BIOLOGY OF REPRODUCTION 48, 595–605 (1993)

Hypophysectomy of the Cyclic Mouse. II. Effects of Follicle-Stimulating Hormone (FSH) and Luteinizing Hormone on Folliculogenesis, FSH and Human Chorionic Gonadotropin Receptors, and Steroidogenesis[1]

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ABSTRACT

This study was designed to determine the effects of FSH and LH on ovarian follicular development in adult hypophysectomized (HX) mice. Twelve days after HX, the animals received s.c. injections of ovine FSH (oFSH; 4 μg/day) or oFSH (4 μg/day) plus ovine LH (oLH; 2 μg/day) twice a day for 1 to 4 days. After 4 days of treatment with FSH alone, the number of preantral follicles (stages 1–3) increased significantly compared to that in HX controls and reached cyclic numbers; however, incorporation of [^{3}H]thymidine into these preantral follicles as compared to HX controls did not increase. The number of healthy antral follicles (stages 4–5) and incorporation of [^{3}H]thymidine into stage 5 follicles started to increase after only 1 day of treatment with FSH, and the number of atretic follicles concomitantly decreased. Treatment with both FSH and LH for 1 to 4 days increased the number of healthy follicles and restored DNA synthesis at all stages (1–5) to normal levels. Two days of replacement with FSH or FSH plus LH was required for follicles to attain preovulatory size (stage 6). FSH alone induced FSH and hCG receptors in granulosa cells, but without the induction of thecal LH/hCG receptors; FSH induced production of progesterone and androstenedione by stage 6 follicles, but not estradiol (E_2) accumulation in the incubation medium or in the serum. Combined FSH and LH induced hCG receptors in the theca and interstitium, and also restored follicular E_2 production to proestrous values. LH alone increased only the number of stage 2–3 follicles. Unexpectedly, LH alone also induced thecal hCG receptors as well as FSH receptors in granulosa cells of preantral and antral follicles. The present results demonstrate that FSH is essential for follicular growth at all stages and for prevention of atretic antral follicles. Both FSH and LH are necessary for regulation of follicular development and differentiation from the earliest preantral to preovulatory stages.

INTRODUCTION

Our previous study [1] demonstrated that hypophysectomy (HX) of adult mice decreased the number of healthy follicles at all stages of development from Day 4 to Day 20 after HX without significant change in the number of atretic follicles. In addition, the rate of DNA synthesis for preantral follicles decreased; the oocytes of preantral follicles were enlarged; and follicular FSH and LH receptors disappeared within 4 days after HX. Thus, the decrease in number of healthy follicles was not due to an increase in atresia, but apparently rather to the smaller number of follicles entering the growing pool in the absence of pituitary gonadotropins. Pituitary gonadotropins therefore appear to be essential for regulating initiation, proliferation, and differentiation of follicles at all stages of development. This conclusion has been supported by many other experiments, including studies in vivo [2] and in vitro [3, 4], studies using hypogonadal models [5], and studies in dwarf mice [6]. However, which pituitary hormones are required for regulation of early stages of follicular development and which ones are essential for later stages of development and differentiation have not been clearly defined.

Accepted October 27, 1992.
Received August 28, 1992.
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FSH plays a pivotal role in gonadal development, differentiation, and maturation via its regulatory actions on granulosa cells. The mechanism of FSH action includes binding to FSH-specific plasma membrane receptors and subsequent activation of adenylate cyclase, leading to increased gene expression of LH receptors and de novo synthesis of steroidogenic enzymes, especially aromatase cytochrome P450; this in turn results in an increased conversion of androstenedione to estradiol [7]. Since androstenedione is produced in the theca under the influence of LH, both FSH and LH are thought to be essential for estrogen biosynthesis [7]. More recently, this "two-cell theory" has been reevaluated because ovarian estradiol biosynthesis is stimulated equally well by FSH and by human menopausal gonadotropins containing an equal ratio of FSH to LH activity [8, 9].

In the present study, therefore, we chose ovine FSH (oFSH) and LH (oLH) and administered them separately or in combination to long-term hypophysectomized (HX) adult mice. Objectives were to evaluate the effects of FSH and LH on (1) regulation of follicular and oocyte growth and regression at different stages of development, (2) induction of their receptors in ovarian tissues, and (3) follicular steroidogenesis.

