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Histological Characteristics and Steroid Concentration of Ovarian Follicles at Different Stages of Development in Pregnant and Non-pregnant Dairy Cows

M. De los Reyes[1], M.L Villagrán[1], R. Cepeda[2], M. Duchens[1], V. Parraguez[3] and B. Urquieta[3]
[1]Laboratory of Animal Reproduction, [2]Laboratory of Histology, [3]Laboratory of Endocrinology, Faculty of Veterinary Sciences, University of Chile, Casilla 2 Correo 15, Santiago, Chile
**Correspondence: E-mail: mdlreyes@uchile.cl*

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ABSTRACT

The aim of the study was to investigate the histological characteristics and steroid concentrations in follicular fluid of different populations of follicles at different stages of development, during pregnancy and the oestrous cycle in cows. Follicles from ovaries collected at a slaughterhouse were allocated into three size categories (small, 2–5.9 mm; medium, 6–13.9 mm; and large, 14–20 mm) in pregnant and non-pregnant cows. Slices were stained with HE and PAS for histological analysis. Follicular fluid was pooled according to size and pregnancy status and estradiol, testosterone and progesterone concentrations in follicular fluid were determined by RIA. Characteristics of healthy follicles did not differ, regardless of follicle size or pregnancy status. Total histological atresia was significantly higher in pregnant cows than in non-pregnant cows ($p < 0.05$). Estradiol increased and testosterone decreased significantly, while follicles increased in size, in both non-pregnant and pregnant cows ($p < 0.05$). Non-pregnant cows had the highest estradiol values in follicles of all sizes. Medium and large follicles from pregnant cows showed the lowest testosterone concentration ($p < 0.05$). Progesterone levels increased with follicle size only in non-pregnant animals. In large follicles, progesterone concentration was significantly higher in non-pregnant cows than in pregnant cows ($p < 0.05$). Considering steroid concentration and histological findings, most large follicles might be atretic during pregnancy in cattle.

Keywords: atresia, cow, ovarian follicles, steroid

Abbreviations: CL, corpus luteum; E_2, estradiol; FF, follicular fluid; HE, haematoxylin–eosin; P_4, progesterone; RIA, radioimmunoassay; T, testosterone

INTRODUCTION

Follicular development is controlled by intriguing relationships between intrafollicular steroids and growth factors, extraovarian factors and the hypothalamic–pituitary system. During follicular growth, the follicle progressively acquires different properties that are essential for further development (Braw-Tal and Yossefi, 1997; Driancourt and Thuel, 1998). Failure to have these properties in the correct and exact sequence usually results in atresia (Hirshfield, 1991; Eppig, 2001). It is well known that very few follicles that begin growth finally ovulate, because most of them undergo atresia before reaching that stage.

In cattle, follicular growth and regression occur in waves throughout the oestrous cycle (Rajakoski, 1960; Sunderland *et al.*, 1994; Fortune *et al.*, 1991; Fortune, 1994; Sartori *et al.*, 2001), with one to four waves per cycle (Sirois and Fortune, 1988; Fortune, 1994; Ginther *et al.*, 2001). During follicular development, granulosa cells are the major source of follicular estradiol, and theca and granulosa cells together determine the intrafollicular concentrations of progesterone and testosterone (McNatty *et al.*, 1984; Bigelow and Fortune, 1998; Soboleva *et al*, 2000). Changes of steroidogenic activity, as measured by changes in concentration of steroids in follicular fluid, occur in cattle when follicles mature or become atretic, and are usually related to a certain size or stage of development (Fortune, 1994; Ginther *et al.*, 1996a; Ginther *et al.*, 2001). In that sense, hormone concentrations in follicular fluid have been used to determine the developmental status of the follicle, where the oestrogen-to-progesterone ratio indicates the degree of atresia in some follicles (Sunderland *et al.*, 1994; Price *et al.*, 1995). Histological studies have defined several degrees of atresia based mainly on the number of pycnotic nuclei, thickness of granulosa cell layer and basement membrane (Grimes *et al.*, 1987; Price *et al.*, 1995).

