Assessment of luteal function by ultrasonographic appearance and measurement of corpora lutea in goats

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Abstract

In order to characterize the evolution pattern of the corpora lutea (CL) and to compare luteal function with their ultrasonographic appearance, 37 estrous cycles of Serrana goats ($n = 22$) were studied during breeding season. A daily transrectal ultrasound scanning was performed through two successive estrous cycles. Both solid and fluid-filled CL were observed and measured in both ovaries of each goat. Additionally, each CL was classified as CL_{ICHE} (CL with irregular contours and heterogeneous echotexture) or CL_{RCGE} (CL with regular contours and granular echotexture). Ovarian cyclic activity and luteal function were evaluated by biweekly plasma progesterone (P4) determination. The CL ($n = 60$) were first visualized on day 2.9 ± 1.0 after the day of ovulation (day 0), showing 7.1 ± 1.8 mm of diameter and reach their maximum size (12.5 ± 1.6 mm) on day 10.7 ± 3.2 ($P < 0.001$). Two days before the following ovulation (day −2), the CL regressed to 8.4 ± 1.3 mm ($P < 0.001$). The central cavity was found in 78.3% of CL, and had a persistence of over 50% until the last days of estrous cycle. The ratio CL length/cavity length was low during the first-third and high during the remaining two-thirds of estrous cycle. On day 2, the percentage of CL_{ICHE} was 33.3%, and began to decrease to 16.7% on day 6, reaching the minimum of 3.3% on day 10 ($P < 0.001$). This proportion increased on day −3 to 48.3% and reached 90% on day −1 ($P < 0.001$). The correlation between CL size and plasma P4 levels was $r = 0.63$ ($n = 87$; $P < 0.001$). A negative correlation between the daily proportion of CL_{ICHE} and plasma P4 levels was found ($r = -0.95$; $n = 18$; $P < 0.001$). These results suggest that the ultrasonographic appearance of CL is a reliable parameter for the assessment of luteal function in goats. Both the characterization of echotexture and size of central cavity could be valuable tools to differentiate between phases of normal estrous cycles.
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1. Introduction

The ultrasonographic characterization of corpora lutea (CL) during the estrous cycle and early pregnancy of ruminants was performed for the first time in cows (Kito et al., 1986; Pierson and Ginther, 1987; Omran et al., 1988; Kastelic et al., 1990b). The comparison between real-time transrectal ultrasonography (RTU), rectal palpation and plasma progesterone (P4) concentration was used to assess the bovine luteal status (Sprecher et al., 1989; Ribadu et al., 1994). Significant correlations between size of CL and plasma P4 were observed during different phases of the estrous cycle in cattle (Kastelic et al., 1990a; Assey et al., 1993; Ribadu et al., 1994).

In goats, the variation of plasma P4 concentration during estrous cycle is well-known from more than 30 years (Heap and Linzell, 1966; Thorburn and Schneider, 1972). Recently, with the development of RTU, the detection and measurement of CL, in this species, were also studied (de Castro et al., 1999; Simões et al., 2005), including their relationship with the P4 levels (Orita et al., 2000; Medan et al., 2003). In ewes, a significant correlation between CL area and P4 concentration was found (Samartzi et al., 1995; Gonzalez de Bulnes et al., 2000). However, there is little information about the echo-characterization of CL with and without central cavities during the estrous cycle of goats.

Although the ultrasonographic appearance (morphology and echotexture) of the CL could be used to evaluate luteal activity in cattle, the functional classification of CL it was not easy when based in only one ultrasonographic examination (Battocchio et al., 1999) or only with the RTU method (Veronesi et al., 2002). However, the ultrasonographic appearance of CL seems to be a more reliable parameter than their size for the assessment of luteal function (Veronesi et al., 2002). To our knowledge, no data about the RTU reliability for this parameter was reported in goats.

The aims of the present study were (1) to describe the evolution of CL during the estrous cycle using RTU and (2) to determine the accuracy of the estimated size and ultrasonographic appearance of the CL in order to identify the different phases of the estrous cycle and to assess luteal activity in Serrana goats.

2. Materials and methods

2.1. Animals

Twenty-two Serrana goats, a local Portuguese breed, with 33.6 ± 8.0 kg live weight and aged 2–9 years were used during breeding season from September to November.

