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Evaluation of Growth, Cell Proliferation, and Cell Death in Bovine Corpora Lutea throughout the Estrous Cycle[1]

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ABSTRACT

To evaluate the kinetics of luteal growth, bovine CL were obtained from four stages (stage I, Days 1–4; stage II, Days 5–10; stage III, Days 11–17; stage IV, Days 18–21) of the estrous cycle, and luteal fresh weight as well as DNA, protein, and progesterone contents was determined. To evaluate the relative rate of cell proliferation, proliferating cell nuclear antigen (PCNA; a specific marker for cell proliferation) was immunolocalized in paraffin-embedded luteal tissue sections. To evaluate the relative rate of cell death, nucleosomal fragmentation of DNA (a specific marker for apoptosis) was detected by agarose gel electrophoresis and also by histochemical localization in paraffin-embedded luteal tissue sections.

Luteal fresh weight and DNA, protein, and progesterone contents increased ($p < 0.01$) from stage I to stage II, were similar between stages II and III, and then decreased ($p < 0.01$) from stage III to stage IV. The ratio of protein to DNA (an index of average cell size) was similar for stages I, II, and III and then decreased ($p < 0.01$) at stage IV. For stage I (corpora hemorrhagica), most proliferating (PCNA-positive) cells were located in or around the core of the tissue infoldings (presumably thecal-derived areas), whereas relatively few proliferating cells were located at the periphery of the tissue infoldings (presumably granulosa-derived areas). For stages II, III, and IV, the majority of proliferating cells appeared to be small cells (i.e., small parenchymal cells, fibroblasts, and endothelial cells). The labeling index (LI; percentage of cells that were PCNA-positive) was greatest at stage I (20.3 ± 1.1%); it then decreased ($p < 0.01$) by stage II and was similar at stages II, III, and IV (3.4 ± 1.1%). Apoptosis, as determined by evaluation of nucleosomal DNA fragmentation by agarose gel electrophoresis and in situ localization, was detectable only at stage IV. These data demonstrate that luteal growth from stage I to stage II resulted from cell proliferation as shown by a high LI at stage I, accompanied by increased luteal DNA content but no change in average cell size, and by similar protein:DNA ratios. Luteal regression from stage III to stage IV was primarily associated with cell deletion and decreased cell size as shown by a decrease in luteal DNA content and the appearance of apoptosis along with a decrease in the luteal protein:DNA ratio. In addition, analysis of the estimated kinetics of cell turnover revealed that the number of proliferating luteal cells remained high until stage III and then decreased at stage IV in association with luteal regression.

INTRODUCTION

The CL is one of the fastest growing tissues in the adult female and also one of the few adult tissues that exhibit periodic growth and regression [1–3]. During the first ten days of the 21-day bovine estrous cycle, luteal weight increases by 20- to 30-fold [2, 4], and if pregnancy does not occur, luteal regression will occur equally rapidly, beginning on Day 16 or 17 after estrus.

Previous studies have demonstrated that the CL undergoes dynamic changes in composition and function throughout the estrous cycle [3–9]. For example, it has been suggested that bovine and ovine large luteal cells proliferate only during the early stages of luteal development and that thereafter little or no mitotic activity is observed [5–8]. Small luteal cells and non-steroidogenic luteal cells, however, may continue to proliferate until the onset of luteal regression [3, 6–8].

In addition to cell proliferation, cell deletion through cell death or migration plays an equally important role in regulating tissue growth and regression [10–12]. Recent studies have shown that apoptosis may play an important role during luteal regression in cows and ewes [13, 14]. Changes in the rate of cell turnover as estimated by evaluating proliferation and apoptosis of luteal cells, however, have not been determined throughout the estrous cycle in cows. The aims of the present study, therefore, were to assess the kinetics of luteal growth throughout the estrous cycle in cows by simultaneously evaluating 1) luteal fresh weight and luteal DNA, protein, and progesterone contents; 2) the relative rate of cell proliferation (by immunohistochemical localization of proliferating cell nuclear antigen [PCNA]); and 3) the relative rate of apoptosis (by detection of nucleosomal fragmentation of DNA by agarose gel electrophoresis and also by histochemical localization in luteal tissue sections).

MATERIALS AND METHODS

Tissue Collection and Preparation

Ovaries were collected from nonpregnant cows at a local slaughterhouse and transported immediately to the laboratory on ice. The stage of the estrous cycle (stage I, Days 1–4; stage II, Days 5–10; stage III, Days 11–17; stage IV, Days 18–21; n = 10 to 12 CL per stage) was determined for each CL on the basis of its morphological appearance

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TABLE 1. Fresh weight and DNA, protein (PTN), and progesterone (P) contents and PTN:DNA ratio of bovine CL at four stages of the estrous cycle.*

Items	Stage I	Stage II	Stage III	Stage IV	SE
Fresh wt (g/CL)	0.9[a]	5.5[b]	6.5[b]	1.6[a]	0.5
DNA content (mg/CL)	1.2[a]	9.2[b]	10.1[b]	2.2[a]	0.7
PTN content (mg/CL)	66.1[a]	461.0[b]	519.3[b]	84.9[a]	33.8
P content (μg/CL)	39.7[a]	369.6[b]	400.4[b]	6.7[a]	34.1
PTN:DNA	56.2[a]	51.0[a]	51.6[a]	37.5[b]	3.3

*Means for stage I (Days 1 to 4), stage II (Days 5 to 10), stage III (Days 11 to 17), and stage IV (Days 18 to 21).
[a,b]Within each row, means with different superscripts differ ($p < 0.01$).

as described previously [15, 16]; CL were then enucleated and weighed. Within a stage, CL were assigned randomly to one of two groups. In the first group (5 to 6 CL per stage), a portion of each CL was minced and stored at −70°C until DNA, protein, and progesterone concentrations could be determined, and the remaining portion was fixed by immersion in Bouin's solution [17]. In the second group (5 to 6 CL per stage), a portion of each CL was minced, frozen in liquid nitrogen, and stored at −70°C until nucleosomal fragmentation of DNA by agarose gel electrophoresis could be evaluated. The remaining portion of each CL was fixed by immersion in 10% buffered formalin [17]. For both groups, fixed luteal tissues were dehydrated by use of a graded series of ethanol, cleared with Americlear solvent (Baxter, McGraw Park, IL), embedded in paraffin (Paraplast Plus, Baxter), sectioned (5 μm), and mounted onto glass slides, as we have described previously [3, 18].

