Action of PMSG on follicular populations in the heifer

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Summary. The short-term action of PMSG on the population of growing follicles in cattle was studied using histological methods. On Day 7 of a synchronized oestrous cycle 10 Friesian heifers were unilaterally ovariectomized. The remaining ovary was immediately stimulated by an injection of PMSG (2000 i.u.) and was removed 48 h after the preovulatory discharge of LH. Control animals did not receive any injection of PMSG. In all ovaries, follicles > 70 µm diameter were counted, measured and checked for atresia. The mitotic index in granulosa cells of follicles of different sizes was estimated in both ovaries of all the PMSG-injected animals.

Unilateral ovariectomy alone had no significant effect on follicular populations. In the interval between PMSG injection and removal of the second ovary (148 ± 22.7 h), PMSG significantly increased the number of normal preantral follicles but did not change the number of normal antral follicles. The mitotic index doubled in preantral and early antral follicles but remained unchanged in large antral follicles. PMSG stimulated slightly the growth of the antrum in large antral follicles but did not stimulate its formation in preantral follicles. The incidence of atresia among antral follicles, particularly the largest ones (diam. > 1.7 mm), was significantly reduced after PMSG, suggesting some 'rescue' of follicles from atresia.

Introduction

In cattle, the number of ovulations that follow a stimulatory treatment always varies considerably among individuals, whatever the choice of breed or of treatment. This variability is an important limit to the development of techniques of embryo transfer. The action of PMSG or FSH on follicular populations has been well studied in different species including the mouse (Ryle, 1969, 1972; Peters, Byskov, Himelstein-Braw & Faber, 1975), rat (de Reviers & Mauléon, 1973; Mauléon & Mariana, 1977; Braw & Tsafiriri, 1980), hamster (Greenwald, 1962, 1973, 1974) and ewe (Hay & Moor, 1978; Dott, Hay, Cran & Moor, 1979), but no precise data are available for cattle. To understand better the mechanisms of superovulation in cattle, we wanted to know what were the effects of PMSG on populations of growing follicles during the interval between injection and ovulation. Owing to the large individual variability of follicular populations in cattle (Rajakoski, 1960; Erickson, 1966; Testart, 1972; Mariana & Huy, 1973; Scaramuzzi, Turnbull & Nancarrow, 1980), it has not been possible to examine these effects by comparing two different groups of animals, i.e. a non-stimulated group and a stimulated group. Follicular populations of the two ovaries of any one cow are, however, qualitatively and quantitatively very similar; intra-animal variation is much lower than between-animal variation (Rajakoski, 1960; Testart, 1972; Mariana & Huy, 1973). This has also been observed in hamsters (Greenwald, 1961), ewes (Cahill, Mariana & Mauléon, 1979) and mares (Driancourt, Paris, Roux, Mariana & Palmer, 1982). Moreover in the cow, after injection of PMSG, the number of large follicles (Testart, 1972) and the ovulation rate

(Saumande & Chupin, 1982) are similar in the two ovaries of any one individual. In the mouse, Falconer, Edwards, Fowler & Roberts (1961) found that, with or without stimulation, the numbers of ovulations were distributed between sides approximately at random, the variation conforming fairly closely to a binomial distribution.

In this work, therefore, the experimental design was chosen such that each animal was its own control. One ovary was removed immediately before the other was stimulated by injecting PMSG. After ovulation, the other ovary was removed and the effect of the PMSG on follicular populations could then be evaluated by comparing the second (i.e. stimulated) ovary with the first (i.e. control) ovary by using histological techniques. Some of the data in this paper have been discussed by Monniaux, Chupin & Saumande (1983).

Materials and Methods

Experimental design

The 18 cyclic Friesian heifers, aged 18 months, were randomly assigned to 2 groups, a control group (8 animals) and an experimental group (10 animals) treated with PMSG. Body weights (mean $\pm$ s.d.) in the two groups were similar, 405 ± 36 kg and 411 ± 59 kg respectively at the beginning of the experiment. Animals were kept in a building with programmed artificial lighting (10 h L:14 h D).

