as well as in newly inseminated sows (groups 1 and 2, experiment B) which is in accordance with other studies on PR in pigs (Koziorowski *et al.* 1984, Kotwica 1986, Stanchev *et al.* 1990, Geisert *et al.* 1994). Studies in different species (Dhaliwal *et al.* 1997, Ing & Tornesi 1997, Bouchard 1999) have described that oestrogen upregulates the progesterone receptor whereas progesterone downregulates both oestrogen receptors and its own receptors but these effects are time- and tissue-specific. Both methods used in the present study support these earlier results on PR as the immunopresence in the glandular epithelium, connective tissue and myometrium of cyclic sows in most cases was high when plasma levels of oestradiol-17 β were high and low when progesterone levels were high. In addition, positive correlations with plasma oestradiol-17 β were found for these compartments in cyclic sows. Negative correlations were found between plasma levels of progesterone and PR-immunostaining in most tissue compartments of both cyclic and inseminated/early pregnant sows. However, in some compartments, such as the surface epithelium of sows from both experiments, positive PR cells did not correlate with the plasma oestradiol-17 β in the present study. Thus, our results show that the mechanisms of PR upregulation by oestradiol-17 β in plasma vary in different uterine tissue compartments and that some other factors or regulatory mechanisms should be involved. Supporting this, our recent findings in anoestrous sows (Sukjumlong *et al.* 2004a) that have low levels of plasma steroid hormones, showed strong staining of PR in all compartments of the uterus except the glandular epithelium.

In the surface epithelium, low intensity and low proportion of PR immunopositive cells were found at prooestrus even though the plasma level of oestradiol-17 β was high. At oestrus as well as early dioestrus/70 h after ovulation, the levels of PR were significantly increased. However, in inseminated sows, a significantly lower mean ratio of PR-positive cells was observed at 20–25 h after ovulation. For the non-inseminated sows, a stage comparable to 20–25 h after ovulation was not studied, thus it was not clear whether this temporary downregulation of PR in the surface epithelium was caused by insemination or only by the lower plasma level of oestradiol-17 β compared with at oestrus. As PR is generally upregulated by oestrogens, the lack of high PR levels in the surface epithelium at prooestrus indicates a delayed effect, possibly

via ER α in the stroma as will be discussed below. This is supported by our previous studies (Sukjumlong *et al.* 2003, 2004b) showing that stromal ER α was high at prooestrus. At later stages (dioestrus, and late dioestrus/d 11 and d 19), the PR levels in the surface epithelium of the present study were low. It has been shown that the morphology of luminal epithelial secretory cells did not differ between cyclic and pregnant gilts up to d10 (Stroband *et al.* 1986). Our present study supports the lack of difference between cyclic and inseminated animals as the pattern of PR presence in the surface epithelium was similar at comparable stages.

Geisert *et al.* (1994) studied PR in the gilt uterus by different methods including immunocytochemistry. Their results showed that PR could not be detected in the surface and glandular epithelia on d 12–18 of the oestrous cycle and early pregnancy but was still present in the stroma and myometrium. Our results differed slightly from this by showing that PR was detected in the epithelia at all stages/groups though it was very low at some stages of the oestrous cycle and pregnancy. It was suggested by another study on porcine cultured endometrial cells (Carnahan *et al.* 2002) that PR may be present in the endometrial epithelium at a lower level than the sensitivity of immunohistochemistry can detect but still permit the response to progesterone. This, and other methodological variations such as sampling time, fixation and type of antibody, could explain the differences in results. Another explanation may be differences in uterine physiology between gilts and sows regarding the presence of PR although that can not be proven by the present study as no gilts were included and differences to the results on gilts by Geisert *et al.* (1994) are not apparent.

Surface and glandular epithelia should be considered as two functionally different cell populations as the pattern of synthesis and release of their products were shown to be different (Fazleabas *et al.* 1985). As progesterone is involved in secretory functions in the uterus (Adams *et al.* 1981, Roberts *et al.* 1987, Vallet *et al.* 1998), differences in PR immunopresence could therefore be expected. Our results support this concept by showing PR differences between surface and glandular compartments. The glandular epithelium can be further divided into functional units as the present study showed that the levels of PR-positive cells differed between superficial and deep layers which is in agreement with other PR studies in ewes (Spencer & Bazer 1995) and bitches (Vermeirsch *et al.* 2000) as well as our earlier studies on ER α using the same sows. (Sukjumlong *et al.* 2003, 2004b). This indicates different roles or functions for glandular levels under the same steroid hormone regulation. In the present study, PR ratios from image analyses were higher in the glandular epithelium of inseminated sows at 5–6 h after AI than in non-inseminated sows at oestrus which might be an effect by insemination. As the semen from boars contains high levels of oestrogen (Claus *et al.* 1987) it could act locally

on the endometrium in addition to plasma oestradiol-17 β and result in the upregulation of glandular PR.