All procedures and experiments involving animals used in this research had the approval of the Animal Welfare Division/Veterinary General Directorate of the Ministry of Agriculture.

2.2. Evaluation of estrous and ovarian activity

Estrous synchronization was performed with two intramuscular injections of $50 \mu g$ of clo-prostenol (Estrumate®, Schering-Plough II) given 10 days apart. Two vasectomised bucks were used for estrous detection. The observation of the flock was continuously performed between 36 and 72 h after the second prostaglandin application, and during the two subsequent natural estrous, in order to detect the onset of estrous in each animal. Out of these periods, and during the whole time of the experiment, at least one male with a marker harness was permanently

with the goats. Observations to identify marked goats were performed every 12 h. The onset of natural or induced estrous was defined as the moment when the female was firstly receptive to mounting.

Ovarian cyclic activity and luteal status were confirmed, in all goats, by plasma progesterone (P4) concentrations, determined by radioimmunoassay analysis (Kubasik et al., 1984) of samples collected biweekly. For each sample, 3–5 ml of blood was extracted, by venipuncture, from alternate jugular veins to heparinized vacuum glass tubes, centrifuged and stored at $-60\,^\circ$C until P4 assay. The normal variations of the day of ovulation and the duration of estrous cycle of each goat allowed the collection of plasma P4 samples in all days of caprine estrous cycle. The P4 was assayed with a direct solid-phase ^{125}I radioimmunoassay (Count-A-Count® Progesterone, Diagnostic Products Corporation, LA). The sensitivity was 0.02 ng/ml. The inter-assay and intra-assay variation were 11.5% ($n = 170$) and 2.2% ($n = 897$), respectively.

The RTU scanning of ovarian structures (Aloka®, model 500 SSD, Japan) was performed with a 7.5 MHz linear transducer (model UST-660-7.5), every day of estrous cycle, between 10:00 and 13:00 h. Both ovaries were observed in each animal, which were held with the animal in a standing position. The sonograms were recorded with a digital video for later computer analysis.

2.3. Identification of number of ovulations by RTU

Number of ovulations was evaluated by the number of follicles larger than 5 mm that disappeared between 20 and 60 h after the onset of estrous and confirmed by the number of CL, after the ovulation, as observed by transrectal RTU.

Time of ovulation was confirmed by the identification of a preovulatory LH peak (Pelletier et al., 1968), 0–24 h after the onset of estrous by serial (every 4 h) blood samples collection.

2.4. Characterization and classification of CL

The number and size of CL, the presence or absence of central cavity, as well as its size, were observed in each ovary by RTU every day. When either the CL or the central cavity was oval, the maximum diameter was measured. All of these retrospective computer analyses were performed using the UTHSCSA Image Tool 3.00 software. A high correlation coefficient ($r^2 = 0.86$) between the diameter of the spherical corpora lutea observed by RTU and by slicing technique was previously found by Simões et al. (2005) using the same methodology.

Each CL was classified in one of two groups, according to their ultrasonographic appearance (Fig. 1) as suggested by Battocchio et al. (1999) and Veronesi et al. (2002) which was devised for use in cattle: CL with irregular contours and heterogeneous echotexture (CL_{ICHE}); CL with regular contours and granular echotexture (CL_{RCGE}).