Although there are many studies on follicular development in cattle, there are few reports that at the same time and under the same conditions describe the concentration of steroids in follicular fluid and histological characteristics of follicles at different stages of development during pregnancy and during the oestrus cycle. The aim of this work was to study the histological and steroidogenic characteristics of bovine ovarian follicles, considering progesterone, testosterone and estradiol concentrations in follicular fluid in follicles of three different size categories, both in pregnant and non-pregnant cattle.

MATERIALS AND METHODS

Ovaries from 323 Holstein cows were collected from a slaughterhouse. They were selected according physiological status of the females (pregnant at any stage of pregnancy, with the presence of a conceptus; or non-pregnant at any stage of the oestrous cycle). Ovaries were placed in plastic bags and transported to the laboratory on ice. All samples were processed within 3 h of collection.

At the laboratory, follicles were measured with a vernier caliper and allocated into three sizes categories (small, 2–5.9 mm; medium, 6–13.9 mm; and large, 14–20 mm) in both pregnant and non-pregnant cattle, with a total of six groups.

Histological study

One hundred and sixteen ovaries from pregnant and 130 from non-pregnant dairy cows (660 and 640 follicles respectively) were studied. Ovaries were fixed in alcoholic

Bouin solution for 24 h. Each sample was obtained using a rotation microtome (Leitz), and samples were stained with haematoxylin–eosin for studying granulosa and theca cells. Periodic acid–Schiff (PAS) was used to observe basal membrane. Histological classification of follicles was performed by two independent observers.

The degree of atresia was assessed histologically by light microscopy into three categories according to Spicer and colleagues (1987): (a) healthy, with intact granulosa cells, well-defined basal layer, theca cells with clearly discernible and no pycnotic nuclei; (b) early atretic, if one or two of the above characteristics described for a healthy follicle was missing; (c) late atretic, with degenerative granulosa cells, several pycnotic nuclei, irregular, discontinuous or absence of the basal membrane, thick theca layers and debris inside the follicular cavity.

Endocrine study

Follicular fluid (FF) was collected from 400 additional ovaries by puncture and aspiration of 1260 follicles (630 each from pregnant and non-pregnant donors). These totals were obtained from five different experimental replicates. In each replicate, FF was pooled according to follicle size (diameter) and the physiological status of the donor; pools were centrifuged at 800*g* at 4°C for 20 min to eliminate debris. From the supernatant, 1.5 ml aliquots were transferred to Eppendorf tubes and maintained at –20°C until hormonal evaluation.

Previously to hormone assay, dilution curves using native FF samples and steroid-free serum as diluent were constructed in order to determine parallelism to each standard curve and the most appropriate dilution factor. Follicular fluid concentrations of progesterone (P_4) and testosterone (T) were determined by solid-phase radioimmunoassay (RIA) (Coat-a-Count, DPC, Diagnostic Products Co., Los Angeles, CA, USA) using pool samples diluted 1:9 (v/v). Inter-assay and intra-assay variation coefficients were 6.2% and 7.5% for P_4 and 4.9% and 5.1% for T assays, respectively. Estradiol (E_2) concentration in FF was determined using a double antibody RIA (KE2, DPC). Pooled samples were diluted 1:100 (v/v) before each assay. Inter-assay and intra-assay variations were 3.8% and 4.6%, respectively. A gamma counter (Nuclear Enterprises, Edinburgh, UK, model NE1612,Turbo) was used for counting. Quality control of assays was assessed using plasma samples from non-pregnant and pregnant cows (Dieleman *et al.*, 1983; Fortune and Hansel, 1985).

Statistical analysis was performed using chi-square test to compare follicular histology according follicular size and physiological status. The endocrine study was analysed using a 2×3 factorial experiment and Tukey test for comparison between means (Sokal and Rohlf, 1981).