The stimulatory treatment was the 'cocktail' treatment of Chupin & Saumande (1979) in which PMSG is injected after first synchronizing the oestrous cycle. On the 9th day of a natural cycle (Day 1 of treatment), each animal received a 6 mg progestagen implant (Norgestomet: Intervet S.A., 49000 Angers, France) and 5 mg intramuscular (i.m.) injection of oestradiol valerate. Six days later (Day 7), the ovary with the corpus luteum of the previous cycle was removed. Just after this unilateral ovariectomy, all the animals received an i.m. injection of a prostaglandin analogue, 0.5 mg cloprostenol (Estrumate: ICI-pharma Vétérinaire, 95880 Enghien, France). One group of animals also received an i.m. injection of PMSG (2000 i.u.). Implants were removed from all the animals at Day 9 of treatment.

Blood samples were obtained every 2 h after the unilateral ovariectomy at Day 7, plasma was collected, and LH concentration measured using a rapid radioimmunoassay. The remaining plasma was then stored at -20°C . All the animals of Group E (unilateral ovariectomy and PMSG) and 5 of the 8 animals of Group C (unilateral ovariectomy but no PMSG) were slaughtered 48 h after the preovulatory discharge of LH was detected and the second ovary was collected. The mean $\pm$ s.d. interval between unilateral ovariectomy and slaughter for Groups E and C was 148 ± 22.7 and 156 ± 12.4 h, respectively. Three animals in Group C did not show any LH peak within the 160 h after unilateral ovariectomy and were excluded from the study.

LH rapid radioimmunoassay

The heterologous radioimmunoassay of Pelletier (1972) was adapted for short-term incubation. A guinea-pig antiserum against ovine LH prepared and tested in the laboratory (Pelletier, 1972) and ^{125}I -labelled ovine LH (CNRS LH M₃, biol. act. $1.8 \times \text{LH-NIH-S1}$, labelled by the chloramine T method, sp. act. $100 \mu\text{Ci}/\mu\text{g}$) were used in the assay which was performed on 100 μl plasma. Incubation was carried out partly at 20°C and partly at 4°C and lasted 5 h 30 min. This assay allowed us to detect the preovulatory discharge of LH but did not give quantitative information. Results were subsequently confirmed using a quantitative LH assay (Pelletier, 1972).

Histological study of ovaries

Both ovaries of the 10 experimental and the 5 control animals were studied by histological techniques. Immediately after removal, ovaries were fixed in Bouin-Hollande solution, and then

dehydrated and embedded in paraffin wax. Serial sections were cut at 7 μm and one section out of 6 was mounted, stained with haematoxylin and examined microscopically.

Follicular counting. In the 30 ovaries studied, all the growing follicles with a maximal area of section greater than 4000 μm^2 (follicles with 2 layers of granulosa cells or more) were counted, measured, and checked for atresia. The oocyte was used as a marker to avoid counting the follicles twice. Follicular profiles were measured using a semi-automatic image analysis system (A.S.M. Leitz); the area of each follicle (limited by the basal layer of granulosa cells) and of its antrum were measured. Using criteria previously proposed for the cyclic cow (Rajakoski, 1960; Erickson, 1966; Marion, Gier & Choudary, 1968) and the stimulated heifer (McKenzie & Kenney, 1973), we defined 3 stages of atresia: early, definite, and late atresia. Early atretic follicles were characterized by a slightly pycnotic or disorganized granulosa with persistence of mitosis, or by a local deformation of the follicle, or by slight anomalies of the oocyte. Definitely atretic follicles were characterized by many pycnotic bodies and no mitosis in granulosa cells, or by substantial deformation of the follicle, or by clear anomalies and deformation of the oocyte. Late atretic follicles were characterized by simultaneously evident anomalies of the granulosa cells and oocyte. Follicles in very late atresia, characterized by invasion by fibroblasts or disappearance of the oocyte, were not counted. Atresia of the oocyte was particularly frequent in preantral follicles, as has been observed in women (Himmelstein-Braw, Byskov, Peters & Faber, 1976), mice (Byskov, 1978) and bitches (Spanel-Borowski, 1981).

Follicles were allocated to size classes according to diameter. The diameter was calculated from the measured area, with the assumption that follicles were spherical. The 11 size classes started at 71 μm diameter (area = 4000 μm^2) and with limits in geometric progression ($d_{i+1}/d_i = 1.578$ where d_i = maximal follicular diameter for follicles in class i).