DNA and Protein Concentrations

Frozen luteal tissues were homogenized in 10 volumes of Tris-HCl buffer (0.05 M Tris, 2 M NaCl, and 2 μM EDTA, pH 7.4) by use of a Polytron (Brinkmann Instr., Westbury, NY) followed by sonication with an ultrasonic processor (Sonics & Materials, Danbury, CT). Tissue homogenates were analyzed for concentrations of DNA by use of the Hoechst dye 33528 procedure [19] and for concentrations of protein by use of the Coomassie Brilliant Blue G assay, as we have reported previously [20]. Standards were DNA type I from calf thymus and BSA (fraction V), respectively (Sigma Chemical Co., St. Louis, MO). Luteal DNA and protein contents were calculated by multiplying their tissue concentrations by luteal fresh weight. Content of DNA was used as an index of cellular hyperplasia, and the protein:DNA ratio was used as an index of cellular hypertrophy [3, 11].

Progesterone RIA

Progesterone concentrations were measured in benzene:hexane extracts of the luteal homogenates mentioned above as previously validated by us in our laboratory [3, 19]. Assay sensitivity was 25 pg/tube. All samples were run in a single assay, and the intraassay coefficient of variation was 5.8%. Recovery of ^{3}H-progesterone in extracted samples was 64 ± 2.7%, and concentrations of progesterone in the extracts were adjusted for recovery. Luteal progesterone contents were calculated by multiplying their tissue concentrations by luteal fresh weight.

PCNA Immunohistochemistry and Morphometry

As an index of the relative rate of cell proliferation, PCNA was immunolocalized in Bouin's-fixed luteal tissue sections by means of a specific monoclonal antibody (Boehringer-Mannheim, Indianapolis, IN) and the modified avidin-biotin-peroxidase complex (ABC) method as described by us previously [3, 18]. The tissue sections were incubated with the PCNA antibody (1:500 in PBS [0.01 M phosphate, 0.14 M NaCl, 0.3% Triton X, 1.5% normal horse serum, pH 7.2]) for 2 h at room temperature. Control staining consisted of omitting the primary antibody. Sections were counterstained briefly (~ 2 sec) with Harris' hematoxylin to visualize unlabeled nuclei. For each CL, photomicrographs of five randomly chosen fields (0.21 mm^2/field) were taken. For each photomicrograph, the labeling index (LI; number of PCNA-stained nuclei expressed as a percentage of total nuclei) was determined for three randomly chosen areas. A total of 187.5 ± 4.2 nuclei were evaluated per photomicrograph; thus approximately 940 nuclei were evaluated per CL.

TABLE 2. DNA, protein (PTN), and progesterone (P) concentrations of bovine CL at four stages of the estrous cycle.*

Items	Stage I	Stage II	Stage III	Stage IV	SE
DNA concentration (mg/g CL)	1.3[a]	1.7[a]	1.6[a]	1.4[a]	0.03
PTN concentration (mg/g CL)	73.7[a]	83.4[a]	81.2[a]	50.1[b]	2.69
P concentration (μg/g CL)	41.4[a]	65.6[a]	65.7[a]	3.1[b]	5.22

*Means for stage I (Days 1 to 4), stage II (Days 5 to 10), stage III (Days 11 to 17), and stage IV (Days 18 to 21).
[a,b]Within each row, means with different superscripts differ ($p < 0.05$).

TABLE 3. Correlations among fresh weight, DNA content, protein (PTN) content, progesterone (P) content, PTN:DNA ratio, and LI of bovine CL throughout the estrous cycle.*

Items	DNA content	PTN content	P content	PTN:DNA	LI
Fresh wt	0.95[a]	0.98[a]	0.87[a]	0.30[e]	−0.47[b]
DNA content		0.98[a]	0.95[a]	0.17[e]	−0.53[a]
PTN content			0.93[a]	0.31[e]	−0.47[a]
P content				0.23[e]	−0.44[c]
PTN:DNA					0.50[d]

*LI, expressed as the percentage of cells exhibiting staining for PCNA (see *Materials and Methods.*)
[a]$p < 0.01$, [b]$p < 0.03$, [c]$p < 0.04$, [d]$p < 0.02$, [e]$p > 0.10$.

Apoptosis Assays

Nucleosomal banding. Genomic DNA was isolated from frozen luteal tissue samples as described previously [21]. Briefly, frozen samples from each CL (~ 0.2 g/CL) were ground with a pestle in a mortar filled with liquid nitrogen. After grinding, 0.8 ml of DNA isolation buffer (10 mM Tris-HCl, 0.1 M NaCl, 0.5% SDS, 0.5 M EDTA, 0.25 mg/ml proteinase K [Sigma], pH 8.0) was added to each frozen sample, and the sample then was incubated at 56°C for 1 h. After incubation, 4 ml of DNase-free RNase (10 μg/ml in 10 mM Tris-HCl, pH 8.0; Pharmacia Biotech, Montreal, Canada) was added, and the mixture was incubated at 56°C for 15 min. Each sample then was extracted twice with 2 ml of phenol (pH 8.0) and 2 ml of chloroform/isoamyl alcohol (24:1 [v:v]). The aqueous phase then was extracted with 10 ml of ice-cold isopropanol. The aqueous phase was recovered, and DNA was precipitated with ice-cold 95% ethanol. After two rinses with ice-cold 70% ethanol, each DNA sample was dried overnight at room temperature.