MATERIALS AND METHODS

Animals and Treatment

Adult female ICR mice (7–8 wk old) were obtained from Harlan Co. (Madison, WI) and maintained under standard

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lighting conditions of 14L:10D (lights-on at 0600 h) at 20°C. The cycle of the mouse was determined as previously described [10], and proestrous mice were used as controls. Cyclic animals at random stages of the estrous cycle were hypophysectomized (Day 0); the maintenance of animals and the criteria for completeness of HX were the same as previously described [1]. At 12 days after HX, animals received an s.c. injection twice a day for 1 to 4 days with either (1) the vehicle solution (20% polyvinylpyrrolidone [PVP] in saline) for an HX control group; (2) NIDDK-oFSH-17 (4 μg/day), with 1.73% LH contamination by weight; (3) oFSH (4 μg/day) and NIDDK-oLH-24 (2 μg/day); or (4) oLH alone (2 μg/day), with less than 0.5% FSH contamination by weight. In addition, another group of HX mice were treated with oFSH (4 μg/day) and oLH-24 (0.2 μg/day). Since there was no significant difference between the results of HX + FSH and HX + FSH + LH (0.2 μg/day), results for the latter group are not considered in detail in the present paper. Sixteen hours after the last injection, following 1 to 4 days of treatment, animals were decapitated at 0900 h under ether anesthesia and serum was saved for steroid assays. Ovaries and uteri were collected and weighed after drying on paper tissues. One ovary from each animal was saved for routine histology (serial sections at 7 μm with hematoxylin-eosin staining) [1], and the other was used for the experimental procedures described.

Isolation of Follicles and In Vitro Incubation with [^{3}H]Thymidine

As described in our previous paper [1], the isolated follicles (four replicates in each stage) were incubated in 24-well culture clusters containing 700 μl Krebs' Ringer buffer containing 25 mM HEPES (KRBH) with 0.5% BSA for 3 h at 37°C in the presence of 0.5 μCi [^{3}H]thymidine [10]. The definition and the number of isolated follicles incubated for each stage were as follows: 50–80 stage 1 follicles (with a single layer of granulosa cells); 20–30 stage 2 follicles (two layers of granulosa cells); 20 stage 3 follicles (more than three layers of granulosa cells); 6 stage 4 follicles (incipient antrum); 5 stage 5 follicles (intermediate antrum); and 4 stage 6 (preovulatory) follicles. The results were expressed as fmol [^{3}H]thymidine per follicle per 3 h.

Autoradiography for FSH and hCG Receptors

Cleaned ovaries were frozen in freon and stored at −80°C. A week before autoradiography, ovaries were sectioned at 8 μm in a cryostat at −20°C. The procedures for iodination, characterization, and other preparation for autoradiography were described previously [1].

Assay of Steroids in Serum and in Incubation Medium

Progesterone (P_4), androstenedione (A), and estradiol (E_2) were assayed by RIA as previously described [11]. The P_4 antiserum and the E_2 antiserum were gifts from Dr. Surve, Sandoz Inc., East Hanover, NJ [12], and Dr. Collins, Emory University, Atlanta, GA [13], respectively. The antiserum to A was purchased from BioTek Research, Lenexa, KS. Rabbit anti-androstenedione cross-reacted with P_4 (0.2%), 17-hydroxyprogesterone (0.1%), testosterone (2.0%), dehydroepiandrosterone (1.0%), dehydrotestosterone (0.5%), and androsterone (4.0%). Nonspecific binding and recoveries were performed for each assay. The lower limits of sensitivity in the assays for P_4, A, and E_2 were 5, 2, and 2 pg per assay tube, respectively.

The spent medium (700 μl/well) from incubation of isolated follicles with [^{3}H]thymidine was used for steroid measurement. A preliminary experiment showed that after ether extraction and charcoal isolation of free and bound [^{3}H]-labeled hormones, free [^{3}H]thymidine in the incubation medium was removed completely and steroid assay results were within the background range in RIA. Therefore, the incubation medium with [^{3}H]thymidine was used for steroid RIAs. Samples of 200 μl of incubation medium were used to measure P_4, A, and E_2. About 400 μl of serum was obtained from each individual mouse; 200 μl of serum was used for E_2 RIA and approximately 100 μl was used for P_4 and A, respectively. All the incubation and serum samples were extracted with 2 ml of diethyl ether. Samples from each group were assayed at one time to avoid interassay variation. The results of in vitro steroid accumulations were expressed as pg/follicle/3 h, and serum steroids were expressed as pg/ml serum. Statistical analysis was performed as previously described [1].

RESULTS

Number of Follicles after FSH and/or LH Replacement in HX Mice

Hypophysectomy (HX) decreased the number of growing follicles at all stages, both preantral and antral follicles [1]. With replacement therapy, the number of preantral follicles (stages 1–3) did not increase significantly until after 3 days of treatment with both FSH and LH (2 μg/day) or 4-day replacement with FSH alone (Fig. 1, left panel). When LH alone (2 μg/day) was injected for 4 days, the number of healthy follicles at stages 2–3 increased to the same level as in the FSH replacement group; but stages 1, 4, and 5 were unaffected (data not shown). There were no atretic follicles at stages 1–2 and very few at stage 3 in the HX control or HX + treatment groups.