2.5. Statistical analysis

The data were analyzed by ANOVA and Bonferroni/Dunn test for comparison of means (mean ± S.D.). The comparison between the number of CL with or without central cavity and the percentage differences between CL_{ICHE} and CL_{RCGE} during the estrous cycle was done using the Chi-square test. Simple or polynomial correlations were used between the CL size or proportion of the CL_{ICHE} (number of CL_{ICHE}/number of total CL) and the plasma P4 concentration.
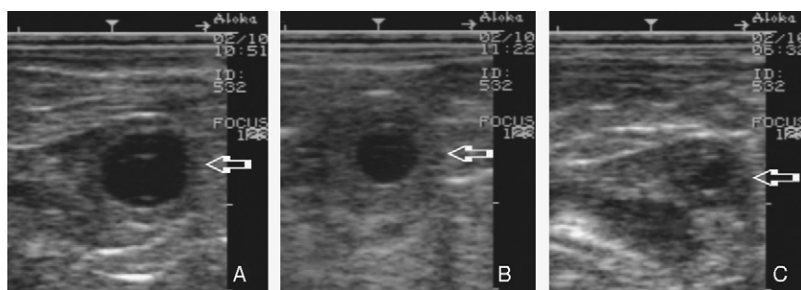
Fig. 1. CL classification according to the ultrasonographic appearance. (A) CL$_{ICHE}$ with fluid-filled cavity (white arrow) in day 4 of estrous cycle: a heterogeneous echotexture is visualized around central cavity with several alternate small areas with different echogenicity and irregular contour. (B) The same CL (white arrow) on day 15 of the estrous cycle representing a characteristic mid-cycle (CL$_{RCGE}$). A uniform granular luteal tissue is present with distinctly and regular contour. The CL$_{ICHE}$ showed in 'C' (white arrow) is the same CL on day 21 (1 day before next ovulation). The length between two lateral bars corresponds to 10 mm.

3. Results

Thirty-seven complete estrous cycles, confirmed by preovulatory LH peak detection and RTU observation, were studied. In the expected third estrous, the time of ovulation was not detected in seven goats.

The mean inter-estrous interval was 20.6 ± 1.0 days. Sixty ovulations were observed between 24 and 48 h after the onset of estrous with a mean of 1.62 ± 0.64 ovulations per cycle. A total of 45 single, 6 double and 1 triple ovulations per ovary in 17 single, 17 double and 3 triple ovulations per goat were observed.

3.1. Evolution of CL in different phases of estrous cycle

The mean time of the first visualization of CL by RTU was day 2.9 ± 1.0 of the estrous cycle ($n = 60$; D0 = day of ovulation; Fig. 2). No differences were found when the ovary had one (day
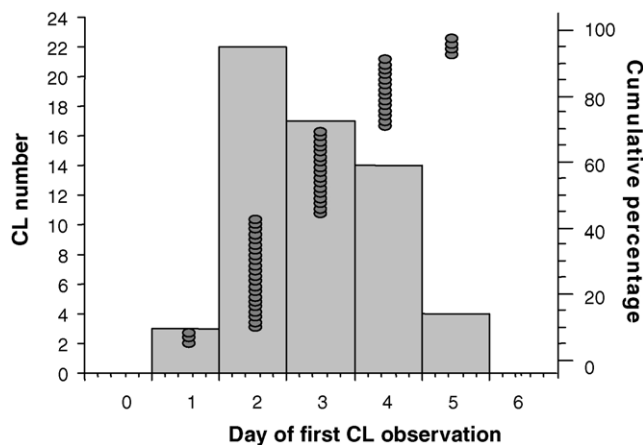

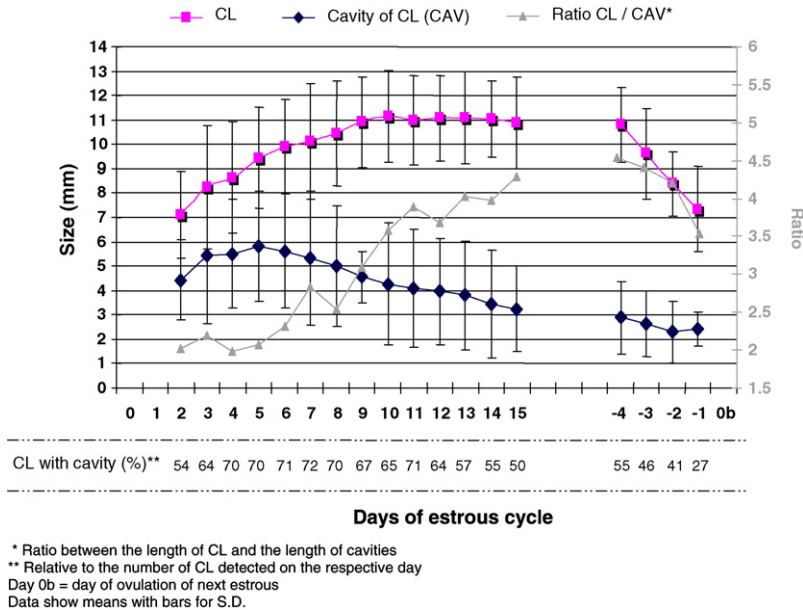Fig. 2. Distribution of the day of first CL visualization by RTU.