Dried DNA was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0), and the resulting DNA concentration was determined by use of a Beckman DU640 spectrophotometer (Beckman Instr., Palo Alto, CA). Isolated DNA from each CL (average 6 μg/lane) was electrophoretically separated by size in 2% agarose gels in TBE buffer (89 mM Tris base, 89 mM boric acid, 0.2 mM EDTA, pH 8.0) at 80 V for 3 h. Gels were stained with ethidium bromide (0.5 μg/ml) and photographed by UV transillumination. For each gel, a 1-kb DNA ladder (BRL, Gaithersburg, MD) was used to estimate the relative size of fragmented DNA.

Apoptosis in situ. Apoptosis was evaluated in formalin-fixed tissue sections by localizing nuclei exhibiting DNA fragmentation with the histochemical procedure of Gavrieli et al. [22] after some modifications. Briefly, luteal tissue sections were treated with 2 N HCl for 30 min to denature nuclear chromatin [3] and then treated with 3% hydrogen peroxide for 10 min to quench endogenous peroxidase activity. Sections then were incubated in buffer (30 mM Tris, 140 mM cacodylate, 1 mM cobalt chloride, pH 7.2) containing terminal deoxynucleotidyl transferase (TdT, 0.3 e.u./μl; Promega, Madison, WI) and biotin-11-deoxyuridine triphosphate (dUTP, 5 μM; Sigma) for 2 h at 37°C. Control staining consisted of incubating tissue sections in buffer containing only TdT or only dUTP (i.e., without substrate or without enzyme). Nuclei exhibiting DNA fragmentation were visualized through use of the modified ABC method as described above. Sections were counterstained briefly (~ 2 sec) with Harris' hematoxylin to visualize unlabeled nuclei. One section was evaluated for each CL, and the percentage of nuclei exhibiting DNA fragmentation (number of apoptosis-positive nuclei expressed as a percentage of total nuclei) was determined for 15 to 20 randomly chosen fields (0.05 mm^2 and 145.8 ± 17.0 nuclei per field) via a computerized image analysis system as described by us previously [3, 18, 20]. Thus, approximately 2550 nuclei were evaluated for each CL.

Statistical Analysis

Data for luteal weight, LI, and luteal DNA, protein, and progesterone contents and concentrations were analyzed by using general linear models [23], with the main effect of stage in the model. When an F test was significant, differences between specific means were evaluated by using Bonferroni's *t*-test [24]. In addition, the association between variables was determined by using linear regression procedures [24].

RESULTS

Luteal Weight and DNA, Protein, and Progesterone Contents and Concentrations

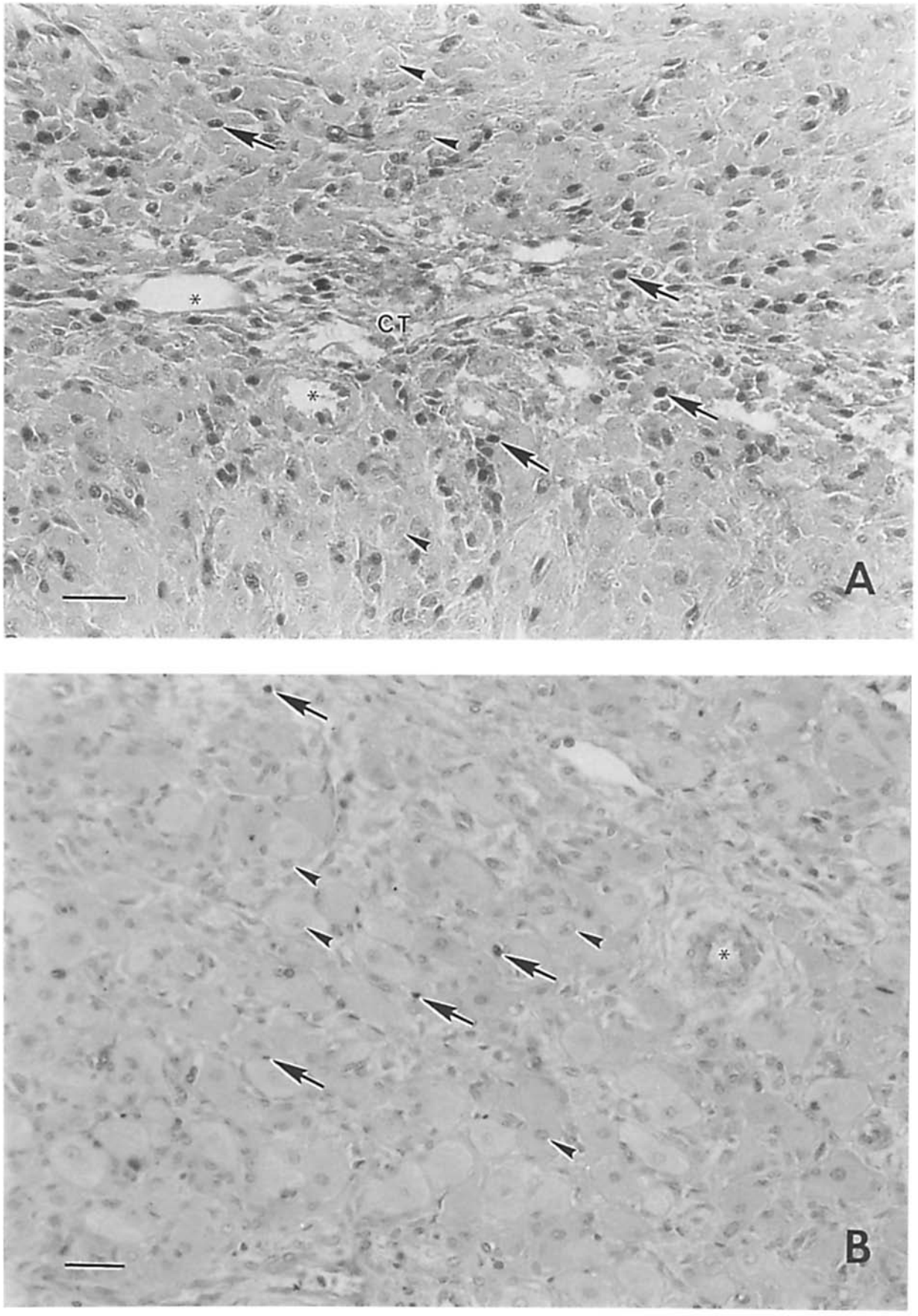
Luteal fresh weight and luteal DNA, protein, and progesterone contents increased ($p < 0.01$) from stage I to stage II, were similar between stages II and III, and then decreased ($p < 0.01$) from stage III to stage IV (Table 1). The ratio of protein to DNA was similar for stages I, II, and III and then decreased ($p < 0.01$) 1.4-fold from stage III to stage IV (Table 1).

