Growth and cellular proliferation of pig corpora lutea throughout the oestrous cycle

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Corpora lutea were obtained from gilts on days 2, 4, 8, 12, 15 or 18 after oestrus. Luteal fresh masses and DNA contents increased linearly ($P < 0.01$) from day 2 to day 12 and day 2 to day 15, respectively. Changes in the ratio of protein:DNA were greatest between days 2 and 4 and days 15 and 18, whereas changes in DNA content were relatively small during the same intervals. Thus, a major component of changes in the size of the corpus luteum during the early and late periods of the luteal phase was cellular hypertrophy. Proliferation of luteal cells *in vivo* (nuclear incorporation of 5-bromo-2-deoxyuridine, a thymidine analogue) was greatest on day 2 and decreased exponentially ($P < 0.01$) throughout the oestrous cycle. Results from co-localization of 5-bromo-2-deoxyuridine and factor VIII (von Willebrand factor), a marker of endothelial cells, or 5-bromo-2-deoxyuridine and 3β -hydroxysteroid dehydrogenase, a marker of steroidogenic cells, indicated that some of the luteal steroidogenic cells proliferated early in luteal development. However, during early and mid-cycle, most of the luteal cell proliferation occurred in the endothelial cells. Thus, during growth of the pig corpus luteum, which is extremely rapid, most of the proliferating luteal cells are vascular endothelial cells. This observation is consistent with the high vascularity and blood flow of the mature corpus luteum and implies a critical role for angiogenesis in luteal development in the pig, as has been proposed for several other mammalian species.

Introduction

The corpus luteum is essential for normal reproductive function in mammals as it is the primary source of circulating progesterone (Niswender and Nett, 1988; Reynolds *et al.*, 1994; Reynolds and Redmer, 1999). The corpus luteum also is one of the fastest growing tissues in the adult (Niswender and Nett, 1988; Jablonka-Shariff *et al.*, 1993; Reynolds *et al.*, 1994). Thus, an improved understanding of the mechanisms regulating luteal growth is important not only because of its implications for regulation of fertility, but also because the corpus luteum makes an excellent model to study growth of normal adult tissues (Reynolds *et al.*, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1998).

In domestic animals, it is thought that large luteal cells, which are presumably derived from the follicular granulosa, either do not proliferate or undergo minimal proliferation only at the beginning of the oestrous cycle (Moss *et al.*, 1954; Priedkalns *et al.*, 1968; McClellan *et al.*, 1975; Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994). In contrast, small luteal cells and non-steroidogenic luteal cells, presumably derived from

the follicular theca, appear to proliferate much more rapidly throughout the oestrous cycle and even into early pregnancy (Priedkalns *et al.*, 1968; McClellan *et al.*, 1975; Jablonka-Shariff *et al.*, 1993, 1997; Zheng *et al.*, 1994; Reynolds *et al.*, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1998, 1999).

Accompanying rapid growth of the corpus luteum, dynamic changes in luteal vascularity occur throughout the oestrous cycle (Clark, 1900; Andersen, 1926; Bassett, 1943; Bruce and Moor, 1976; Reynolds, 1986; Reynolds *et al.*, 1992; Zheng *et al.*, 1993). The mature corpus luteum is highly vascular and receives one of the greatest rates of blood flow, per unit of tissue mass, of any organ (Reynolds, 1986; Niswender and Nett, 1988; Reynolds *et al.*, 1992, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1998, 1999). Conversely, decreased luteal vascularity is associated with inadequate luteal function and early embryonic loss (Hansel, 1981; Garverick and Smith, 1986; Niswender and Nett, 1988; Reynolds *et al.*, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1998, 1999).

Although the patterns of growth, vascular development and cellular proliferation of the corpus luteum are well understood in several mammalian species, this information is not yet available for the corpus luteum of domestic pigs (Reynolds *et al.*, 1994; Redmer and Reynolds, 1996; Reynolds

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and Redmer, 1998). Therefore, the objectives of this study were to evaluate luteal growth and development, as well as the relative rates of proliferation of the various types of luteal cell, throughout the oestrous cycle in pigs.

