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# Blockade of recruitment of ovarian follicles by suppression of the secondary surge of follicle-stimulating hormone with porcine follicular fluid*

(ovarian inhibin/folliculostatin/luteinizing hormone/estradiol/progesterone)

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**ABSTRACT** The increased serum concentration of follicle-stimulating hormone (FSH; follitropin) between proestrus and estrus in the rat has been hypothesized to recruit the follicles destined to ovulate in the next cycle. Injection of porcine follicular fluid (PFF) late in proestrus suppresses the secondary FSH surge; injection early in proestrus suppresses the primary FSH surge without affecting the secondary FSH surge. Thus, it is possible to use PFF to test the FSH/follicular recruitment hypothesis and to distinguish between the contributions of the primary and secondary FSH surges to this recruitment. The normal recruitment of follicles occurs in the diameter range 350–499 μm between the day of proestrus and the day of estrus. When the secondary FSH surge was suppressed by injection of PFF late in proestrus, PFF, but not porcine serum (PS), blocked follicular recruitment into size groups of 350–499 μm on the morning of estrus. The number of ova ovulated did not differ between PFF- and PS-treated animals. When we suppressed only the primary FSH surge, by injecting PFF early in proestrus, there were no differences between PFF- and PS-treated animals in the number of ova ovulated, follicle size distribution, or hormones. In the last experiment, the secondary FSH surge was blocked with PFF but was replaced with exogenous ovine FSH which caused a dose-related increase in follicular recruitment, substantiating the interpretation that the follicular fluid suppressed recruitment by suppressing FSH secretion. Thus, in mammals with short reproductive cycles, the gonadotropin surges provide a "fail-safe" mechanism whereby luteinizing hormone triggers ovulation, thus ending one cycle, and the secondary increase in FSH levels recruits follicles for the next cycle.

During the afternoon of the day known as proestrus in the rat estrous cycle the two gonadotropic hormones, luteinizing hormone (LH; lutropin) and follicle-stimulating hormone (FSH; follitropin), surge between 1400 and 2000 hr (1, 2). By midnight between proestrus and the next day, estrus, the serum LH level returns to baseline but FSH remains increased throughout the night until 1200 hr on estrus (1–3). This continued secretion of FSH has been called the "secondary FSH surge."

Reflecting the marked alterations in serum gonadotropins, between the mornings of proestrus and estrus there is also a discontinuous growth of ovarian follicles into the "pre-Graafian" size class, 390–500 μm in diameter (4, 5). McClintock and Schwartz (6) first proposed that, although the LH surge terminated the cycle in progress by inducing ovulation, the FSH secretion at proestrus initiated the recruitment of the cohort of follicles that would ovulate in the next cycle.

Recently an experimental tool has become available for selectively suppressing serum FSH without altering serum LH or interfering with radioimmunoassay of blood levels of gonadotropin, as does antiserum to gonadotropic hormones (7, 8).

Porcine follicular fluid (PFF) contains a protein called "folliculostatin" or "ovarian inhibin" which inhibits the secretion of FSH specifically in both intact and ovariectomized female rats (9–13). In fact, Schwartz and Channing (11) have shown that injection of PFF late in proestrus (1545 and 1830 hr), after the primary LH and FSH surges have taken place, specifically suppresses the secondary FSH surge. Moreover, PFF administered earlier on the day of proestrus (1200 hr) suppresses the primary FSH surge without affecting the LH surge; however, after the effect of PFF wears off, the secondary FSH surge occurs (12, 13). Thus, by using PFF it is possible to test the hypothesis that either the primary or the secondary FSH surge in proestrus and estrus is necessary for the recruitment of ovarian follicles.

In the present study ovarian follicles in 50-μm diameter size classes from 250 to >500 μm (preovulatory) were measured in four protocols. In experiment I, ovaries from untreated control rats were removed on the mornings of proestrus and estrus, to establish the timing of the phenomenon of follicular recruitment in our rats showing 4-day estrous cycles. In experiment II, rats were treated with PFF late in proestrus to block the secondary FSH surge (11) and were autopsied on the morning of estrus. In experiment III, rats were treated with PFF early in proestrus, to block the primary FSH surge (12), and were autopsied on the morning of estrus. Follicular recruitment was blocked when the secondary, but not the primary, FSH surge was suppressed. Moreover, in experiment IV, replacement with ovine FSH restored follicular recruitment in a dose-dependent manner.