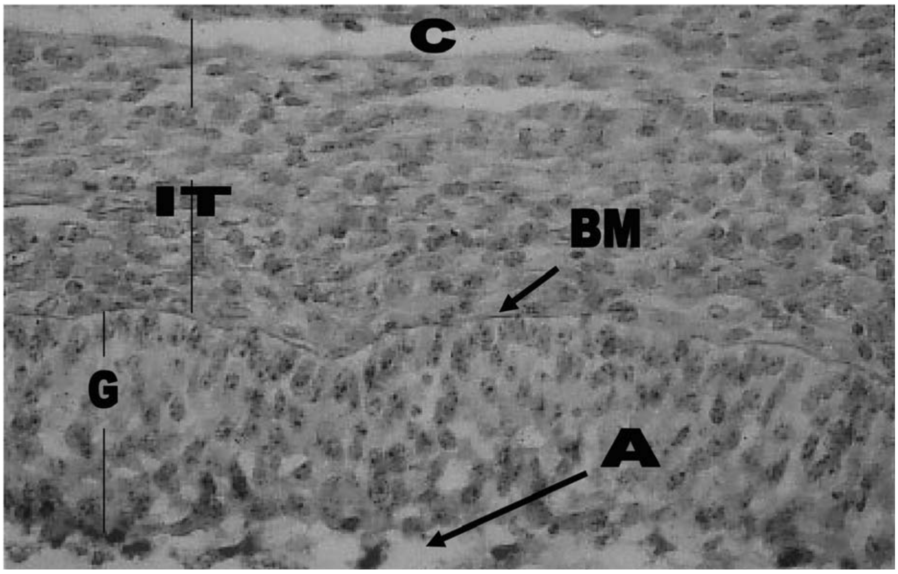

Figure 1. Photomicrograph of a normal cow follicle (medium size). IT, internal teca; BM, basal membrane; G, granulosa; A, antrum; C, capillary. Haematoxylin–eosin– PAS (original magnification ×300)

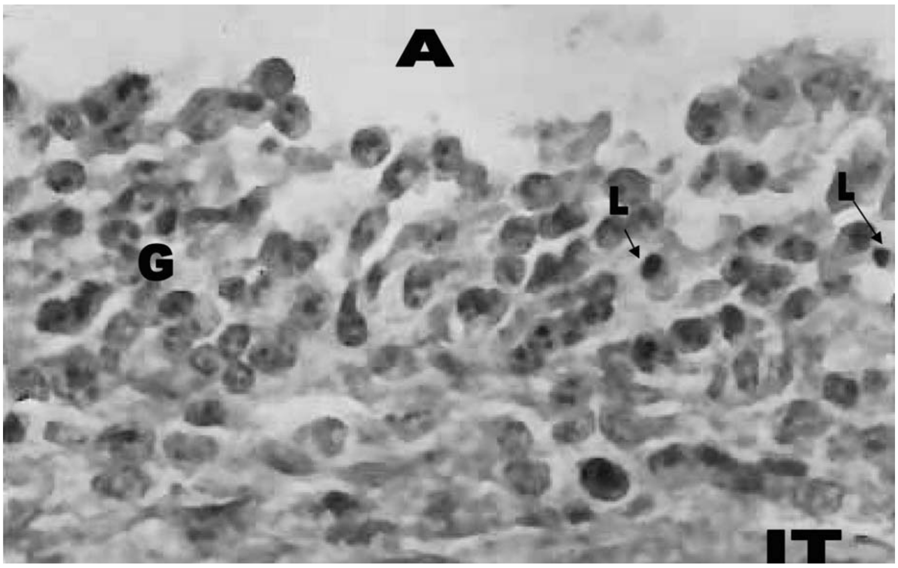

Figure 2. Photomicrograph of a early atretic cow follicle (medium size). IT, internal teca; BM, basal membrane; G, granulosa; A, antrum; L, leucocytes. Haematoxylin–eosin–PAS (original magnification ×600)