Materials and Methods

Tissue collection

Crossbred (Yorkshire $\times$ Duroc) gilts that had shown at least one oestrous cycle of normal duration (18–23 days) were randomly assigned to be killed on days 2, 4, 8, 12, 15 or 18 of the oestrous cycle ($n = 6$ –8 gilts per day). For each gilt, 10 ml of blood was collected into a heparinized test tube via puncture of the vena cava (Reynolds and Ford, 1984) at 1 h before the animal was killed. After blood was drawn, each pig received an i.v. injection of 5-bromo-2-deoxyuridine (BrdU, 5 mg kg⁻¹ body weight; Aldrich, Milwaukee, WI), a thymidine analogue (Jablonka-Shariff *et al.*, 1993, 1994, 1997; Jin *et al.*, 1994). After the animals were killed, the ovaries were removed and all corpora lutea from both ovaries ($n = 13.4 \pm 0.3$ corpora lutea per gilt) were dissected and weighed. For one gilt on day 15 after oestrus, a large (1320 mg) fluid-filled corpus luteum was found, which was not analysed further and was not included in the data for this gilt. For each gilt, four corpora lutea were chosen at random and were fixed in Carnoy's solution (Jablonka-Shariff *et al.*, 1992, 1994, 1997; Zheng *et al.*, 1993; Jin *et al.*, 1994); these corpora lutea were used for immunohistochemistry and analysis of the labelling index, as described below. Additional samples of all of the remaining corpora lutea were snap frozen on dry ice and stored at -80°C until analysis of DNA, protein and progesterone contents. Thus, DNA, protein and progesterone analyses were conducted only on the remaining corpora lutea ($n = 9.4 \pm 0.3$ per gilt) and not on those obtained for immunohistochemistry and labelling index.

DNA and protein concentrations and contents

DNA and protein concentrations of corpora lutea were determined as described by Reynolds *et al.* (1990), Jablonka-Shariff *et al.* (1993, 1997) and Zheng *et al.* (1994). Briefly, 100 mg of each corpus luteum was homogenized in 10 volumes of buffer containing 3 mmol Na₃I⁻¹ and 1 mmol EDTA I⁻¹ (pH 7.2) using a Polytron (Brinkmann, Westbury, NY). Tissue homogenates were analysed for DNA concentration using the diphenylamine procedure and for protein concentration using the method of Lowry *et al.* (1951) (see also Reynolds *et al.*, 1990). Standards were DNA type I from calf thymus and BSA (fraction V), respectively (Sigma, St Louis, MO). Tissue DNA and protein contents were calculated by multiplying the concentrations by total luteal mass. The DNA content was used as an index of tissue hyperplasia, and the ratio of protein:DNA was used as an index of tissue hypertrophy (Baserga, 1985; Reynolds *et al.*, 1990; Jablonka-Shariff *et al.*, 1993, 1997; Jin *et al.*, 1994; Zheng *et al.*, 1994).

Progesterone radioimmunoassays

Progesterone concentrations were measured in benzene–hexane extracts of plasma by radioimmunoassay as previously validated by Taraska *et al.* (1989) and Redmer *et al.* (1991). The assay sensitivity (90% B/B₀) was 25 pg per tube. All samples were assayed in a single assay; the intra-assay coefficient of variation was 4.5%. The recovery of [³H]progesterone in extracted samples was 85.8%, and progesterone concentrations in the extracts were adjusted for recovery.

Progesterone concentrations were also measured in benzene–hexane extracts of the luteal tissue homogenates using the same radioimmunoassay described above as reported by Jablonka-Shariff *et al.* (1993) and Zheng *et al.* (1994). All of the tissue homogenates were run in a single assay, and the intra-assay coefficient of variation was 5.2%. Recovery of [³H]progesterone in extracted samples was 75.7%, and progesterone concentrations in the extracts were adjusted for recovery. Luteal progesterone contents were calculated by multiplying the tissue concentrations by luteal fresh mass (Zheng *et al.*, 1994).