## MATERIALS AND METHODS

**Animals.** Sprague–Dawley female rats (ARS, Madison, WI), 60 days old, were housed in a 14-hr light/10-hr dark environment (lights on 0500–1900 hr) with free access to food and water. Vaginal cytologic examination was performed daily by means of saline lavage. All rats had shown at least two consecutive 4-day cycles before being used. Terminal blood samples were taken by decapitation. Oviducts from animals sacrificed in estrus were examined for the presence of ova. In experiment I, animals autopsied on the morning of proestrus had to show ballooned uteri (intraluminal water, ≥100 mg) as a confirmation of proestrus (2). All groups contained 5 animals except where noted otherwise.

**Injected Materials and Hormone Radioimmunoassays.** PFF was collected and treated with charcoal to remove virtually all steroids, as described (9, 11). Control animals received

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Abbreviations: LH, lutropin (luteinizing hormone); FSH, follitropin (follicle-stimulating hormone); PFF, porcine follicular fluid; PS, porcine serum.

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porcine serum (PS) (GIBCO), also extracted with charcoal. All injections were intraperitoneal.

Serum was frozen and assayed for LH by radioimmunoassay (ovine/ovine system, with NIH-LH-S16 standard), FSH (rat/rat system, with NIH-FSH-RP1 standard), estradiol (GDN antiserum no. 244), and progesterone (GDN antiserum no. 869). The radioimmunoassays have been described in detail (14).

**Histology.** One ovary with attached oviduct was removed from each animal, fixed in Bouin's solution, embedded in paraffin, serially sectioned at 7 μm, and stained with Azan trichrome. Ovarian sections were examined under ×100 magnification, and follicular diameter was determined by using a calibrated ocular micrometer. With the basement membrane of the granulosum as the boundary of the follicle, two diameters at right angles to each other were measured and averaged. To avoid counting the same follicle twice, the section containing the nucleolus of the oocyte was measured. All follicles with an average diameter ≥250 μm were measured and classified according to degree of atresia (Fig. 1). Only data from healthy (nonatretic) follicles are presented in this paper.

**Experiment I. Demonstration of Follicular Recruitment in Untreated Rats.** Untreated proestrous and estrous rats were decapitated at 1100 hr. One ovary from each rat was examined as described above. Data in each size category were analyzed by Student's $t$ test; $P < 0.05$ was considered to be significant.

**Experiment II. Effect of Blocking Secondary FSH Surge with PFF on Follicular Recruitment at Estrus.** Proestrous rats were injected with 0.5 ml of PFF or PS at 1545 and 1830 hr, a treatment that will suppress only the secondary FSH surge (11), and they were decapitated at 1100 hr in estrus. An ovary from each rat was examined as described above. In the 450- to 499-μm-diameter group, a $P$ value could not be calculated by Student's $t$ test because there were no follicles of this size in PFF-treated animals. For this group, the Wilcoxon ranking method for unpaired replicates was used and yielded a value of $P < 0.05$ (15).

**Experiment III. Effect of Blocking Primary FSH Surge with PFF on Follicular Recruitment at Estrus.** Proestrous rats were injected with 1 ml of either PFF or PS at 1200 hr, a treatment known to suppress only the primary FSH surge. They were autopsied at 1000 hr on the next morning (estrus). These are ovaries from animals whose hormone levels have been reported (12).