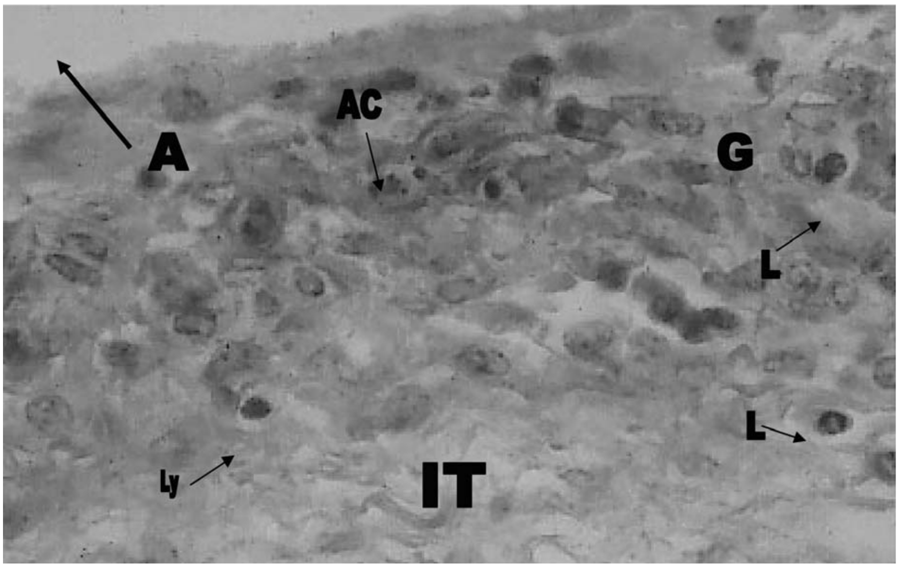

Figure 3. Photomicrograph of a late atretic cow follicle (medium size). IT, internal teca; BM, basal membrane; G, granulosa; A, antrum; L, leucocytes; Ly, lymphocytes; AC, apoptotic cell. Haematoxylin–eosin–PAS (original magnification ×600)

RESULTS

In healthy follicles (Figure 1), there were many concentric layers of normal granulosa cells. The basal membrane was well defined in the whole perimeter, with well defined limits between granulosa and theca cells. In the internal theca layer many blood capillaries were observed. No cellular detritus was detected in the follicular antrum; these characteristics were the same for every normal follicle, irrespective of the physiological status or follicle size. Nevertheless, the number of granulosa cells layers (7–8) was slightly greater in the small and medium-size follicles than in the large follicles (4–5), both in pregnant and non pregnant cows ($p > 0.05$). Follicles in early atresia (Figure 2), showed a thin and disorganized external granulosa layer. Polymorphism and some pycnotic nuclei were also observed. Many lymphocytes were detected in the internal theca of some follicles. Cells and detritus were also detected in the follicular cavity. Follicles in late atresia (Figure 3) showed a total destruction of the basal membrane; either this was not observed or just a few pieces were detected sometimes under PAS reaction. The granulosa layer was irregular and disorganized with many pycnotic nuclei and apoptotic cells, and degenerative cells were also observed. Many follicles showed complete destruction of the granulosa layer. These follicles had lymphocyte infiltration in theca and granulosa layers and much detritus was present in the follicular antrum.

TABLE I
Numbers and percentages of follicular status according histological evaluation for three sizes of follicles in non-pregnant and pregnant cows

Follicular size	Follicular status	Physiological status: Non-pregnant		Pregnant	
		n	%	n	%
Small (2–5.9 mm)	Healthy	160	59.3	110	50
	Early atretic	90	33.3	60	27.3
	Late atretic	20	7.4	50	22.7
	Total atresia	110	40.7	110	50
	Total follicles	270	100	220	100
Medium (6–13.9 mm)	Healthy	90	45	140	42.4
	Early atretic	50	25	120	36.4
	Late atretic	60	30	70	21.2
	Total atresia	110	55	190	57.6
	Total follicles	200	100	330	100
Large (14–20 mm)	Healthy	50	29.4	10	9.1
	Early atretic	110	64.7	70	63.6
	Late atretic	10	5.9	30	27.3
	Total atresia	120	70.6	100	90.9
	Total follicles	170	100	110	100

Significant differences according to the statistical analysis are described in the text

The proportions of each follicle type according histological study in non-pregnant and pregnant cows are described in Table I. A corpus luteum (CL) was present in all pregnant cows, and CL at different stages were observed in most of the cycling animals. The morphological observation of the ovaries revealed that small and medium follicles represented the most frequent sizes. Follicles larger than 14 mm in diameter made up 21% of the total number of follicles histologically classified. Percentages of healthy and total atretic (corresponding to the total percentage of early and late atresia) in small and large follicles were significantly different ($p < 0.05$) between the two physiological statuses. The greatest proportion of total atresia was observed in pregnant cows. Early atresia was the most common degree of atresia observed in large follicles from all animals. Late atresia, however, was significantly higher in pregnant than in non-pregnant cows ($p < 0.05$). Percentage of total atresia increased as follicle status increased in size, but this difference was only significant ($p < 0.05$) in large follicles compared to medium and small follicles, in both pregnant and non-pregnant females. Small follicles showed the highest proportion of healthy follicles.