Immunohistochemistry (BrdU, BrdU and factor VIII, BrdU and 3 β -HSD)

BrdU, a thymidine analogue that is incorporated into cellular DNA during the S phase of the cell cycle, was immunolocalized in paraffin-wax-embedded tissue sections (5 μm) using a specific BrdU antibody and the modified avidin–biotinylated peroxidase complex (ABC) method (Schutte *et al.*, 1987; Jablonka-Shariff *et al.*, 1992, 1993, 1997; Jin *et al.*, 1994) to visualize proliferating cells. Briefly, after 15 min treatment with 3% (v/v) H₂O₂ in absolute methanol to quench endogenous peroxidase, followed by 30 min treatment with 2 mol HCl I⁻¹ to denature nuclear chromatin, tissue sections were treated for 20 min with blocking buffer consisting of PBS (0.01 mol phosphate I⁻¹ and 0.14 mol NaCl I⁻¹, pH 7.3) containing 0.3% (v/v) Triton-X100 (Mallinckrodt, Paris) and 1% (v/v) normal horse serum (Vector Laboratories, Burlingame, CA).

Sections were incubated for 60 min at 22°C with a mouse monoclonal anti-BrdU antibody (Boehringer Mannheim, Indianapolis, IN; 1 mg ml⁻¹ in blocking buffer). Control sections were incubated with normal horse serum (Vector Laboratories) or mouse ascites fluid (ICN Biochemicals, Costa Mesa, CA) in place of the primary antibody. Detection of the primary antibody was accomplished using a biotinylated secondary antibody (horse anti-mouse IgG; Vector Laboratories) and the ABC method (Vectastain; Vector Laboratories). The colour reaction was developed by incubating the sections for 3–4 min with a peroxidase substrate (SG; Vector Laboratories) that produces a grey–black colour. Tissue sections were rinsed twice in distilled H₂O and counterstained briefly (10 s) with Harris' haematoxylin (Luna, 1968; Jablonka-Shariff *et al.*, 1992, 1993, 1997; Jin *et al.*, 1994).

Paraffin-wax-embedded tissue sections were stained for BrdU and also for 3 β -hydroxysteroid dehydrogenase (3 β -HSD), the enzyme that catalyses the conversion of

Table 1. Number, fresh masses, DNA content, and protein:DNA ratios of pig corpora lutea throughout the oestrous cycle

	Day of the oestrous cycle					
	2	4	8	12	15	18
Number	13.1 ± 0.7	14.6 ± 1.2	13.9 ± 0.8	12.5 ± 0.8	13.6 ± 0.7	12.8 ± 0.7
Fresh mass (mg)	134 ± 5.1 ^a	336 ± 73.9 ^{ab}	466 ± 37.8 ^{ab}	525 ± 37.5 ^b	513 ± 34.3 ^b	228 ± 31.0 ^a
DNA content						
(mg per corpus luteum)	0.40 ± 0.04 ^a	0.58 ± 0.01 ^{ab}	0.89 ± 0.10 ^{bc}	0.92 ± 0.11 ^c	1.20 ± 0.18 ^c	1.00 ± 0.12 ^c
Protein:DNA	28.1 ± 2.8 ^{ab}	63.4 ± 13.6 ^c	52.4 ± 6.2 ^c	49.9 ± 3.7 ^{bc}	45.9 ± 7.5 ^{bc}	17.2 ± 1.6 ^a

^{abc}Within a row, means with different superscripts are significantly different ($P < 0.05$).