Luteal DNA concentration was similar for all four stages. In contrast, luteal protein and progesterone concentrations were similar for stages I, II, and III and then decreased ($p < 0.05$) from stage III to stage IV (Table 2).

Luteal fresh weight was positively correlated ($p < 0.01$) with DNA, protein, and progesterone contents and was negatively correlated ($p < 0.03$) with LI (Table 3). Similarly, DNA content was positively correlated ($p < 0.01$) with protein and progesterone contents and was negatively correlated ($p < 0.01$) with LI. Protein content was positively correlated ($p < 0.01$) with progesterone content and was negatively correlated ($p < 0.01$) with LI (Table 3). Progesterone content was negatively correlated ($p < 0.04$) with LI, and the protein:DNA ratio was positively correlated ($p < 0.02$) with LI (Table 3).

PCNA Immunochemistry and Morphometry

PCNA was immunolocalized in CL from all stages of the estrous cycle (Fig. 1); however, PCNA staining was greater



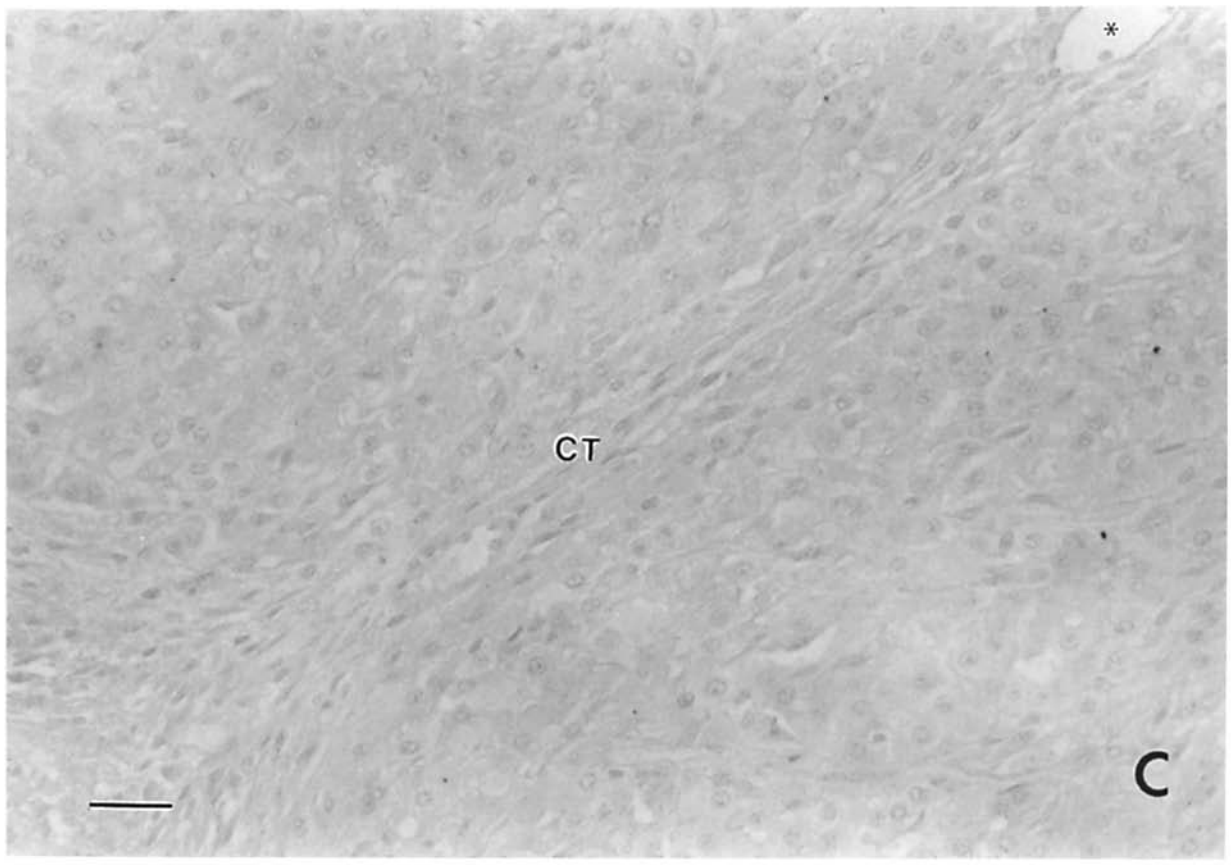


FIG. 1. (Left and above.) Immunohistochemical localization of PCNA in sections of bovine CL from stages I (A) and III (B) of the estrous cycle. Both sections were stained with anti-PCNA antibody. Note that at stage I (A), many proliferating cells were present in or around the cores of the tissue infoldings; at stage III (B), most of the proliferating cells were small cells. Representative control staining for a stage I CL is shown in (C). CT, connective tissue tracts. Arrows indicate PCNA-positive nuclei; arrowheads indicate PCNA-negative nuclei; asterisks indicate the lumen of some larger microvessels. Bars = 50 μm.

for CL at stage I than for CL at any other stage. The distribution of PCNA-positive cells at stage I also was heterogeneous, with most proliferating cells located in or around the cores of the tissue infoldings (presumably thecal-derived areas) and relatively few proliferating cells located at the periphery of the tissue infoldings (presumably granulosa-derived areas; Fig. 