TABLE II
Steroids concentrations in follicular fluid of follicles of each size in non-pregnant and pregnant cows

	Physiological status					
	Non-pregnant			Pregnant		
Follicular size	Estradiol (nmol/L)	Testosterone (nmol/L)	Progesterone (nmol/L)	Estradiol (nmol/L)	Testosterone (nmol/L)	Progesterone (nmol/L)
Small (2–5.9 mm)	$17.6^{a} \pm 6.0$	$190.8^{a} \pm 24.8$	$247.1^{a} \pm 34.9$	$3.3^{a} \pm 1.2$	$164.5^{a} \pm 17.2$	$274.0^{a} \pm 32.9$
Medium (6–13.9 mm)	$149.4^{b} \pm 27.0$	$110.3^{b} \pm 6.8$	$304.2^{a} \pm 21.0$	$36.4^{b} \pm 8.6$	$63.9^{b} \pm 7.5$	$277.9^{a} \pm 25.2$
Large (14–20 mm)	$204.1^{c} \pm 4.6$	$51.0^{c} \pm 12.9$	$475.6^{b} \pm 62.8$	$47.6^{c} \pm 16.1$	$26.5^{c} \pm 4.0$	$135.9^{b} \pm 41.4$

Values are mean $\pm$ SEM

[a,b,c]Within a column, means with different superscripts are significantly different ($p < 0.05$)

Estradiol, testosterone and progesterone concentrations in follicular fluid in non-pregnant and pregnant cows are shown in Table II. Estradiol concentration in follicular fluid increased significantly ($p < 0.05$) as the follicle status increased. Regarding physiological status, significant differences ($p < 0.05$) were found between same-size follicles. Non-pregnant cows showed the highest estradiol concentrations in follicular fluid at all follicular sizes. Testosterone concentration decreased ($p < 0.05$) with increasing follicle status size. Medium and large follicles from pregnant cows had lower ($p < 0.05$) testosterone concentration than those from non-pregnant cows. Progesterone concentration increased ($p < 0.05$) with increasing follicle status size only in non-pregnant females. Large follicles from pregnant cows showed the lowest ($p < 0.05$) values of progesterone concentration. Estradiol/progesterone ratio (E/P) was calculated (based on concentration values expressed in nmol/L) for each follicular group. The E/P value in large follicles was higher ($p < 0.05$) than that in small follicles in both non-pregnant and pregnant cows.

DISCUSSION

Overall analysis of the histological data showed that an important percentage (57%) of follicles in each size class in both physiological statuses had signs of atresia, which is consistent with previous reports (Spicer *et al.*, 1987; Hazeleger *et al.*, 1995). Patterns of follicular growth and atresia in different species have revealed that atresia does not occur equally across all stages of follicular development (Hirshfield, 1991; Thatcher *et al.*, 1996; Eppig, 2001). In the present study, atresia was more evident as follicles increased in size, which has also been reported in other studies (Silván *et al.*, 1993; Price *et al.*, 1995). It has been reported that follicular growth up to a certain point can occur readily in the presence of basal conditions, such as basal concentrations of gonadotrophins, metabolic hormones and growth factors, but that when follicles reach the 8th–10th granulosa cell generation they reach the end of their normal lifespan under these basal conditions (Hirshfield, 1991; Fortune, 1994). Thus, only follicles that are able to respond to specific additional signals, such as those given by the surge of gonadotrophins and the intrafollicular IGF system, will continue to develop (Fortune *et al.*, 2004). The study presented here showed that the vast majority of total histological atresia (early and late atresia) was evident in advanced follicular development.