Values are the means ± SE.

pregnenolone to progesterone (Gore-Langton and Armstrong, 1994; Conley *et al.*, 1995) to evaluate proliferation of steroidogenic luteal cells. The first portion of this dual labelling consisted of staining for BrdU as described above. Subsequently, slides were incubated (22°C) for 20 min in blocking buffer (PBS, Triton-X100 with 1–2% (v/v) goat serum; Vector Laboratories), followed by incubation for 90 min at 22°C with a specific rabbit polyclonal antibody against 3β-HSD (1:250 in blocking buffer; Oxygene, Dallas, TX) as reported by Conley *et al.* (1995). Control sections were incubated with normal rabbit serum (Vector Laboratories) in place of the 3β-HSD primary antibody. Sections were rinsed twice in PBS and incubated with a biotinylated secondary antibody (goat anti-rabbit IgG; Vector Laboratories) for 30 min. After rinsing twice in PBS, sections were incubated with ABC complex (Vector) for 30 min and rinsed twice in PBS. The colour reaction was developed by incubating sections (3–4 min) in a second peroxidase substrate (AEC; Vector Laboratories), which produces a yellow–brown deposit.

Paraffin-wax-embedded tissue sections were stained for BrdU and for factor VIII (von Willebrand factor), which is a specific marker for endothelial cells (Shepro and D'Amore, 1984; Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1993; Reynolds *et al.*, 1994), to evaluate proliferation of endothelial cells. The first portion of this dual labelling was staining for BrdU as described above. Factor VIII was immunolocalized using a specific rabbit anti-human factor VIII antibody (Sigma) and indirect immunoperoxidase detection via the ABC method as reported by Reynolds *et al.* (1992), Jablonka-Shariff *et al.* (1993) and Zheng *et al.* (1993). For this procedure, factor VIII antibody (final dilution 1:100 in blocking buffer) was incubated with tissue sections for 30 min at 22°C. Control staining consisted of replacing the factor VIII primary antibody with the same dilution of normal rabbit serum (Vector Laboratories). The factor VIII primary antibody was then visualized using the same procedures as described above for visualization of the 3β-HSD primary antibody, except that the peroxidase substrate used (VIP; Vector Laboratories) produces a purple deposit.

Labelling index

For each corpus luteum, seven randomly chosen fields were evaluated in a randomly chosen tissue section ($n = 1-4$

corpora lutea per animal). The labelling index, which provides an index of the rate of cell proliferation, was calculated as the area of positively stained (BrdU-labelled) nuclei as a percentage of the total area stained (BrdU- plus haematoxylin stained) using an image analysis system (VIDAS v.2.5; Roche Image Analysis Systems, Elon College, NC) as reported by Jablonka-Shariff *et al.* (1993), Jin *et al.* (1994) and Fricke *et al.* (1997). It has been demonstrated that this procedure compares favourably with the use of tritiated thymidine for determining the labelling index *in vivo* (Jablonka-Shariff *et al.*, 1993; Lin and Allison, 1993; Jin *et al.*, 1994; Fricke *et al.*, 1997).

Statistical analysis

Data were analysed using the general linear model (GLM) (SAS, 1985) analysis of variance; the day of the oestrous cycle was the main effect. When the F test was significant ($P < 0.05$), differences among means were evaluated with Duncan's multiple-range test (SAS, 1985). In addition, linear and non-linear regression procedures were used to evaluate the relationships among the variables (SAS, 1985). Unless otherwise noted, the data are reported as means ± SE.

Results

The average numbers of corpora lutea per gilt did not vary across the stages of the oestrous cycle (Table 1). Fresh masses of corpora lutea increased ($P < 0.01$) linearly ($y = 135.8 + 37.2x$; where y is mass (mg) and x is day of the oestrous cycle; $r = 0.93$) and by more than fourfold from day 2 to day 12 of the oestrous cycle (Table 1). In contrast, luteal DNA contents increased ($P < 0.01$) linearly ($y = 0.32 + 0.058x$; $r = 0.94$) from day 2 to day 15 (Table 1). Nevertheless, luteal fresh masses were highly correlated ($r = 0.96$; $P < 0.05$) with luteal DNA contents. Changes in the size of luteal cells, as indicated by the ratio of protein:DNA, were greatest between days 2 and 4 and days 15 and 18 (Table 1), and these changes were also apparent histologically (Fig. 1). Although the size of the cells was smaller on day 2 compared with days 4–12, the cells appeared to be normal and healthy on the basis of their nuclear morphology (Fig. 1a). In contrast, on days 15 and 18 the cells were not only smaller but many of the nuclei