Mitotic index. Both ovaries of the 10 animals of Group E were studied. For each ovary, mitotic indices were estimated in follicles at about the size when the antrum forms (see Text-fig. 3), i.e. between 115 μm , when 10% of follicles have an obvious antral cavity, and 280 μm (90% with a cavity). Ten follicles in this size range with an antrum and 10 others without an antrum were selected at random for study. In addition, larger (> 0.5 mm) follicles were similarly studied: 15 normal follicles (10 of 0.5–1 mm, 5 of > 1 mm) and 10 early atretic follicles were selected from each ovary, except in a few cases when the ovary contained too few such follicles. For each follicle, 3 successive profiles containing the oocyte were examined. On each, the area of granulosa was measured, the number of mitotic figures was counted, and the number of granulosa cells in the profile was calculated using an estimate of the cellular density made using a circular reticule (30 measurements per follicle). The total number of cells per follicle was then estimated, according to Pedersen (1970). To estimate the mean number of cells in follicles of different sizes, we used regression lines of the form: $N = a + bS$ where N = total number of granulosa cells per follicle, and S = area of (largest) follicular profile.

For each type of ovary (removed before or after stimulation), three such regression lines have been calculated from the data for individual follicles: one for follicles at the size of antrum formation but still lacking an obvious antral cavity, one for follicles of this size but with an antral cavity, and one for follicles > 0.5 mm diameter.

Statistical methods

Due to the non-normality of data relating to numbers of follicles and mitotic index, some non-parametric statistics have been used: tests of association were made using the Spearman rank coefficient (r_s) and the effects of PMSG and unilateral ovariectomy were tested using the paired test of Wilcoxon (Siegel, 1956). Slopes of regression lines were compared using the t test (Steel & Torrie, 1980). χ^2 analysis was used to compare the distributions of follicles in non-stimulated and stimulated ovaries.

Results

Effect of PMSG on numbers of growing follicles

The mean number of follicles per animal was higher in Group C heifers ($P < 0.05$), but the total numbers of normal follicles and of atretic follicles were very variable amongst the individual heifers in Groups C and E (Table 1). This large between-animal variation was evident in the first (before PMSG) and second (after PMSG in Group E) ovary removed. However, within each heifer the number of follicles in the two ovaries agreed closely (Text-fig. 1; overall $r_s = 0.85$; slopes of regression lines ≈ 1) and so the first ovary served as a good control for the effect on the second ovary of unilateral ovariectomy in Group C and of unilateral ovariectomy plus PMSG in Group E.

In the heifers in Group C, there was no significant effect of surgery on the numbers of normal preantral or antral follicles. Nor did it affect the numbers of atretic follicles in these size classes. This indicated that the first ovary removed was, in fact, a good control for the effect of PMSG on the second ovary in Group E cows. In these cows, the mean $\pm$ s.e.m. number of normal preantral

Table 1. Mean numbers of normal and atretic follicles in ovaries of heifers in Group C (unilateral ovariectomy (ULO) only) and in Group E (ULO + PMSG)

	Group C (N = 5)		Group E (N = 10)	
	Before ULO	After ULO	Before ULO + PMSG	After ULO + PMSG
Normal follicles	616 (333–1593)	609 (286–1621)	340 (53–755)	409 (73–881)
Early atretic follicles	42 (20–103)	33 (14–92)	15 (0–41)	13 (0–57)
Definite atretic follicles	40 (14–103)	32 (12–85)	38 (9–93)	40 (4–139)
Late atretic follicles	73 (11–227)	65 (8–194)	42 (0–143)	46 (4–160)

Values in parentheses indicate the ranges in each group.

Text-fig. 1. Data showing the relationship between numbers of follicles in the first ovary (N_1), removed at unilateral ovariectomy, and numbers in the second ovary (N_2), removed at slaughter in (a) Group C heifers (no PMSG) and (b) Group E heifers (PMSG). Each datum point represents one heifer and the lines are regression lines fitted by least squares. Data for atretic follicles include all stages of atresia. (b) Reproduced from Monniaux *et al.* (1983).

follicles increased significantly after PMSG injection (342 ± 79 compared with 277 ± 70 , $P < 0.05$; Wilcoxon paired test, $T = 6$, $n = 10$) while the number of atretic preantral follicles remained unchanged. The number of normal antral follicles was unchanged but the mean $\pm$ s.e.m. number of atretic (early + definite) follicles decreased significantly (8.6 ± 2.1 compared with 11.5 ± 2.4 , $P < 0.05$; Wilcoxon paired test, $T = 7$, $n = 10$).