1A). In contrast, PCNA-positive cells at stages II, III, and IV were distributed throughout the CL (i.e., in parenchymal areas and connective tissue tracts; Fig. 1B). Nevertheless, for stages II, III, and IV, staining was distributed heterogeneously within a tissue section, with some areas containing many proliferating cells and others containing comparatively few proliferating cells. For CL from stages II, III, and IV, the majority of PCNA-positive cells appeared to be small cells (i.e., small parenchymal cells, fibroblasts, or endothelial cells). The intensity of control staining at each of the four stages was negligible (Fig. 1C). The percentage of cells exhibiting PCNA staining was great-

TABLE 4. Kinetics of cell turnover in bovine CL throughout the estrous cycle.*

Items	Stage I	Stage II	Stage III	Stage IV
Number of cells ($\times 10^6$)[a]	176.6 ± 14.7	1393.8 ± 126.4	1631.3 ± 98.9	328.5 ± 57.4
LI (%)	20.3 ± 1.1	3.5 ± 1.1	3.5 ± 1.0	3.3 ± 1.0
Proliferating cells ($\times 10^6$/day)[b]	77.2 ± 7.1	102.2 ± 9.4	128.7 ± 34.9	27.3 ± 8.7
Apoptosis (%)	ND	ND	ND	3.0 ± 0.4
Dying cells ($\times 10^6$/day)[c]	ND	ND	ND	77.8 ± 8.5

*Means ± SEM for stage I (Days 1 to 4), stage II (Days 5 to 10), stage III (Days 11 to 17), and stage IV (Days 18 to 21).

[a]Estimated by multiplying DNA content (Table 1) by estimated DNA per cell (6.6×10^{-12} g/cell [11]).

[b]Estimated by multiplying number of cells by LI (%), assuming that the duration of the S-phase in vivo is ≈11 h [11].

[c]Estimated by multiplying number of cells by the incidence of apoptosis (%), assuming that the duration of apoptosis as observed histologically is ≈3 h [25].

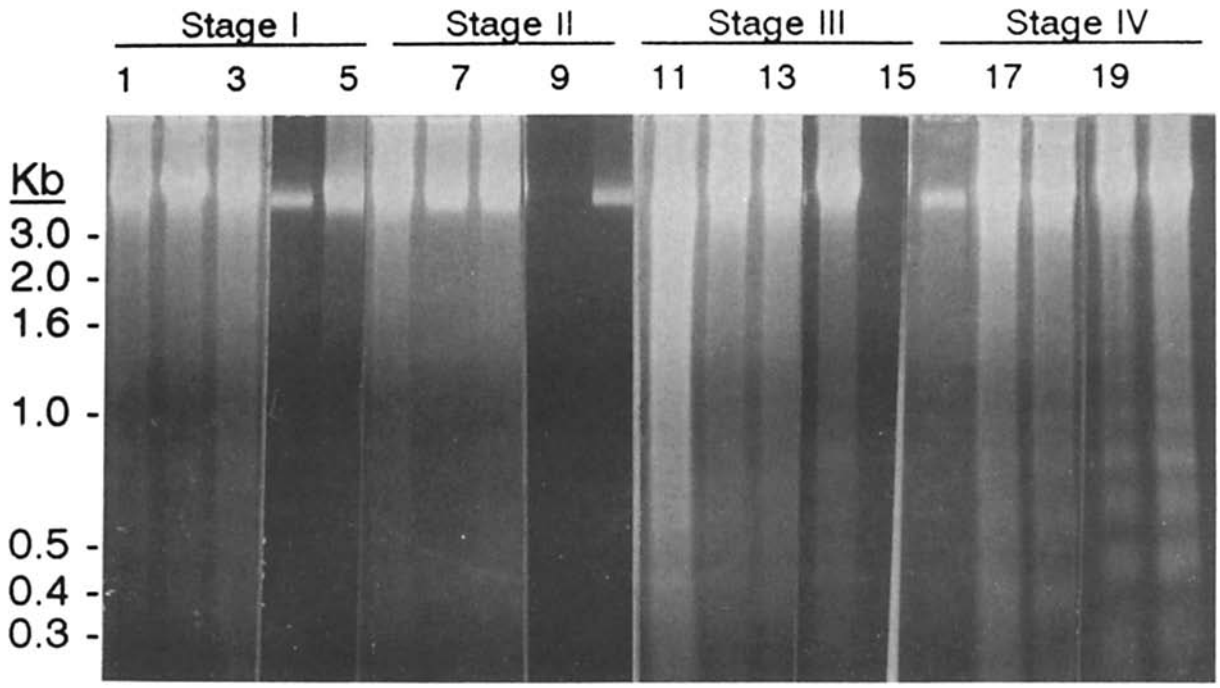


FIG. 2. Ethidium bromide staining of agarose gel (2%) electrophoresis of DNA isolated from bovine CL from stages I (lanes 1–5), II (lanes 6–10), III (lanes 11–15), and IV (lanes 16–20) of the estrous cycle. One-kilobase DNA standards are indicated on the left. Note that genomic DNA fragments were detectable only at stage IV (4 of 5 CL).