Fig. 1. Immunolocalization of 5-bromo-2-deoxyuridine (BrdU) in sections of corpora lutea from pigs on days 2 (a), 4 (b), 8 (c), 12 (d), 15 (e) and 18 (f) of the oestrous cycle. Two BrdU-labelled nuclei are indicated (arrowheads) in each field. Note the marked increase in the size of cells from day 2 to day 4 (a,b); also note on days 15 and 18 (e,f) the marked reduction in the size of cells as well as the 'pockets' of pyknotic nuclei (small arrows) dispersed among regions of apparently healthy cells (large arrows). Scale bar represents 50 μ m.

appeared pyknotic, and 'pockets' of these pyknotic nuclei were dispersed among regions of apparently healthy cells (Fig. 1e,f). Luteal progesterone content and plasma progesterone concentrations followed a pattern similar to that of luteal masses (Table 2).

Immunohistochemical staining of pig corpora lutea for

BrdU showed that the relative rate of luteal cell proliferation (that is, the proportion of cells showing incorporation of BrdU into the nucleus) was greatest on days 2 and 4 of the oestrous cycle and was less on all of the other days (Fig. 1). These observations were in agreement with the quantitative evaluation of luteal labelling index, which was greatest

Table 2. Luteal progesterone contents and plasma progesterone concentrations throughout the oestrous cycle in pigs

	Day of the oestrous cycle					
	2	4	8	12	15	18
Luteal progesterone (mg per corpus luteum)	6.7 ± 1.3 ^a	18.6 ± 6.4 ^b	36.1 ± 3.2 ^{bc}	52.1 ± 4.1 ^c	27.4 ± 10.4 ^b	1.0 ± 0.3 ^a
Plasma progesterone (ng ml ⁻¹)	5.0 ± 1.6 ^a	21.6 ± 2.0 ^b	33.2 ± 2.7 ^{bc}	35.7 ± 2.7 ^c	22.8 ± 7.2 ^b	0.7 ± 0.2 ^a

^{abc}Within a row, means with different superscripts are significantly different ($P < 0.05$).

Values are the means ± SE.

Fig. 2. Labelling index (LI) of pig corpora lutea throughout the oestrous cycle. These values are based on the area of 5-bromo-2-deoxyuridine (BrdU)-labelled nuclei expressed as a percentage of the total stained area (haematoxylin + BrdU) for seven randomly chosen fields in each of four corpora lutea per gilt ($n = 6-8$ gilts per day). The labelling index on days 2 and 4 was greater ($P < 0.05$) than on all other days. The labelling index decreased exponentially ($P < 0.01$) throughout the oestrous cycle (regression line: $LI = 34.51807e^{(-0.214008 + 0.006969t)}$, $r = 0.70$, where LI = the labelling index at any given time, LI_0 = the labelling index at time 0, k_1 = the initial instantaneous growth rate (proportion per day), k_2 = the change in the instantaneous growth rate per unit of time, and t = time in days). Pooled SEM = 2.23%.

($P < 0.05$) on days 2 and 4 of the oestrous cycle ($21.0 \pm 4.2\%$; Fig. 2) but reduced thereafter ($8.6 \pm 1.1\%$ across days 8–18). Overall, the decrease in labelling index throughout the oestrous cycle could be described ($P < 0.01$) by the exponential growth model (Jablonka-Shariff *et al.*, 1993; Reynolds and Redmer, 1999):

$$LI = LI_0 \times e^{(-k_1 + k_2 \times t)t}$$

where LI = the labelling index at any given time, LI_0 = the labelling index at time 0, k_1 = the initial instantaneous growth rate (proportion per day), k_2 = the change in the instantaneous growth rate per unit of time, and t = time in days.