**Experiment IV. Effect of Suppressing Endogenous Secondary FSH Surge and Then Replacing with Exogenous FSH.** Proestrous rats were injected with 0.5 ml of PFF or PS at 1545 and 1830 hr, as in experiment II. PS-treated animals were then injected subcutaneously (under red light) at 2100 hr proestrus with either saline (0.75 ml) or ovine FSH (40 μg) (NIH FSH S-5 in sterile saline). PFF-treated animals were injected

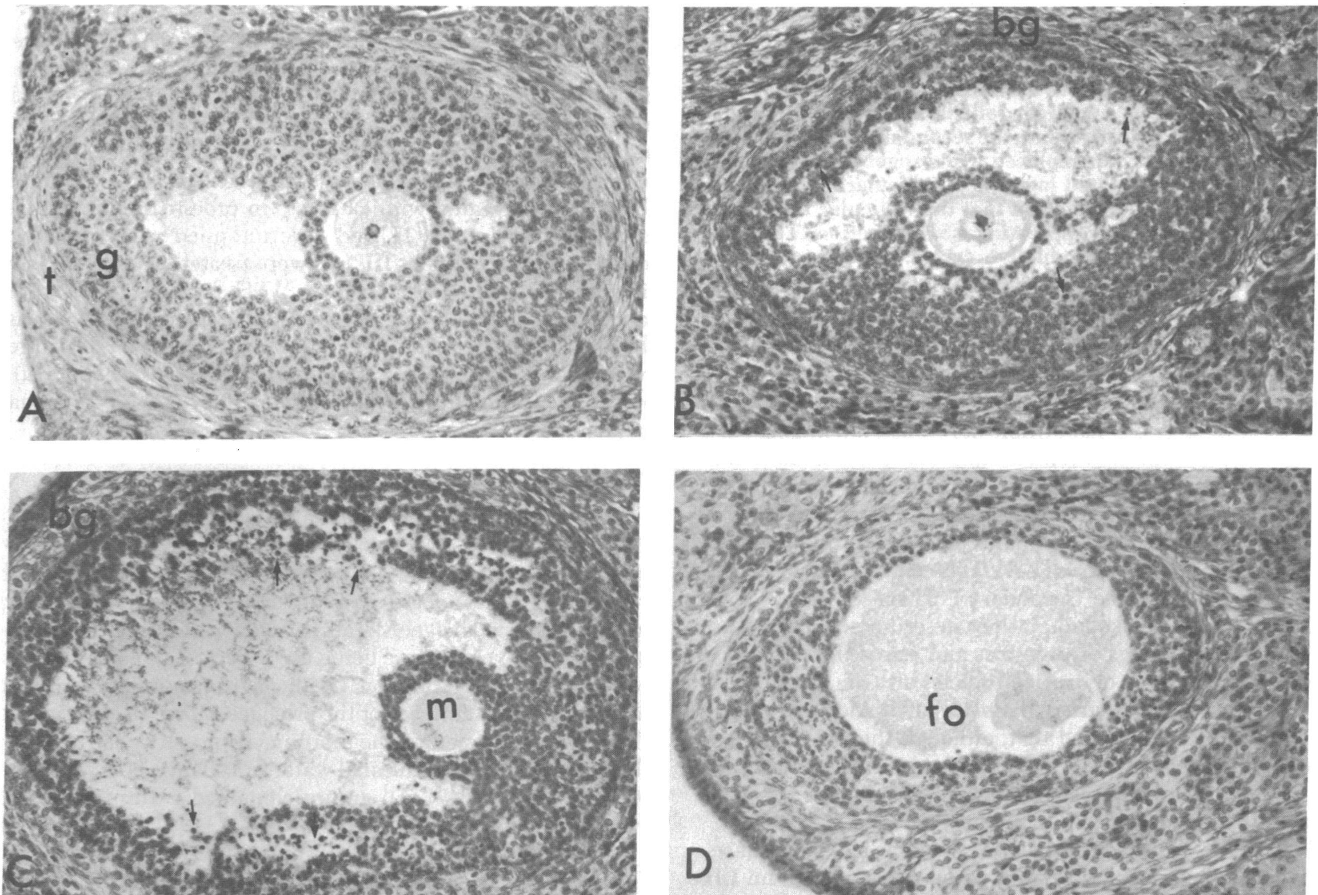


FIG. 1. Follicles in untreated animals. (Azan trichrome; ×190.) (*A*) Healthy follicle; average diameter, 300 μm. g, Granulosa layer; t, theca layer. (*B*) Stage I (presumed beginning) atresia. The outer granulosa layer has become palisaded or "beaded" around the entire circumference (bg) and there are three or more pyknotic granulosa cells (arrows) in the section measured. The nucleus still retains its nucleolus. The inner granulosa cells are loosely adherent to each other, giving a lacy or moth-eaten appearance. (*C*) Stage II atresia. The oocyte has resumed meiosis. The nuclear membrane and nucleolus have disappeared and either chromosomes (m) or the first polar body is present. There are many pyknotic granulosa cells. (*D*) Stage III atresia. The oocyte is grossly misshapen, or fragmented (fo) into several pieces. The granulosa layer has become thin and the basement membrane may be disrupted. The theca layer has hypertrophied.

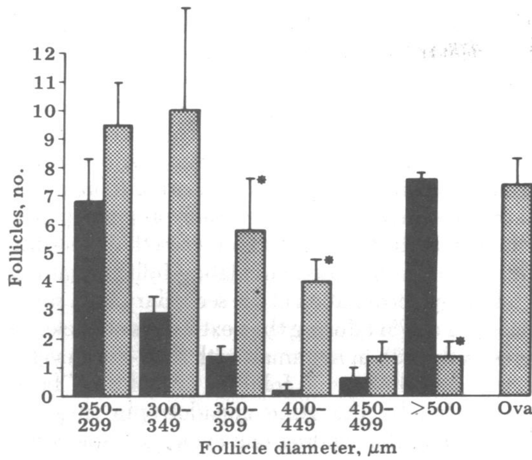


FIG. 2. Distribution of healthy follicles classified in 50-μm size classes (and the number of fresh ova found in the oviduct) found in one ovary of untreated rats in proestrus and estrus (experiment I). Results are mean ± SEM in five animals. Solid bar, proestrus; stippled bar, estrus; *, $P \leq 0.05$ for difference between proestrous and estrous rats.

subcutaneously at 2100 hr proestrus with saline or 40, 80, or 160 μg of ovine FSH. All animals were decapitated at 1100 hr estrus. Ovaries were examined as described above.