est ($p < 0.01$) at stage I, was decreased ($p < 0.01$) by stage II, and was similar at stages II, III, and IV (Table 4).

Apoptosis

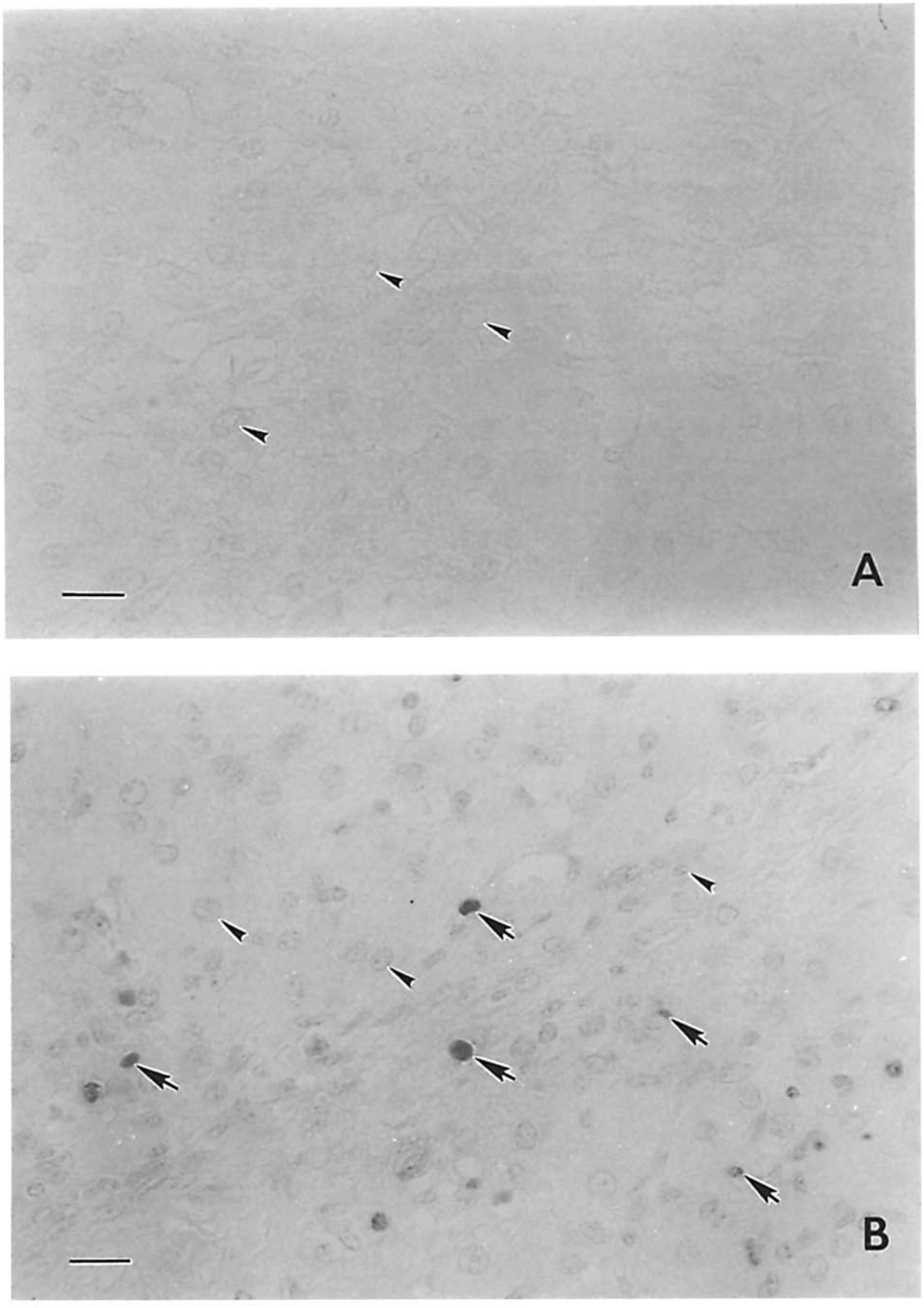
Evaluation of genomic DNA for apoptosis by agarose gel electrophoresis revealed that nucleosomal fragmentation of DNA was detected in 4 of 5 CL from stage IV but was not detectable in any CL from stages I, II, or III (Fig. 2). Consistent with these results, histochemical localization of apoptosis demonstrated that apoptosis-positive cells were present in CL only at stage IV (Fig. 3, A and B, Table 4). In addition, most positively stained cells appeared to have shrunken nuclei and a low cytoplasmic to nuclear ratio (Fig. 3B). The intensity of control staining at each of the four stages was negligible (Fig. 3C).