Factor VIII was present in the luteal tissues on all days of the oestrous cycle and was immunolocalized to the cytoplasm of the endothelial cells of the larger microvessels, as well as to those of the capillaries (Fig. 3). On day 2, localization was primarily in the larger microvessels within the connective tissue (presumably invading thecal) tracts

(data not shown). By day 4 and continuing to day 15, factor VIII was localized in the capillaries throughout the luteal parenchyma as well as in the larger microvessels that were located primarily in the connective tissue tracts (Fig. 3a,b). In addition, during mid-cycle (days 4–15), endothelial cells formed a dense network surrounding the luteal parenchymal cells (Fig. 3a,b), thus, in the mature corpus luteum most of the parenchymal cells were in contact with one or more capillaries. However, by day 18, which represents the period of luteal regression, factor VIII expression was confined almost exclusively to the larger microvessels (Fig. 3c).

Dual staining for BrdU and factor VIII demonstrated that most (approximately 60–75%) of the proliferating cells on days 2–4 of the oestrous cycle were endothelial cells (Fig. 3a). Thus, co-localization of BrdU and factor VIII was greatest early in the oestrous cycle. By mid-cycle, although many of the BrdU-labelled cells still appeared to be endothelial cells, an equal number of BrdU-labelled cells were negative for factor VIII (Fig. 3b). By day 18, most of the cell proliferation was in factor VIII-negative cells (Fig. 3c); when present, the BrdU-labelled endothelial cells were associated with the larger microvessels. However, only approximately one-third of the corpora lutea on day 18 showed cell proliferation, whereas cell proliferation in most of the day 18 corpora lutea was relatively low.

The steroidogenic enzyme 3β -HSD was present in the luteal tissues on all days of the oestrous cycle and was localized to the cytoplasm of the luteal parenchymal cells (Fig. 4). On day 2, although 3β -HSD was found throughout the luteal parenchyma, the concentrations were relatively low (data not shown). By day 4, and continuing to day 15, the intensity of 3β -HSD staining remained relatively high (Fig. 4b). By day 18, the intensity of 3β -HSD staining had again decreased (Fig. 4c).

Dual staining for BrdU and 3β -HSD demonstrated that although relatively few of the BrdU-positive cells also expressed 3β -HSD, most of the proliferating steroidogenic cells were present early in the oestrous cycle (Fig. 4a). By day 8 and thereafter, proliferating steroidogenic cells were observed only rarely (Fig. 4b,c).

Discussion

The results of the present study demonstrate that the pig corpus luteum grows extremely rapidly and shows

Fig. 3. Dual immunohistochemical staining for factor VIII (purple) and 5-bromo-2-deoxyuridine (BrdU) (grey-black) in sections of corpora lutea from pigs on days 4 (a), 12 (b) and 18 (c) of the oestrous cycle. Note the cytoplasmic labelling for factor VIII (purple), nuclear labelling for BrdU (arrowheads), and frequent dual labelling of cells (arrows); also note factor VIII staining of capillaries early in the oestrous cycle (a) and at mid-cycle (b), but primarily larger microvessels (*) late in the oestrous cycle. Scale bar represents 25 μ m.

Fig. 4. Dual labelling for 3 β -hydroxysteroid dehydrogenase (3 β -HSD) (yellow-brown) and 5-bromo-2-deoxyuridine BrdU (grey-black) in sections of corpora lutea from pigs on days 4 (a), 12 (b) and 18 (c) of the oestrous cycle. Note the cytoplasmic labelling for 3 β -HSD (yellow-brown) and nuclear labelling for BrdU (arrowheads); some dual-labelled cells (arrows) are present early in the oestrous cycle (a) but none later in the oestrous cycle (b,c). Scale bar represents 25 μ m.

extremely high rates of cellular proliferation from day 2 to day 12 of the oestrous cycle. The results of the immuno-histochemistry show that although both parenchymal (steroidogenic) and non-parenchymal (that is, primarily endothelial cells, pericytes and fibroblasts) luteal cells proliferate throughout the oestrous cycle, most of the cell proliferation in corpora lutea at the early stage of the oestrous cycle occurs in the non-steroidogenic cells.