## RESULTS

**Experiment I.** On the morning of proestrus many healthy follicles >500 μm in diameter were present (Fig. 2). These are the large Graafian follicles that will ovulate 10–12 hr after the primary surge of LH. There were few follicles 350–499 μm in diameter. By the morning of estrus there had been a significant increase in the number of healthy follicles in the size groups 350–399 μm ($P < 0.05$) and 400–449 μm ($P < 0.01$). There were few follicles >500 μm in estrus, but freshly ovulated ova had appeared in the oviduct.

**Experiment II.** The injection of PFF at a time known to inhibit the secondary surge of FSH (11) significantly decreased the number of follicles on the morning of estrus in size groups 350–399 μm ($P < 0.05$), 400–499 μm ($P < 0.001$), and 450–499 μm ($P < 0.05$) (Fig. 3). The number of ova ovulated at estrus did not differ between PS- and PFF-treated animals. Serum LH, FSH, and estradiol levels were not different in PS- and PFF-treated rats (data not shown) or from values previously obtained on the morning of estrus (1–3, 12, 14). However, progesterone levels were significantly suppressed in the PFF-treated rats (15.8 ± 2.2 ng/ml in PS, 6.6 ± 0.5 ng/ml in PFF, $P < 0.05$). The same phenomenon has been seen in another group of similarly treated rats (unpublished observations). This progesterone suppression may result from an action, on the ovary, of a "luteinization inhibitor" found in PFF (16).

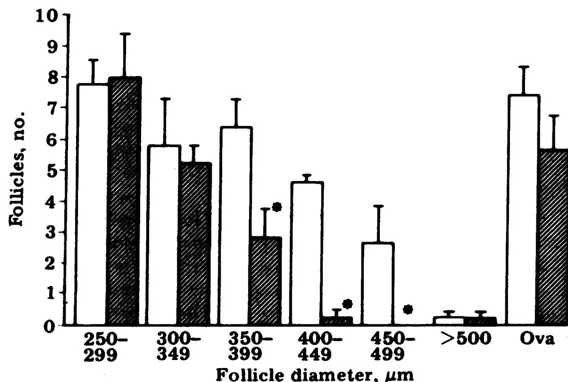


FIG. 3. Distribution of healthy follicles (and the number of fresh ova) in rats autopsied at estrus, after treatment with PFF or PS at 1545 and 1830 hr (experiment II). See legend to Fig. 2 for further explanation. Results are mean ± SEM in five animals. Open bar, PS; hatched bar, PFF; *, indicates a significant difference in number of follicles between PS and PFF treated rats.