Fig. 3. Evolution of CL size, central cavity size and ratio CL/CAV during the estrous cycle.

2.8 ± 1.1; $n = 45$) or more (day 3.0 ± 1.0; $n = 7$; $P > 0.05$) CL. However, only on day 6 it was possible to visualise all CL.

On day 2, the diameter of the CL was 7.1 ± 1.8 mm ($n = 24$). This diameter increased during the following days and reached their maximum size on day 10.7 ± 3.2 ($n = 56$) with a mean diameter of 12.5 ± 1.6 mm ($P < 0.001$). No differences were observed ($P > 0.05$) when the ovary had one ($n = 44$) or two ($n = 6$) CL. Between day 10 of the estrous cycle (11.2 ± 1.9 mm) and day -4 before the following ovulation (10.8 ± 1.6 mm) the CL size remained constant (Fig. 3; $P > 0.05$). Between days -4 and -1, CL diameter decreased to 7.3 ± 1.8 mm ($P < 0.001$). Due to the variation of the inter-estrous interval (19–24 days), the last 5 days of each estrous cycle were reported to the time of the next ovulation.

The central cavity was observed in 78.3% (47/60) of CL originated from the 37 interovulatory intervals. These cavities could be observed at the same time of first CL detection. During the estrous cycle, some minor cavities were only observed in alternate days.

During metaestrous (days 2–6) and initial diestrous (days 7–9), the size of the fluid-filled cavities was similar to day 4 (5.5 ± 2.3 mm) and decreased to 3.5 ± 2.2 mm on day 14 ($P < 0.001$). After day 15, their size remained constant (3.3 ± 1.8 mm) until the last day (day -1) of RTU examination (2.4 ± 0.7 mm; $P > 0.05$). However, in this last day only 27.7% (13/47) of the total cavities observed during the estrous cycle were detected and measured.

The mean ratio between the length of CL and the length of cavities (ratio CL/CAV) was low ($P < 0.001$) during the first-third of estrous cycle (CL/CAV = 2.0 on day 4) and increased during the middle of luteal phase (CL/CAV = 4.3 on day 15), showing no differences ($P > 0.05$) until the last day of observations in the estrous cycle (CL/CAV = 3.5 on day -1).

Between days 2 and 6 of estrous cycle, 73.9% (116/157) of fluid-filled CL observations had the CL/CAV ratio smaller than 2.2 (the central cavity tends to represent half or more of the total
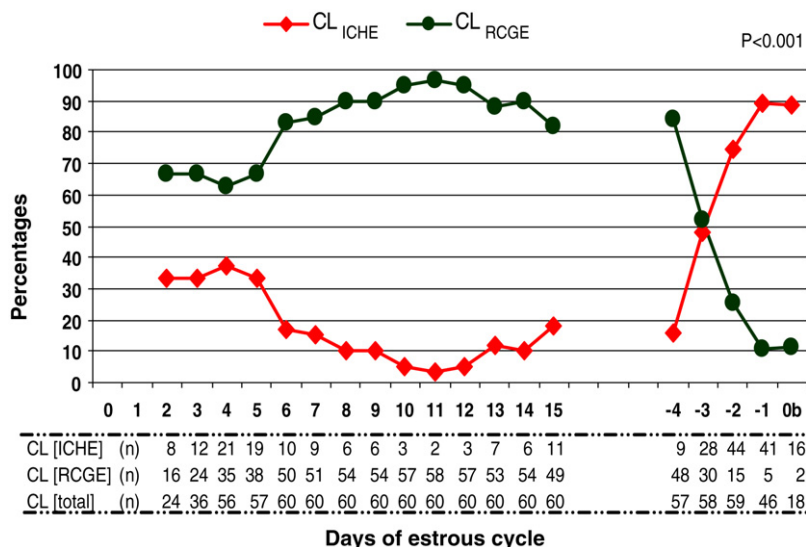
Fig. 4. Variations of CL_{ICHE} and CL_{RCGE} proportions during the estrous cycle.

diameter of CL). This ratio was ≥ 2.2 in 70.2% (260/370) of observations of CL with cavity, performed between days 6 and -4 and 90.5% (57/63) between days -3 and -1.