The number of healthy stage 4 follicles started to increase after only 1 day of treatment with either FSH or FSH + LH as compared to the HX control group ($p < 0.05$). Atretic stage 4 follicles decreased significantly after 2 days of replacement with FSH and disappeared completely after 2 days of FSH + LH. The total number of follicles at stage 4 did not significantly increase until after 4 days of treatment (Fig. 1, right panel). Healthy stage 5 follicles increased after only 1 day of treatment with either FSH alone

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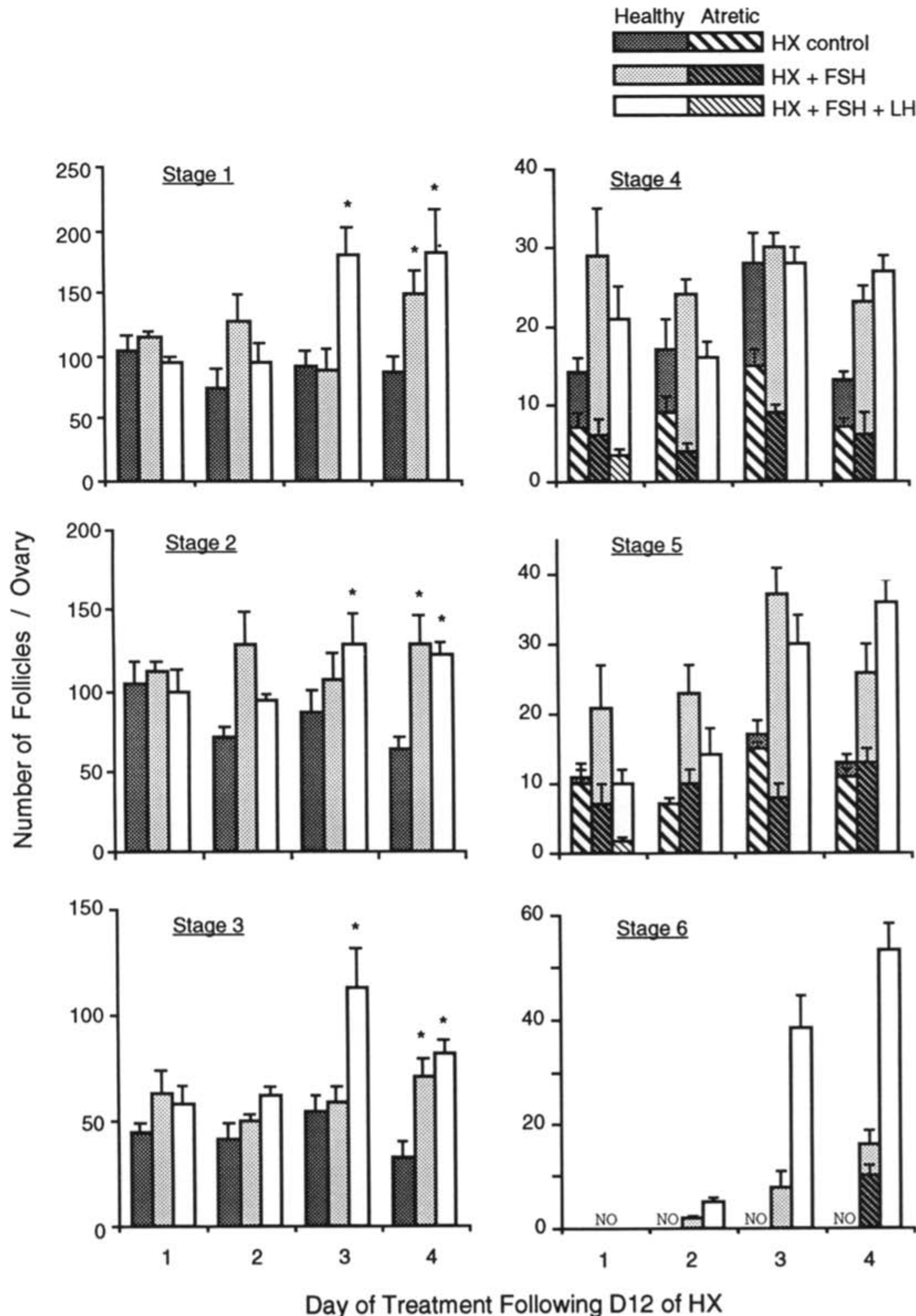