In comparison with our previous study on ER α using the same cyclic (Sukjumlong *et al.* 2003) and inseminated/early pregnant sows (Sukjumlong *et al.* 2004b), some differences could be observed between ER α and PR immunostaining. In the glandular epithelium of cyclic sows, PR staining intensity was higher at oestrus than at early dioestrus while ER α staining intensity was vice versa. Furthermore, in the surface epithelium of inseminated/early pregnant sows, high presence of PR (intensity and proportion) was observed at oestrus and at 70 h after ovulation (groups 1 and 3) whereas ER α intensity and proportion was low in those groups (Sukjumlong *et al.* 2004b). This shows that the high level of PR presence occurred before ER α in the uterine glands which indicates that the appearance of ER α and PR in the epithelia are differently regulated at the same reproductive stage.

Studies in the mouse have demonstrated that expression of epithelial PR requires the presence of PR in the stroma (Kurita *et al.* 2000a,b, 2001). Moreover, it has been reported in primates that uterine epithelia were more sensitive to downregulation by progesterone than stromal cells (Lessey *et al.* 1988, Brenner *et al.* 1990, Hild-Petito *et al.* 1992). Similarly, in pregnant women and ewes, endometrial stroma remained PR-positive throughout pregnancy while the epithelial PR was downregulated which indicated that the actions of progesterone on endometrial epithelia at gestation might be mediated by the stroma (Perrot-Applanat *et al.* 1994, Spencer & Bazer 2002). The results of the present study support these concepts of stromal PR by showing (after oestrus) that the presence of PR positive cells was maintained in the connective tissue during the oestrous cycle as well as in inseminated/early pregnant sows.

In the sow myometrium, uterine contractions have been shown to increase around oestrus (Scheerboom *et al.* 1987, Langendijk *et al.* 2002). In the myometrium at oestrus we found high levels of ER α in both cyclic and inseminated sows in our previous studies (Sukjumlong *et al.* 2003, 2004b) and high levels of PR in both experimental groups of sows in the present study. Furthermore, Scheerboom *et al.* (1987), reported that the characteristics of uterine myometrial activity during pregnancy were similar to those of a cyclic sow until day 12, and were thereby independent of the presence of blastocysts. Our present study supports this finding by showing a similar decrease of PR in the myometrium from oestrus until late dioestrus in cyclic sows/d 19 in early pregnant sows. From these results, we speculate that PR in the myometrium is upregulated by high plasma levels of oestradiol-17 β during oestrus in order to prepare for the progesterone regulation via PR at following stages of the oestrous cycle/pregnancy. Increasing and high levels of progesterone have been shown to provide a quiescent uterus with low myometrial activity (Porter & Watts 1986, Ding *et al.* 1994) and to decrease PR levels (Batra & Iosif 1989, Bouchard 1999),

which was supported by our present results with low PR levels at d 19 of early pregnant sows (group 5, experiment B) compared with high PR levels at prooestrus in cyclic sows (experiment A).

From the previous studies of ER α and proliferative activities in the uteri of the same sows (Sukjumlong *et al.* 2003, 2004b), significant correlation was found between ER α stromal cells and a proliferative marker, Ki-67, in the epithelia. Though no significant correlation between ER α and Ki-67 cells was found for the other tissue compartments, a similar pattern was noticed. However, there may be other factors than oestrogen involved in the regulation of proliferation as progesterone has also been shown to promote proliferation via the induction of growth factors (Reynolds *et al.* 1990, Taketani & Mizuno 1991) and a high correlation between PR expression and endometrial cell proliferation was found in early pregnant goats (Flores *et al.* 2001). In addition, downregulation of PR resulted in inhibition of uterine stromal cell proliferation in pregnant rats (Rider & Psychoyos 1994). In the present study, a high presence of PR in the epithelia was observed at oestrus, while high proliferation, as studied previously (Sukjumlong *et al.* 2003, 2004b), was observed in the glandular epithelium at early dioestrus/70 h after ovulation in cyclic and early pregnant sows respectively. It is therefore suggested that high proliferation in the glandular epithelium may be due to the high presence of PR at oestrus, being occupied by low but increasing levels of progesterone and resulting in an induction of proliferation at following stages. An alternative is that proliferation is stimulated indirectly via receptors in the stroma and by growth factors. However, the mechanisms of this regulation need to be elucidated by further studies.

In conclusion, this study shows that immunopresence of PR in the sow uterus differed between uterine compartments at the same reproductive stage. Differences were also found for some uterine compartments when comparing cyclic and inseminated/early pregnant sows at corresponding stages. The relatively consistent immunostaining of PR in the stroma of both cyclic and inseminated/early pregnant sows strengthens a stromal role in the regulation of physiological activities in the sow uterus during the oestrous cycle as well as early pregnancy.

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