3.2. Daily evolution of CL_{ICHE} and CL_{RCGE} proportions

During the estrous cycle, daily variations of CL_{ICHE} percentages were observed (Fig. 4). On the initial phase of the cycle, at day 5, the proportion of CL_{ICHE} was 33.3% and began to decrease on day 6 (16.7%; $P < 0.001$). On the middle of the cycle (days 10–12), the proportion of CL_{ICHE} reached their lowest value (3.3%; $P < 0.001$). From day -4 and to the day of new ovulation (last day of the CL observation recorded), this percentage increased from 15% (day -4) to 48.3% at day -3 and to about 90%, at estrous (days -1 and 0; $P < 0.001$). Some of them, especially in the last days of estrous cycle, presented high irregular and indistinct contours and could not be measured. However, these observations were included in the CL numbers for proportion determination of CL_{ICHE}. During the first days of estrous cycle, the proportion of CL_{RCGE} was higher than 60% and represents $\geq 90\%$ of CL during the middle of diestrous (days 10–14). After day -4, marked ultrasonographic alterations of these CL were observed.

3.3. Evolution of plasma P4 levels during the estrous cycle

At the onset of the estrous cycle (days 0–3), the plasma P4 concentration is low (<0.5 ng/ml; Fig. 5). A significant increase of plasma P4 levels from day 4 (3.8 ± 0.9 ng/ml) to day 9 (9.3 ± 1.8 ng/ml; $P < 0.001$) was identified. After day 9, these levels presented no significant variations until day -4 (7.1 ± 1.0 ng/ml). After day -4, the plasma P4 concentrations quickly decreased to levels under 0.5 ng/ml on day -2 (0.2 ± 0.1 ng/ml; $P < 0.001$). A significant higher variation of the plasma P4 was observed on day -3 than on the previous or following day.
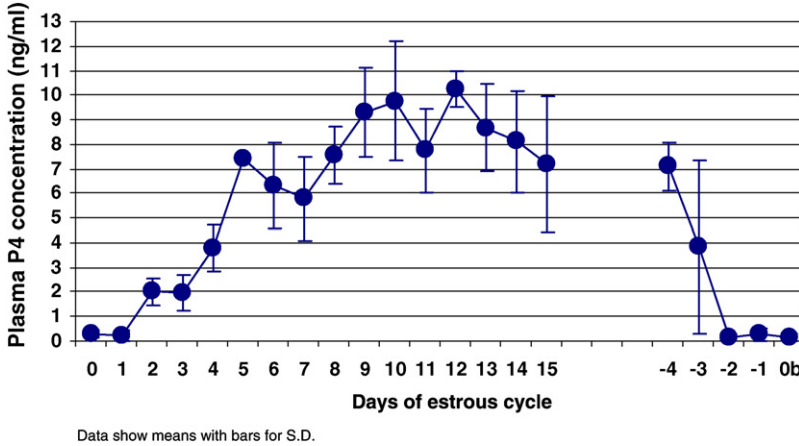
Data show means with bars for S.D.

Fig. 5. Plasma P4 levels evolution during the estrous cycle.

3.4. Relationship between corpora lutea and the plasma P4 levels

It was possible to associate CL with the respective P4 levels in 190 observations by RTU. The plasma P4 concentration was lower ($P < 0.05$) in goats with one CL (5.4 ± 3.0 ng/ml; $n = 87$) than two or three CL (6.4 ± 3.7 ng/ml; $n = 103$), independently of the day of estrous cycle.

In goats with one CL per estrous cycle (17 cycles), the correlation between the size of CL and the plasma P4 levels was significant ($r = 0.63$; $r^2 = 0.40$; $n = 87$; $P < 0.001$; Fig. 6).

The correlation between the size of CL and the plasma P4 concentration was higher for CL_{RCGE} ($r = 0.60$; $r^2 = 0.36$; $n = 62$; $P < 0.001$) than for CL_{ICHE} ($r = 0.48$; $r^2 = 0.23$; $n = 25$; $P < 0.05$).