Follicular growth occurs in a wave-like manner during pregnancy in cattle (Guilbault *et al.*, 1986; Pierson and Ginther, 1986; Ayoub and Hunter, 1993; Ginther *et al.*, 1996b; Naton *et al.*, 1999). Pregnancy did not affect the diameter of large follicles, since the maximum diameter of the dominant follicle was similar to that in non-pregnant females (15–20 mm); this diameter was larger than the maximum diameter of dominant follicles of the first or second wave in pregnant cows reported previously (Bergfelt *et al.*, 1991; Ginther *et al.*, 1996b). However, the percentage of large follicles obtained here was lower in pregnant than in non-pregnant cows. Dominguez (1995), reported that the number of follicles larger than 10 mm was reduced during the last two trimesters of pregnancy. This could account for a decline in follicular activity observed after the third or fifth month of pregnancy (Rexroad and Casida, 1975; Gibbons *et al.*, 1999). It has been demonstrated that the anterior pituitary has a decreased content of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in late pregnancy and the early postpartum period (Roche *et al.*, 1992). Since the growth of follicles to ovulatory size is dependent on FSH (Campbell *et al.*, 1995), this could explain the decreased follicular activity. Furthermore, the decrease in the diameter of the largest follicles during the last stage of pregnancy may reflect a decrease in LH pulse frequency; therefore, fewer follicles are able to reach preovulatory size. We did not classify the stage of pregnancy, but a large number of ovaries from pregnant cows came from cows in advanced pregnancy. This may explain the low numbers of large follicles in pregnant females. The proportion of total histological atresia was significantly higher in pregnant cows. It has been demonstrated that the presence of a conceptus appears to favour a more rapid turnover of follicles from small to large classes, but further growth is limited by increased atresia (Guilbault *et al.*, 1986; Thatcher *et al.*, 1991). The proportion of small non-atretic follicles was lower in pregnancy as well as among all follicles in each class; this result agrees with those of Ginther and colleagues (1996b) and could corroborate that pregnancy is associated with an increased proportion of atresia. It is possible that prolonged exposure to high progesterone concentrations during pregnancy may be responsible for suppressing follicular development directly or selectively altering LH concentrations (Gibbons *et al.*, 1999). In the current study, a high proportion of early atresia was observed, especially in large follicles. Guilbault and colleagues (1986) also observed a high percentage of early atresia in non-pregnant cows, among all atretic follicles in a class, but in large follicles of pregnant females they reported a major proportion of late atresia. In our study, early atresia was predominant in both physiological statuses. Early atresia observed in these follicles may be due to a failure to stimulate enzymatic activity preceding the more evident degenerative changes in follicles, like those observed in late atresia.

Steroid content in follicular fluid reflects the synthetic capabilities of the granulosa and theca layers (Bigelow and Fortune, 1998; Driancourt and Thuel, 1998; Soboleva *et al.*, 2000; Fortune *et al.*, 2001). In the present study, the steroid concentration of follicular fluid changed as follicular size increased. In the cow, granulosa cells are the main site of follicular androgen aromatization, with estradiol being the major product (Roche, 1996; Hendriksen *et al.*, 2000). Estradiol concentration increased in follicular fluid and testosterone concentration decreased as follicle size increased in both statuses. This could be in agreement with histological findings; since the lifespan of large dominant follicles is controlled by LH secretion, signs of atresia could be initiated as androgenic substrates for aromatization become limiting within the dominant follicle. The increase of estradiol during follicular development is comparable with other data obtained in cattle (Einspanier *et al.*, 1993; Gibbons *et al.*, 1999). An increase in granulosa cell number and aromatase activity could account for the fall in follicular concentration of androgen and the rise of estradiol concentration associated with increasing follicular size. The physiological status affected the estradiol concentration in all follicles sizes. The lowest values, registered in pregnant cows, probably account for a decrease of steroidogenic capability of granulosa cells, which may be concordant with a major proportion of atresia in follicles of pregnant cows. Testosterone concentration in follicular fluid was also significantly lower in pregnancy than in non-pregnancy in follicles of all sizes. The level of testosterone secretion is dependent on the LH responsiveness of theca cells (Melvin *et al.*, 1999). There is evidence that both estradiol and LH may be important in regulating theca cell function during antral follicular development; circulating LH would interact with estradiol and result in an additive stimulation of androgen production by theca cells (Roberts and Skinner, 1990a,b; Eppig, 2001; Manikkam *et al.*, 2001). However, during the luteal phase as well as during pregnancy, LH pulse frequency is low because the concentration of circulating progesterone may inhibit LH secretion, which in turns affects the steroidogenesis (Manikkam and Rajamahendran, 1997; Calder *et al.*, 1999; Melvin *et al.*, 1999).