In this study, steroidogenic activity of luteal parenchymal cells was determined by measuring progesterone concentrations in plasma and luteal tissues. In agreement with previous reports (Masuda *et al.*, 1966), the profile of plasma progesterone concentrations was similar to that of luteal progesterone content; there was an increase from the early to the mid-luteal phase. Thus, luteal progesterone content appears to provide a good index of normal function of the corpus luteum in pigs. In addition, luteal progesterone content was reflected by changes in the intensity of 3 β -HSD staining, which was least on days 2 and 18 and greatest on days 4–15, in agreement with other studies of luteal 3 β -HSD (Conley *et al.*, 1995).

On the basis of the linear increases in both luteal mass and DNA content, the doubling time for tissue mass or number of cells between days 2 and 12 was approximately 122–194 h, which is slower than that reported for sheep (57–78 h; Jablonka-Shariff *et al.*, 1993). Nevertheless, the pig corpus luteum, as reported for other species, grows as fast as even the most aggressive tumours, such as Burkitt's lymphoma (Baserga, 1985; Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Christenson and Stouffer, 1996). The labelling index of the early corpus luteum also was high (24%) compared with that of normal adult tissues, which is typically < 0.5%. Therefore, the rates of growth and cellular proliferation of pig corpora lutea are comparable with those of the fastest growing tissues reported.

The data for luteal mass obtained in the present study are in agreement with previous reports by Cavazos *et al.* (1969) and Conley and Ford (1991). However, in addition, it was shown that between days 2 and 4 of the oestrous cycle, the marked increase in luteal fresh masses was accompanied by only a 45% increase in luteal DNA content. In contrast, during this same interval the luteal protein:DNA ratio increased by more than twofold. Thus, the early growth of the pig corpus luteum is associated primarily with cellular hypertrophy (that is, an increase in the size of cells) and not with cellular hyperplasia (that is, an increase in the number of cells). Similarly, between days 15 and 18, the more than twofold decrease in the size of the corpus luteum was associated with a 2.7-fold decrease in cell protein:DNA ratio but only a 20% decrease in DNA content. Thus, during early growth (days 2–4) and regression (days 15–18) of the pig corpus luteum, changes in the size of the corpus luteum appear to be primarily a consequence of changes in the size of cells rather than changes in the number of cells. In contrast, from day 4 to day 15, growth of the pig corpus luteum was associated primarily with increased DNA content, indicating continued hyperplasia, but little or no change in the protein:DNA ratio.

This pattern of growth is in contrast to those observed in corpora lutea from cows and sheep (Jablonka-Shariff *et al.*,

1993; Zheng *et al.*, 1994; Reynolds *et al.*, 1994; Reynolds and Redmer, 1999). In both cows and sheep, luteal growth depends primarily on changes in the number of cells and not on changes in the size of cells. Furthermore, in corpora lutea from cows and sheep, the ratio of protein:DNA remains constant throughout the oestrous cycle until it decreases in association with luteal regression (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994). However, the protein:DNA ratio only provides an indication of the average size of cells, and these data should therefore be interpreted with this in mind. In sheep, for example, although the overall size of cells does not change, the size of the large luteal cells, which represent 11% of the total number of cells, increases by threefold; conversely, the size of the non-steroidogenic cells (fibroblasts, endothelial cells and pericytes), which represent 68% of the total number of cells, decreases by 1.2- to 2-fold (Farin *et al.*, 1986). Nevertheless, the results of the present study indicate that an increase in the average size of cells is an important component of early luteal growth in pigs but not in cows and sheep.

Assuming that there is a constant amount of DNA per nucleus (~ 6.2 pg; Baserga, 1985; Reynolds *et al.*, 1990), the total number of cells per corpus luteum was approximately 67, 97, 150, 155, 203 and 163 $\times 10^6$ on days 2, 4, 8, 12, 15 and 18, respectively. Therefore, on the basis of the labelling index and the estimated number of cells per corpus luteum, the total number of cells per corpus luteum that were synthesizing DNA remained relatively constant throughout the oestrous cycle, and was 35, 38, 33, 27, 32 and 30 $\times 10^6$ per day on days 2, 4, 8, 12, 15 and 18, respectively. This pattern of cellular proliferation also differs from those of cows and sheep, in which significant increases in the total number of proliferating cells occur early in the oestrous cycle (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Reynolds and Redmer, 1999).