Kinetics of Luteal Cell Turnover

To evaluate the kinetics of luteal cell turnover throughout the estrous cycle, we used DNA content to estimate the total number of luteal cells and used LI and the incidence of apoptosis (%) to estimate the rates of cell proliferation and cell death for CL from each stage of the estrous cycle (Table 4). The number of luteal cells was estimated by multiplying the DNA content of each CL by the estimated amount of DNA per cell (6.6×10^{-12} g DNA per cell [11]). The number of proliferating luteal cells per day was estimated by multiplying the number of luteal cells by the LI (%) and assuming that the duration of the S-phase in vivo is about 11 h [11]. The number of dying cells per day was estimated by multiplying the number of luteal cells by the incidence of apoptosis (%) and assuming that the duration of apoptosis as observed histologically is about 3 h [25]. The estimated number of proliferating luteal cells increased from stage I to stage II, remained relatively high through stage III, and then decreased dramatically at stage IV. Dying cells, however, were detectable only at stage IV, and their number was 3-fold greater than the number of proliferating cells.

DISCUSSION

In the present study, bovine CL exhibited dynamic changes in fresh weight and in DNA, protein, and progesterone contents throughout the estrous cycle. These data were generally in agreement with previous findings on fresh weight and progesterone content of bovine CL [4, 15]. The present data for DNA and protein contents, however, extend our knowledge of bovine luteal growth and also may provide a good index of functional differentiation, since both DNA and protein contents were highly correlated with luteal fresh weight and progesterone content. Luteal growth was extremely rapid from stage I to stage II as reflected by a 6-fold increase in fresh weight. On the basis of DNA content (an index of cell number) and the ratio of protein to DNA (an index of cell size), results from the present study indicate that this rapid increase in luteal tissue mass is due primarily to hyperplasia, as reflected by an 8-fold increase in DNA content but no change in the ratio of protein to



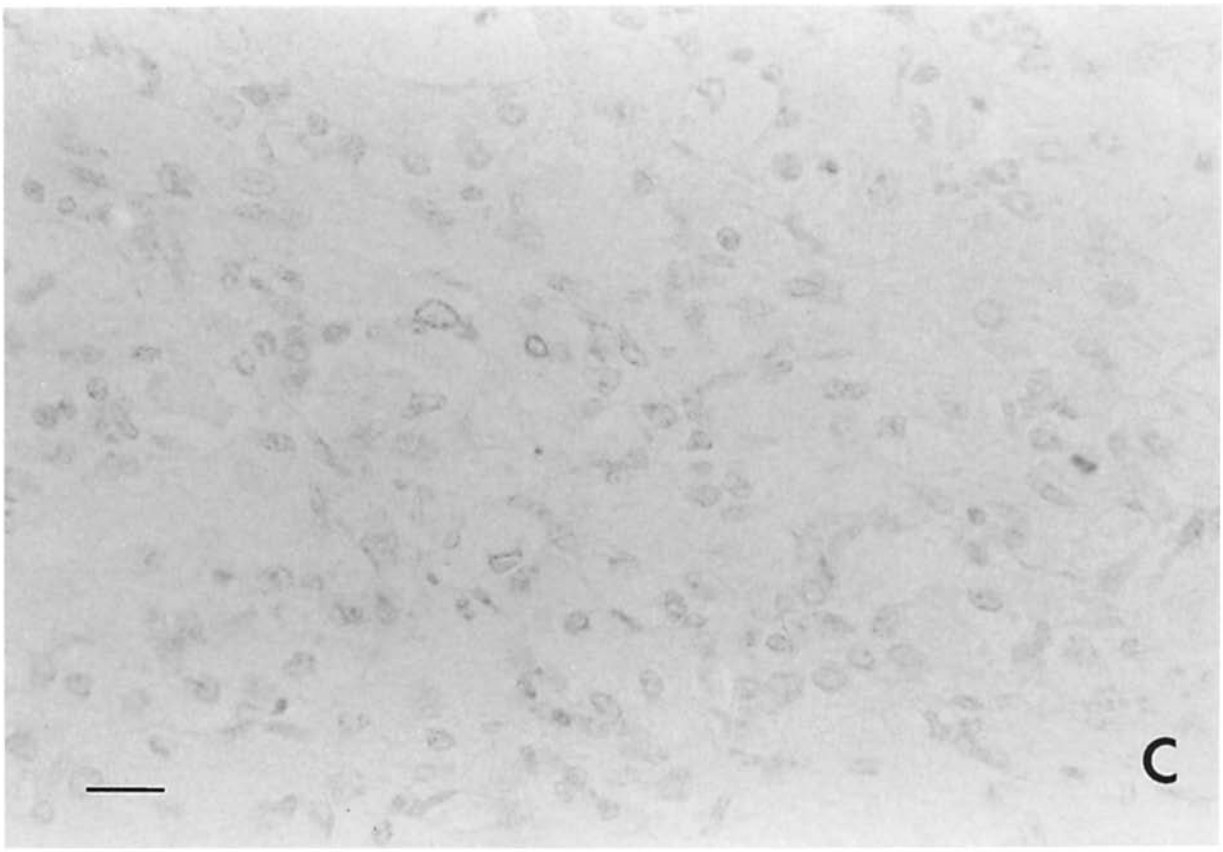


FIG. 3. (Preceding page and above.) Labeling of fragmented DNA in sections of bovine CL from stages III (A) and IV (B) of the estrous cycle. Note that cells undergoing apoptosis were present only at stage IV. Representative control staining for a stage IV CL is shown in (C). Arrows indicate apoptosis-positive nuclei; arrowheads indicate apoptosis-negative nuclei. Bars = 25 μm.