Considerable variation has been reported in cattle, particularly in progesterone concentrations between follicles of the same size (Einspanier *et al.*, 1993; Jolly *et al.*, 1994, 1997; Manikkam and Rajamahendran, 1997). The higher concentration of progesterone in medium and large follicles compared to small follicles in non-pregnant cows accords with other studies (McNatty *et al.*, 1984; Kruip and Dieleman, 1985; Einspanier *et al.*, 1993). An association has been described between the increased concentration of progesterone and the atresia of the largest follicles collected during repetitive oestrous cycles in cattle (Ireland and Roche, 1982; McNatty *et al.*, 1984; Meidan *et al.*, 1993; Jolly *et al.*, 1994). The high progesterone levels observed in atretic follicles may indicate a minor activity of enzymes that degrade progesterone, such as

17α-hydroxylase, responsible for the conversion of progesterone to androgen. On the other hand, there is evidence that LH could increase progesterone production by granulosa cells of the dominant follicle, since experimental reduction of circulating LH concentration resulted in decreased intrafollicular concentration of progesterone (Thatcher *et al.*, 1996; Ginther *et al.*, 2001; Manikkam *et al.*, 2001). In that sense, an increase in progesterone concentration in follicular fluid may also indicate that the steroidogenic activity in large follicles is greater than that in smaller ones (Beg *et al.*, 2002), as in preovulatory post-gonadotrophin-surge follicles, which initiate differentiation of theca cells to luteal cells. Progesterone concentration in follicular fluid of large follicles was higher in non-pregnant than in pregnant cows. Compared to small and medium follicles in these latter females, large follicles, in contrast, showed the lowest concentrations of progesterone. The reason for this decrease in progesterone in pregnant cows is unclear. Previous reports have found high levels of progesterone in follicular fluid of pregnant cows (Thatcher *et al.*, 1991), which is coincident with an increase of atresia (Guilbault *et al.*, 1986; Ginther *et al.*, 1996b). Other studies have demonstrated that concentrations of progesterone of intermediate atretic follicles are similar to those of non-atretic follicles (Grimes *et al.*, 1987). In pregnant cows, the largest follicles showed the major proportion of early atresia and it is known that histological signs of atresia in a follicle precede loss of steroidogenic capacity (Takagi *et al.*, 1993). Thus it is possible that the concentration of progesterone in these large follicle, which were mainly in early atresia, did not reach the levels of atretic ones; and, in contrast to large non-pregnant cows' follicles, they were not exposed to gonadotrophin surge.

Estrogen-to-progesterone (E/P) ratio is often used to characterize follicles as healthy or atretic (Meidan *et al.*, 1993; Takagi *et al.*, 1993; Jolly *et al.*, 1994; Price *et al.*, 1995) because it should show the progression from estradiol- to progesterone-dominated follicles as follicles become more atretic. Nevertheless, in small follicles the E/P ratio is confusing because the ability of theca cells to respond to LH and produce androgen precedes the ability of granulosa cells to aromatize androgen to oestrogen (McNatty *et al.*, 1984; Roberts and Skinner, 1990a); the cAMP response to LH in granulosa cells was low or not detected in granulosa cells in 2–8 mm follicles, and increased linearly with increasing follicle diameter >8 mm (Jolly *et al.*, 1994). Otherwise, in the largest follicles of cycling cows it is difficult to differentiate between healthy preovulatory post-gonadotrophin-surge and atretic follicles according the E/P ratio. In large follicles of pregnant females, in contrast, this ratio may be useful as an index of atresia, because all these follicles are larger than 8 mm and they are not exposed to the LH surge. In large follicles of pregnant cows, the E/P ratio averaged 0.35. Previous studies have described atretic follicles as those with an E/P ratio less than 1 (Silván *et al.*, 1993; Sunderland *et al.*, 1994), but others have classified follicles as atretic when the E/P ratio is under 0.1, and if the ratio is between 0.1 and 1 they could be in atresia depending on other parameters (Kruip and Dieleman 1985; Grimes *et al.*, 1987). Considering these data together with the histological findings, it is possible that most of the large follicles of pregnant cows were atretic.

Although interpretation of the results of the present study is limited in terms of possible physiological events by the fact that slaughterhouse material was used, in which the peripheral hormonal concentrations and the exact stages of the cycles were unknown, the results provode evidence that the steroid concentration and histological characteristics of follicles are different between non-pregnant and pregnant dairy cows during follicular development.

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