The pig corpus luteum also contains a substantial number of proliferating cells during luteal regression, which is not observed in either cows or sheep (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Reynolds and Redmer, 1999). A portion of this sustained high rate of cell proliferation may be due to cell turnover in the portion of the luteal vasculature that is maintained during luteal regression. In addition, it may be due to an increased content of fibroblasts in association with resorption of the luteal parenchymal tissue and increased fibrous tissue content of the regressing corpus luteum (Reynolds *et al.*, 1994; Reynolds and Redmer, 1999). However, such high rates of cell proliferation are not observed during luteal regression in other species that show similar morphological changes during luteal regression (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Reynolds and Redmer, 1999).

Studies in which morphometric techniques were used to evaluate luteal development in several species demonstrated that corpora lutea undergo dynamic changes in cellular composition throughout the oestrous cycle (Parry *et al.*, 1980; Niswender *et al.*, 1985; Farin *et al.*, 1986, 1989; Schwall *et al.*, 1986; Lei *et al.*, 1991; Wiltbank and Niswender, 1992). On the basis of histological observations of mitotic figures in ovine and bovine corpora lutea, it was suggested that steroidogenic luteal cells proliferate only early in the oestrous cycle

(McClellan *et al.*, 1975; O'Shea *et al.*, 1980, 1986). However, the present study and several other studies have demonstrated that at least a few steroidogenic cells proliferate throughout most of the oestrous cycle (Priedkalns *et al.*, 1968; Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Reynolds and Redmer, 1999), albeit at a relatively low rate, especially after mid-cycle.

The observation that a large proportion of the proliferating cells in the pig corpus luteum are endothelial cells is consistent with data from other species. In rats, the greatest labelling index of luteal endothelial cells (26–36%) was observed within 7–24 h after ovulation, but the labelling index remained relatively high (2–4%) at the later stages of luteal development (Gaede *et al.*, 1985; Meyer and McGeachie, 1988). More recent studies have confirmed that a major portion of the proliferating luteal cells in cows, rats, primates and sheep are endothelial cells (Jablonka-Shariff *et al.*, 1993; Zheng *et al.*, 1994; Christenson and Stouffer, 1996). These observations are not unexpected because, by mid-cycle, endothelial cells form $\geq 50\%$ of the cells of the mature corpus luteum (Farin *et al.*, 1986; Lei *et al.*, 1991; Reynolds and Redmer, 1999).

During its rapid growth phase, the corpus luteum of domestic animals shows dynamic structural and functional changes that must be tightly regulated to ensure normal growth and development (Baserga, 1985; Reynolds *et al.*, 1992, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1999). After ovulation, the corpus luteum is formed from the granulosa and thecal cells of the ruptured follicle (Zheng *et al.*, 1993; Reynolds *et al.*, 1994; Redmer and Reynolds, 1996; Reynolds and Redmer, 1999). Thecal invasion brings with it a host of blood vessels and proliferating endothelial cells, and the number of these non-steroidogenic cells increases continually (Reynolds and Redmer, 1999). These observations are consistent with the present study, in which most of the proliferating cells during early and mid-cycle were endothelial cells. As a result of this rapid endothelial proliferation and vascular growth, the steroidogenic cells were closely associated with capillaries by mid-cycle. These observations are similar to reports for corpora lutea of several other species (Dharmarajan *et al.*, 1985; Zheng *et al.*, 1993; Jablonka-Shariff *et al.*, 1994; Reynolds *et al.*, 1994; Redmer and Reynolds, 1996) and highlight the importance of angiogenesis in normal luteal growth and development (Redmer and Reynolds, 1996; Reynolds and Redmer, 1999).

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