When follicular size classes were considered (Text-fig. 2), PMSG increased ($P < 0.05$) the number of normal follicles of diameter $< 180 \mu\text{m}$ (classes 1 and 2) and decreased ($P < 0.05$) the number of atretic (early + definite) follicles of diameter $180\text{--}280 \mu\text{m}$ (class 3) and the number of atretic, large antral follicles of diameter $1740\text{--}2740 \mu\text{m}$ (class 8). PMSG had no detectable effect on the numbers of late atretic follicles.

Effect of PMSG on follicular growth

The changes observed in the numbers of follicles after PMSG suggest that the action of PMSG differs amongst follicles of different sizes, in particular between preantral follicles and large antral follicles. To study this further, the action of PMSG on proliferation of granulosa cells and on formation and enlargement of the antrum was evaluated in all 20 ovaries of Group E heifers. Two types of follicle were selected for study: those in the size range of antrum formation ($115\text{--}280 \mu\text{m}$; some with an obvious antrum and some without) and large antral follicles (diameter $> 0.5 \text{ mm}$).

Follicles at antrum formation: action of PMSG on multiplication of granulosa cells. The regression lines relating the number of granulosa cells per follicle to the area of the maximal profile were calculated and used to estimate the mean numbers of granulosa cells per follicle. These calculations were carried out separately for follicles $115\text{--}280 \mu\text{m}$ with an obvious antrum and for those without. For the follicles without an antrum, the slope of the regression line was slightly higher in ovaries taken after PMSG treatment than in those taken before PMSG (0.092 versus 0.077 ; $0.05 < P < 0.1$, d.f. 136). The mean number of cells per follicle (Table 2) was unchanged. For follicles with an antrum, PMSG had no significant effect on the slope (0.096 versus 0.104 , N.S., d.f. 107) or on the mean number of cells per follicle.

In follicles at antrum formation ($115\text{--}280 \mu\text{m}$), mitotic indices were low before PMSG injection and increased 2-fold after injection: this stimulation by PMSG was evident for antral and preantral follicles of this size (Table 3).

Follicles at antrum formation: appearance of the antrum in relation to follicular size. An antrum was never present in follicles of diameter $< 100 \mu\text{m}$ but had always appeared by the time they were $> 450 \mu\text{m}$. In this range, especially the part of it between 180 and $330 \mu\text{m}$ (Text-fig. 3), the percentage of follicles with an antrum was lower after PMSG treatment than before PMSG

Table 2. Mean numbers of granulosa cells in follicles of different sizes in heifers treated with PMSG (Group E)

Follicular diam.		Diameter (μm)	Mean no. of granulosa cells per follicle	
			Before ULO + PMSG	After ULO + PMSG
115–280 μm	Without antrum	115	390 (± 90)	240 (± 100)
		195	1920–2040 (± 120) (± 160)	2000–2070 (± 100) (± 120)
	With antrum	280	5150 (± 260)	4890 (± 320)
$> 0.5 \text{ mm}$		500	30 490 (± 16320)	53 640 (± 20820)
		1000	141 700 (± 14020)	150 900 (± 19800)
		5000	3 702 000 ($\pm 165 000$)	*3 264 000 ($\pm 108 000$)
		10000	14 830 000 ($\pm 689 000$)	*12 990 000 ($\pm 453 000$)

Values in parentheses are 95% confidence intervals. About half of the follicles with a diameter of $195 \mu\text{m}$ have an antrum and the 2 numbers given for them are respectively for follicles with and without an antrum.

* $P < 0.05$, compared with before ULO + PMSG.

Text-fig. 3. Relationship between size of follicles and the percentage of them that contained a distinct antrum in heifers treated with PMSG (Group E). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Table 3. Mitotic index (%) of normal follicles and follicles in early atresia in heifers treated with PMSG (Group E)

Follicular diam.		Mean mitotic index (%)	
		Before ULO + PMSG	After ULO + PMSG
115–280 μm	Normal follicles without antrum	0.31 (0.07)	*0.67 (0.11)
	Normal follicles with antrum	0.36 (0.07)	**0.76 (0.05)
0.5–1 mm	Normal follicles	0.77 (0.10)	0.85 (0.07)
	Early atretic follicles	0.69 (0.13)	0.99 (0.09)
> 1 mm	Normal follicles	1.52 (0.14)	1.75 (0.08)
	Early atretic follicles	0.83 (0.10)	1.12 (0.13)

Values in parentheses are standard errors of the means.