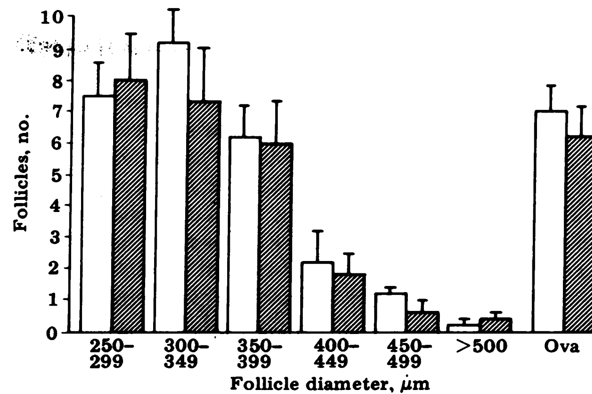


FIG. 4. As in Fig. 3, with PS or PFF treatment at 1200 hr in proestrus (experiment III).

**Experiment III.** The injection of PFF at 1200 hr in proestrus, which is known to suppress the primary FSH surge but not the secondary FSH surge (12), did not block either the recruitment

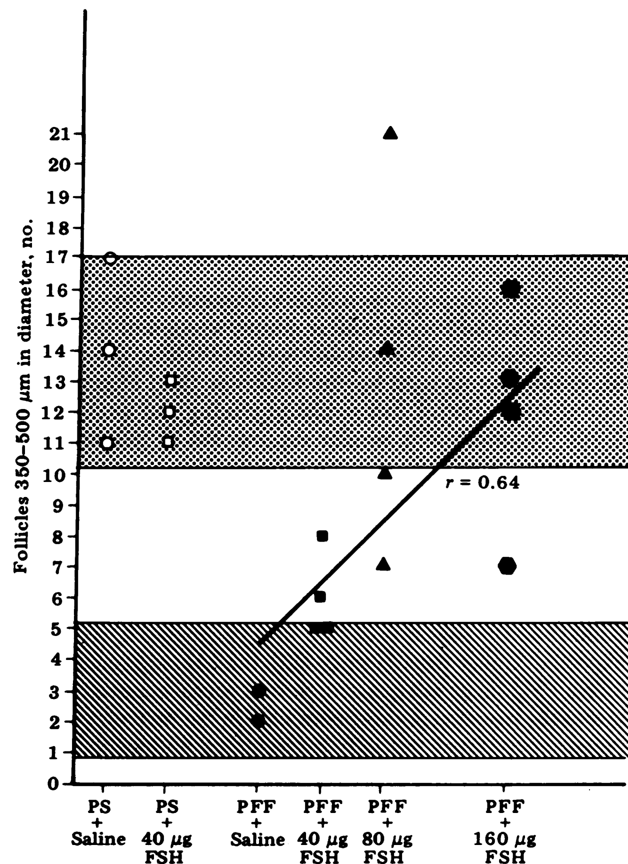


FIG. 5. Follicular recruitment after treatment with exogenous FSH and PS (open symbols) or PFF (solid symbols). The 95% confidence intervals for all follicles 350–500 μm (in one ovary at 1100 hr in estrus) is represented for PS-treated animals (stippled area) and for PFF-treated animals (hatched area) (calculated from experiment II). The number of follicles 350–500 μm in diameter from one ovary of each individual animal in the treatment groups in experiment IV is plotted: ○, PS + saline; □, PS + 40 μg of FSH; ●, PFF + saline; ■, PFF + 40 μg of FSH; ▲, PFF + 80 μg of FSH; ●, PFF + 160 μg of FSH. For line, $r = 0.64$.

of follicles by 1000 hr estrus or ovulation (Fig. 4). There also were no differences in serum LH, FSH, estradiol, or progesterone levels at the time of autopsy (data shown in ref. 12).