DNA. These results, however, must be interpreted with caution, because the protein:DNA ratio is a crude estimate of cell size and does not take into account changes in the proportion of large and small luteal cells that may occur during luteal development or changes in the size of specific cell types (e.g., the increase in size of large luteal cells) [3, 9, 26–28]. Although no attempt was made to identify the proliferating cell type(s), the majority of PCNA-positive cells at stage I were located in thecal-derived areas from which most small cells are believed to originate [28]. Furthermore, relatively few large parenchymal cells exhibited positive staining for PCNA at stages II, III, and IV. Thus, the vast majority of PCNA-positive cells appeared to be small cells (i.e., small parenchymal, fibroblasts, or endothelial cells). This observation agrees with previous data indicating that in ovine and bovine CL, the majority of luteal cells proliferating throughout the estrous cycle are small cells [3, 8, 26, 29].

In contrast to luteal DNA, protein, and progesterone contents, the changes in luteal DNA, protein, and progesterone concentrations showed different patterns throughout the estrous cycle. Luteal DNA concentration remained unchanged throughout the estrous cycle, whereas luteal protein and progesterone concentrations were similar for stages I, II, and III and then decreased by stage IV. These data again suggest that the increase in tissue mass and progesterone content during stages I and II results primarily from cell hyperplasia.

In support of this observation, luteal LI, an index of the rate of cell proliferation, also was extremely high in early-cycle (stage I) CL. In the present study, we immunolocalized PCNA in luteal tissue sections to identify proliferating cells. Proliferating cell nuclear antigen is a non-histone acidic nuclear protein that is essential for DNA synthesis [30–33]. Because PCNA is most abundant during the S-phase, it is generally considered to be a reliable marker for estimation of cell proliferation [32–36]. In addition, we have recently shown close agreement between the percentage of ovine luteal cells incorporating exogenous bromodeoxyuridine (BrdU, a thymidine analog) and those staining for PCNA at two stages of the estrous cycle (Day 4 and Day 8 after estrus) (J. Sherman, D.A. Redmer, and L.P. Reynolds, unpublished observations).

In the present study, the LI was 20.3% for stage I CL; it then decreased to approximately 3.5% for CL at stages II, III, and IV. In comparison, most normal adult tissues pro-

liferate slowly and exhibit an LI of less than 0.5%, whereas adult tissues that constantly renew, such as epidermis, intestinal epithelium, and bone marrow, can exhibit an LI as great as 1 to 10% [11, 37]. In addition, the LI of rapidly growing tumors have been estimated to be between 11 and 35% [11, 38]. Thus, the LI from stage I CL in the present study is comparable to or greater than those of rapidly growing tumors. These data agree with our previous report of a high LI (~ 34% on Day 2 after estrus) during the rapid growth phase of ovine CL [3].

Apoptosis, also known as programmed cell death, plays a complementary but opposing role to mitosis in regulating normal and neoplastic tissue growth and regression [10–12], and has been reported to occur during normal as well as prostaglandin $F_{2\alpha}$-induced luteal regression [13, 14]. Apoptosis is characterized biochemically by the cleavage of DNA into fragments that are multiples of approximately 185 bp in length [10, 39]. When separated by agarose gel electrophoresis, these DNA fragments form a laddering pattern that is the hallmark of apoptosis [22]. In the present study, the presence of DNA laddering in agarose gels could be detected only in stage IV CL. Similarly, histochemical localization of DNA fragmentation in tissue sections was observed only in stage IV CL. However, because most apoptosis-positive cells appear to have shrunken nuclei and little cytoplasm, it is impossible to classify these cell types as parenchymal, endothelial, fibroblastic, etc.

Because apoptosis is a rapid process, i.e., from several minutes to several hours in duration, even a low incidence of histologically detectable apoptosis probably is indicative of a substantial rate of cell deletion [10, 25, 40, 41]. The percentage of bovine luteal cells exhibiting apoptosis as detected by histochemical labeling of fragmented DNA was 3% at stage IV. These data, however, must be interpreted with caution, because during apoptosis, one nucleus can be broken into several pieces and this could result in an overestimation of the number of cells undergoing apoptosis. On the basis of the percentage of dying cells, the estimated number of luteal cells, and the estimated duration of apoptosis, we estimated that approximately 77.8×10^6 cells were dying per day in stage IV CL. This relatively high rate of cell deletion was accompanied by a 4.5-fold decrease in luteal DNA content from stage III to stage IV. Using similar calculations, we estimated that approximately 27.3×10^6 cells were proliferating per day in stage IV CL. These estimates demonstrate that structural luteal regression occurs when the rate of cell deletion exceeds the rate of cell proliferation, thereby resulting in a net loss of cells and a concomitant reduction in luteal tissue mass. In addition, the ratio of protein to DNA decreased from stage III to stage IV, suggesting that an overall decrease in cell size contributed to the 3-fold decrease in luteal fresh weight. Whether the cell deletion observed in the present study occurred in a specific population of cells, such as small or large parenchymal cells, endothelial cells, or fibroblasts, was not determined and will require further study.

In summary, results of the present study demonstrate that the rate of tissue growth and DNA accumulation is extremely rapid during early luteal development and that a high rate of cell proliferation is the primary mechanism by which this growth occurs. Unexpectedly, although cell proliferation decreased substantially after the initial period of rapid luteal growth, it remained relatively high throughout the estrous cycle. Luteal regression from stage III to stage IV was characterized by a dramatic decrease in luteal weight and DNA content and was accompanied by a dramatic increase in the number of dying cells as well as a decrease in cell size. These data support previous observations by us and others showing that the ruminant CL undergoes rapid tissue growth and cell proliferation throughout the estrous cycle [2, 3, 18, 26, 27]; the findings also extend the hypothesis that cell deletion through apoptosis plays an important role in structural luteal regression. Data such as these will provide us with a better understanding of the kinetics of cell turnover during luteal growth and regression in cows and will aid in the elucidation of mechanisms that regulate luteal function.

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