* $P < 0.05$, ** $P < 0.01$, compared with before ULO + PMSG.

Text-fig. 2. Distribution of sizes of follicles in heifers treated with PMSG (Group E). Mean values for 10 animals. Size ranges: ① = preantral follicles; ② = follicles at antrum formation; ③ = antral follicles. * $P < 0.05$, compared with corresponding group in ovaries taken before ULO and PMSG.

treatment, indicating that PMSG did not provoke any hastening of antrum formation or, if it did, then the effect was concealed by an accelerated movement of small antral follicles into larger size classes, through acceleration of granulosa cell proliferation or growth of the antrum.

Large antral follicles: action of PMSG on multiplication of granulosa cells. In follicles > 0.5 mm diameter, the slope of the regression line, used to estimate the mean number of granulosa cells per follicle, was significantly lower after PMSG treatment (0.165 versus 0.189, $P < 0.001$, d.f. 152). Consequently, the number of cells in follicles about 5 mm diameter or larger was significantly decreased ($P < 0.05$) (Table 2). This suggests that the antrum in these follicles would be larger than in follicles of the same size before stimulation.

In the normal follicles of ovaries removed before PMSG injection, the mitotic index increased progressively with increase in follicular size (Table 3); it was higher ($P < 0.01$) in follicles of 0.5–1 mm than in (smaller) follicles at antrum formation and was yet higher ($P < 0.01$) in those > 1 mm. Early atresia in large (> 1 mm) follicles was associated with reduced mitotic index ($P < 0.02$).

PMSG had no significant effect on mitotic index in any category of the large antral follicles.

Large antral follicles: action of PMSG on growth of antrum. There was a linear relationship between the logarithm of follicular diameter and the logarithm of antrum diameter in each ovary (Text-fig. 4). Slopes of the regression lines for different animals were heterogeneous ($P < 0.05$). In 9 of the 10 Group E heifers, the slope was slightly higher for the ovary taken after PMSG than for the other ovary (Text-fig. 4) and in one of these the difference was statistically significant ($P < 0.01$). Overall this effect was significant ($P < 0.05$; χ^2 (Bartlett's correction) = 4.9) and suggests that there was a weak stimulatory effect of PMSG on growth of the antrum. After PMSG treatment the tendency was for the larger follicles (> 0.5 mm) to have a larger antrum and for the smaller follicles (< 0.5 mm) to have a smaller antrum. This is in accord with the above-mentioned apparent

Text-fig. 4. Growth of the antrum. Example of the relationship between antral size and follicular size in a heifer treated with PMSG (Group E). Scales on both axes are logarithmic. Datum points are for individual follicles of Heifer 62 and the lines are regression lines fitted to the log-transformed data.

inhibition of antrum formation (Text-fig. 3) and (in large follicles) the reduced number of cells (Table 3) after PMSG.

Effect of PMSG on formation of luteal structures

Fresh corpora lutea or luteinized follicles, or both (in 7 of the 10 ovaries), were found in the ovaries removed 48 h after the LH peak of the Group E heifers. In the fresh corpora lutea, some follicular fluid still remained and granulosa cells showed only the first signs of luteinization. Some of these corpora lutea displayed many pycnotic bodies. Luteinized follicles were large (diameter about 1 cm) and their granulosa cells and thecal cells showed the first signs of luteinization. Local luteinization, particularly in cumulus cells, was observed in some follicles in definite atresia. The wall of luteinized follicles, unlike that of a true corpus luteum, was not disrupted, and the oocyte was always found enclosed in dispersed cumulus cells. Resumption of meiosis was often observed in oocytes of luteinized follicles, and only once in an atretic non-luteinized follicle.

After unilateral ovariectomy and PMSG injection, the number of corpora lutea per ovary varied between 0 and 10 and the number of luteinized follicles between 0 and 7. Only one control animal had more than one (2) corpora lutea in the ovary removed after unilateral ovariectomy and no control animal had any luteinized follicles.