No differences ($P > 0.05$) in the plasma P4 levels were found between CL with central cavity (5.9 ± 2.9 ng/ml; $n = 48$) or not (4.9 ± 3.1 ng/ml; $n = 43$).

A negative correlation between the daily proportion of CL_{ICHE} and the mean plasma P4 concentration (Fig. 7) of all goats was found ($r = -0.95$; $r^2 = 0.90$; $n = 18$; $P < 0.001$).
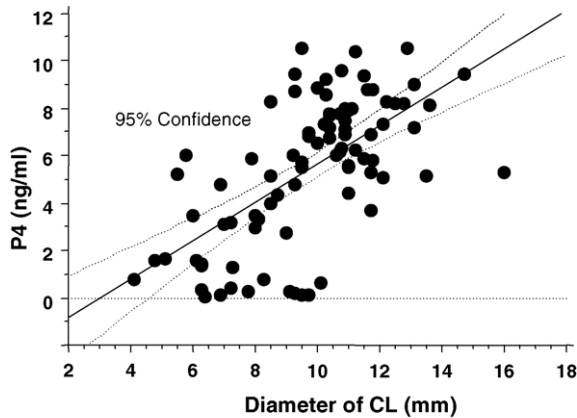

$Y = -2.48 + 0.81 * X$; $r^2 = 0.40$; RSD = 2.4; $n = 87$; $P < 0.001$

Fig. 6. Relationship between the size of CL and plasma P4 concentration.

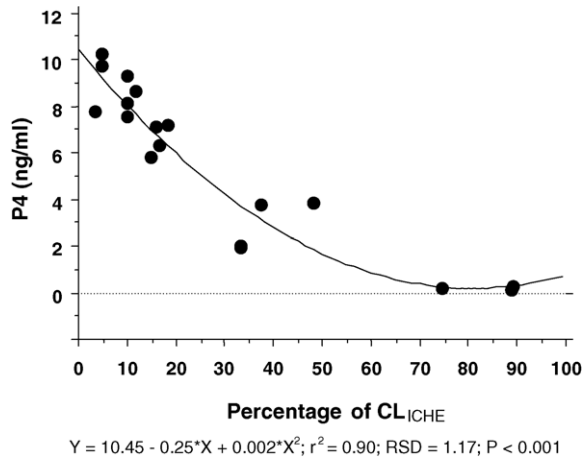$$Y = 10.45 - 0.25*X + 0.002*X^2; r^2 = 0.90; RSD = 1.17; P < 0.001$$

Fig. 7. Relationship between the proportion of CL_{ICHE} and the plasma P4 concentration.

4. Discussion

4.1. Evolution of CL pattern

The observations of all CL from day 6 onwards were in agreement with the data reported by Dickie et al. (1999) and Bouttier et al. (2000), regarding the accuracy in RTU detection of CL on day 10 in ewes and on day 9 in goats, respectively. The accuracy and sensitivity of RTU to quantify the CL number per ovary declines when CL numbers increase (Dickie et al., 1999; Viñoles et al., 2004). However, in our work, no differences were found in the first day of observation in ovaries with different number of CL, presumably, because only six double and one triple ovulations per ovary were found.

The first day of CL identification by RTU observed in our study (2.9 ± 1.0), agrees with the results obtained in Shiba goats (Orita et al., 2000; Medan et al., 2003). In our study, the CL was detected in the first day or even in the second day. In cows, the first detection of these CL was possible in the day of ovulation (Omran et al., 1988). Although this possibility also occurs in ewes (Gonzalez de Bulnes et al., 2000; Duggavathi et al., 2003), the detection is better performed from day 3 or 4 of estrous cycle onwards (Gonzalez de Bulnes et al., 2000). In the present study, CL attain their maximum diameter (12.5 ± 1.6 mm on day 10.7 ± 3.2) later than that observed by Medan et al. (2003) in Shiba goats (12.1 ± 0.3 mm on day 8). In Nubian goats, the CL observed by laparoscopy, reached a maximum visible diameter of 9.4 ± 0.6 mm on day 11 (Camp et al., 1983). However, a high variation of the day of maximum diameter of CL was observed in our study.

On day 17, a change in vascularization of CL was observed, with a marked reduction of size in the following days (Camp et al., 1983).