**Experiment IV.** The number of follicles between 350 and 500 μm in diameter recruited by 1100 hr in estrus in animals treated with PS plus saline or PS plus 40 μg of ovine FSH fell within the 95% confidence interval for PS-treated animals calculated from Experiment II (Fig. 5). The number of follicles recruited in animals treated with PFF plus saline fell within the 95% confidence interval for PFF-treated animals calculated from experiment II. PFF-treated animals given exogenous FSH showed a significant ($P < 0.05$) dose-related increase in the recruitment of follicles 350–500 μm in diameter (Fig. 5). The highest dose of exogenous FSH (160 μg) appears to have overcome the effects of PFF treatments by recruiting a normal number of follicles.

## DISCUSSION

Measurement of follicle size distribution in the ovaries of control rats in our colony confirms that there is a recruitment of follicles into the medium-size class (≥350 μm) between the mornings of proestrus and estrus (4, 5). This cohort of follicles, minus those which proceed into the later stages of atresia (Fig. 1 *B–D*), will go on to ovulation 4 days later. In the present communication we have shown that injections of PFF, in a protocol previously demonstrated to suppress the secondary surge of FSH (11), can block this recruitment. Superimposition of exogenous ovine FSH overrides the blockade of recruitment (Fig. 5), implicating the suppression of serum FSH levels in the blockade of follicular growth rather than a coincidental toxic effect of the fluid on the ovary. Exogenous FSH can also recruit follicles into the same size categories in rats in which the preovulatory gonadotropin surges were prevented with phenobarbital (17). Because PFF is highly specific in suppressing only FSH, and not LH, the present data are evidence for the specificity of FSH in follicular recruitment.

This experiment provides a direct demonstration of an alteration of gonadal function after administration of follicular fluid. In the hamster, Chappel and Selker (18) have shown that multiple treatments with bovine follicular fluid at proestrus reduce the number of ova ovulated during the next cycle, probably the result of a similar suppression of follicular recruitment.

The failure of suppression of the primary FSH surge to block recruitment (Fig. 4) strengthens the interpretation that the secondary surge is necessary *and* sufficient for follicular recruitment on estrus. It is known that endogenous or exogenous LH surges will stimulate a secondary increase in FSH (3, 19). Because the early injection of PFF in experiment III would not be expected to alter the LH surge (12), the LH surge could then induce a FSH surge adequate to permit recruitment. It is possible that the injection of exogenous FSH in the final experiment induced recruitment partially by inducing endogenous FSH secretion (3, 19). The essential function of the primary FSH surge is obscure because it is known to be redundant to the LH surge for ovulation (3, 8) and triggering of the secondary FSH increase (3, 19) and unnecessary for follicular recruitment (Fig. 4). It may be that, once the pituitary has been sensitized by estrogen and stimulated by hypothalamic gonadotropin-releasing hormone, FSH is simply released as a part of the LH surge (20).

The present findings and those in the literature provide evidence for the following theorized cascade of events at the end of the estrous cycle. The primary surges of LH (and FSH) cause the resumption of oocyte meiosis (21) and ovulation in the most mature cohort of follicles. Synchronously, the primary LH surge suppresses the secretion from the ovaries of a peptide hormone, folliculostatin, which has been holding tonic levels of FSH in check throughout the cycle (3, 11, 22). With the decrease in circulating folliculostatin, serum FSH increases before midnight (1–3) and remains increased until the morning of estrus. This prolonged increase in FSH recruits a cohort of follicles (Fig. 2) that will ovulate during the next cycle if pregnancy does not supervene. It may be that the sudden growth or recruitment of follicles produces increased circulating folliculostatin levels which leads to the termination of the secondary FSH surge and low tonic levels of FSH during the next 2 days of cycle. As discussed previously (23), in mammals with short-lived cycles, like the rat, this recruitment of follicles provides a "fail-safe" mechanism whereby the same gonadotropin surge(s) that triggers ovulation, thus ending one cycle, provides follicular recruitment for the next cycle.

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