Discussion

In control animals, unilateral ovariectomy alone had no significant effect in the short term of this experiment (around 6 days) on the numbers of growing follicles. This result differs from those in rodents but is in accord with those in the ewe. In the hamster, 4 h after unilateral ovariectomy, preantral follicles with 6 layers of granulosa cells formed an antrum (Chiras & Greenwald, 1978a) and, 20 h later, the number of large follicles had increased 2-fold in the remaining ovary (Greenwald, 1961). In the rat, 12 h after unilateral ovariectomy, an increase in the number of large follicles was evident (M. M. de Reviers & W. R. Gibson, unpublished). In the ewe, on the other hand, Dufour, Cahill & Mauléon (1979) showed that the compensation was much less marked: 70 days after unilateral ovariectomy, there was a clear increase only in the number of preantral follicles. The apparent inertia of the follicular populations of the ewe and cow may be related to their longer oestrous cycles and is probably due to slower follicular growth rates.

Changes in the numbers of follicles in ovaries removed after treatment in Group E were, in this experiment, attributed to PMSG alone since unilateral ovariectomy was without effect. The main findings were that PMSG increased the number of normal preantral follicles (with a diameter $< 180 \mu\text{m}$) and decreased the number of atretic (early + definite) larger follicles, particularly those at the size of antrum formation and those antral follicles with a diameter $> 1.7 \text{ mm}$.

The observation that PMSG increased the number of normal preantral follicles ($< 180 \mu\text{m}$) could be explained in several ways. The hypothesis that PMSG acted by preventing some of these follicles from becoming atretic is unlikely because the rate of atresia was $< 1\%$ (Text-fig. 2) and was not affected by PMSG. In these follicles, the observed doubling of the mitotic index, on the other hand, indicates that PMSG probably increased growth rates in preantral follicles. This would be expected to increase the numbers of preantral follicles. Since PMSG did not hasten antrum formation in this short-term experiment, the increase was not offset by any acceleration in the transfer of follicles from preantral to antral.

Our observations on heifers differ in two ways from those on stimulation of follicular growth by PMSG or FSH in rodents. Firstly, in the heifer a response was detected only in the preantral follicles, since neither the numbers of normal antral follicles nor their mitotic index were affected. In rodents, antral follicles are also stimulated (mouse: Ryle, 1972; rat: de Reviers & Mauléon, 1973; hamster: Chiras & Greenwald, 1978b). It is possible that in the heifers PMSG also stimulated the

growth of large antral follicles but that this effect was transient, being terminated by the preovulatory discharge of LH. Secondly, formation and growth of the antrum are stimulated in rodents (Mariana & Machado, 1976; Mauléon & Mariana, 1977) but there was no such change in the heifers of the present, short-term experiment. It is possible that a weak stimulation by PMSG, detected in the very large antral follicles, would have become much clearer some days later. For the smaller follicles, the antrum formed at a larger size after PMSG treatment, presumably because the stimulation of growth through cellular multiplication predominated over any stimulation of secretion of antral fluid.

To explain the effect of PMSG on antral follicles, i.e. decrease in the number of atretic follicles without changing the number of normal follicles, two hypotheses can be made: PMSG either prevented some normal follicles from becoming atretic or it 'rescued' some atretic follicles and returned them to a normal state. These hypotheses have been proposed previously following various studies *in vivo* on mice (Peters *et al.*, 1975) and rats (Braw & Tsafiri, 1980) and *in vitro* on ewes (Hay, Moor, Cran & Dott, 1979). Whatever the exact mechanism may be, our observations on the heifer again suggest the existence of a delicate equilibrium between normal follicles, 'rescued' follicles, and follicles that ovulate or luteinize. We suggest that large antral follicles prevented from becoming atretic or 'rescued' from early stages of atresia would be able to ovulate or to luteinize after the LH preovulatory discharge. The presence of pycnosis, sometimes abundant, in some fresh corpora lutea, and particularly in many luteinized follicles, could indicate that follicles which ovulate or luteinize after PMSG are not always normal. We propose that some large follicles rescued from atresia by PMSG could ovulate or, more commonly, only luteinize following the preovulatory discharge of LH.

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