In the present study, the decrease of CL diameter and increase of CL_{ICHE} proportion observed in days -3, -2 and -1, are in agreement with the CL regression. The regression of CL size started before and was slower than the decrease in P4 levels. The association of these events reflects the temporal difference between functional and structural luteolysis. According to Orita et al. (2000), the decrease of plasma P4 concentration seems to begin 2 days before the start of CL regression.

The first day of central cavities detection by RTU (54% of total CL observed on day 2) in our study is in agreement with the 2.0 ± 0.3 days observed in ewes by Gonzalez de Bulnes et al. (2000). Regarding the proportion of central cavities identified, the value obtained in our study with Serrana goats (78.3%) was higher than the values reported by Kito et al. (1986) in cows (37.2%) and Gonzalez de Bulnes et al. (2000) in ewes (33%).

In ewes, Gonzalez de Bulnes et al. (2000) observed that the CL cavities did not persist during all days of the estrous cycle and disappeared between days 6 and 12. In our study, a decrease of the size of central cavities was observed after day 6, but most of them persisted until the last days of the estrous cycle (41% of total CL observed on day -2). More studies are necessary for CL morphogenesis comprehension, associating the RTU with histomorphological and hormonal tools.

The significant difference between the proportion of CL_{ICHE} present during the follicular phase (days -1 and 0; 90%), the onset of the luteal phase (days 2–5; up to 30%) and the middle of the luteal phase (day 11; 3%) suggests that it is possible to distinguish between this last phase on the basis of ultrasound appearance. Moreover, in CL with central cavity, the ratio CL/CAV observed in the first-third was lower than in the remaining days of estrous cycle, with the exception of the last day. This can provide a technique that allows differentiating the several phases of the estrous cycle. In the last day of estrous cycle, the structural luteolysis led to marked alterations in the appearance of all CL making them difficult to detect.

Between days 2 and 6 of estrous cycle, more than 70% of CL with cavity, independently of their ultrasonographic appearance, had a ratio <2.2. In this stage, the misleading observations between CL with cavity and another's anechoic structures (ovarian cysts and anovulatory follicles) could be higher, due to the relative great diameter of central cavities. On other hand, between days 7 and -4, more than 70% of this type of CL had a ratio ≥ 2.2 and were, predominantly, CL_{RCGE}. Between days -3 and -1, this last proportion increase to 90% and CL_{ICHE} were predominant.

4.2. Relationship between CL pattern evolution and P4 levels

In the present study, the plasma P4 concentration was higher when there were more than one CL (5.4 ± 3.0 ng/ml for one CL and 6.4 ± 3.7 ng/ml for more than one CL) and this supports the evidence of higher P4 levels observed in Alpine goats with higher ovulation rate in induced estrous (Chemineau et al., 1982). The low value of the correlation between the diameter of CL and plasma P4 levels ($r = 0.63$) observed in our study could be due to the very low values of P4 (about 0 ng/ml) observed with CL smaller than 10 mm.

No significant differences of plasma P4 levels were found in our study between CL with or without fluid-filled cavity. These data are in agreement with other results obtained in ewes (Gonzalez de Bulnes et al., 2000) and heifers (Kastelic et al., 1990a). In fact, the CL with cavity does not seem to interfere with fertility (Kito et al., 1986) or with the length of estrous cycle both in heifers (Kastelic et al., 1990b) and in ewes (Gonzalez de Bulnes et al., 2000).

The negative correlation found between the CL_{ICHE} percentage and P4 levels in goats ($r = -0.95$) supports the results obtained in cows by Veronesi et al. (2002), who considered the ultrasonographic appearance more reliable than the CL size for the assessment of luteal status.

In conclusion, the significant variation of CL_{ICHE} percentage during estrous cycle and its relationship with the plasma P4 levels suggests that the CL ultrasonographic appearance could be a reliable parameter to assess the luteal function in goats. A large proportion of CL with fluid-filled central cavities was observed in goats that continues to near the end of estrous cycle. In this last type of CL, one RTU examination could provide a differentiation between the metaestrous, middle

diestrous, late diestrous or proestrous of normal cycles. However, more studies are necessary for confirmation of reliability of assessment of luteal function by CL ultrasonographic appearance in larger numbers of animals.

Acknowledgements

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