FIG. 1. Effects of FSH and LH on number of follicles at different developmental stages in HX mice (mean ± SE). Ovine FSH-17 (4 μg/day), FSH + oLH-24 (2 μg/day), or vehicle solution (20% PVP) was injected s.c. twice a day for 1–4 days. Animals were killed 16 h after the last injection. The bars on the left panel show healthy preantral follicles at stages 1–3; the number of follicles in asterisk-marked groups was significantly increased ($p < 0.05$) as compared to that in the HX control group on the same day of treatment. The right panel represents total antral follicles, with stacked bars showing the number of both healthy (upper portion) and atretic follicles (lower portion). NO (in panel for stage 6 follicles) signifies no such follicles at the preovulatory stage in the groups marked. The statistical difference between control and treatment groups for antral follicles (stages 4–6) is not shown in the figure, but is given in the text.

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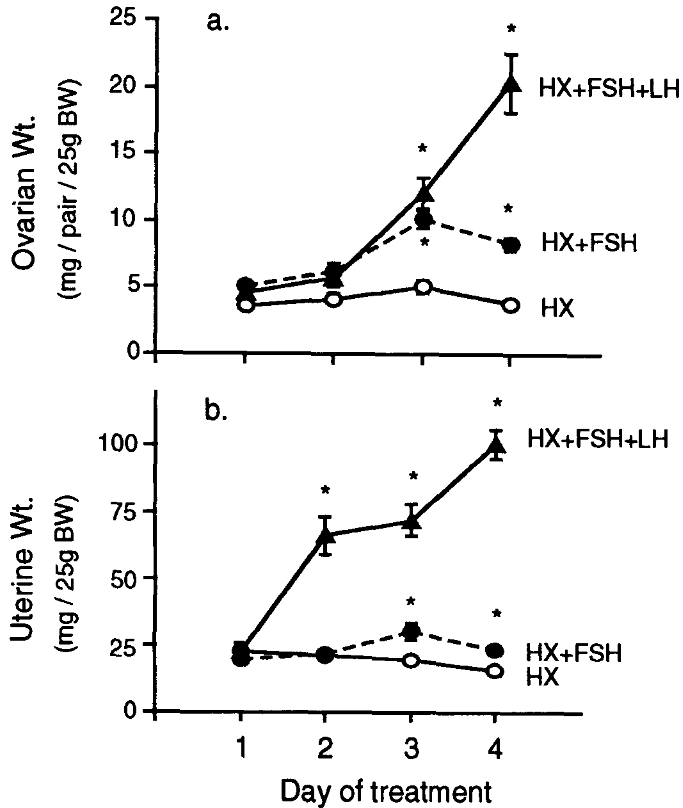

FIG. 2. Effects of FSH and LH replacement on the weight of the ovary (a) and the uterus (b) in HX mice. The mean and the standard errors were based on 25 g of body weight from 4–6 animals in each group. The asterisks represent significant difference ($p < 0.05$) between treatment groups and the HX control group within the same day of hormonal treatment.

or FSH + LH; atretic follicles at this stage decreased significantly after 1 day of FSH and LH injections and disappeared completely after 2 days. The total number of stage 5 follicles increased significantly ($p < 0.01$) after 3 days of FSH alone or FSH + LH (Fig. 1). Therefore, for stage 4 and 5 follicles, during the first 2 days of FSH or FSH + LH replacement, the decrease in atretic follicles was the main reason for the increase in healthy follicles.

The follicles grew to stage 6 (preovulatory follicles) within 2 days of treatment with FSH or FSH + LH in HX mice (Fig. 1). By 3 and 4 days of replacement with FSH alone, the number of healthy preovulatory follicles was close to normal proestrous values; but about two thirds of total follicles at stage 6 were atretic after 4 days of treatment with FSH alone (4 μg/day), whereas all were healthy on Days 2 and 3 of treatment. In contrast, the same dose of FSH + LH (2 μg/day) induced many more follicles, as compared to HX + FSH, to attain the preovulatory stage (53 ± 3 follicles/ovary) after 4 days of treatment without any sign of atresia. Furthermore, although the ovarian weight of the HX + FSH group was significantly greater than that of HX controls, the ovarian weight in the HX + FSH + LH groups was further increased as compared to that of the HX + FSH group (Fig. 2a).

DNA Synthesis of Follicular Cells after FSH and/or LH Replacement in HX Mice

The method for evaluating DNA synthesis of follicular cells was in vitro incorporation of [^{3}H]thymidine into isolated follicles for 3 h. Since there were no significant differences in DNA content per follicle before and after hormonal treatments, the patterns of [^{3}H]thymidine incorporation were identical when expressed as [^{3}H]thymidine per follicle or [^{3}H]thymidine per μg DNA. Therefore, the rate of DNA synthesis was expressed as [^{3}H]thymidine per follicle.

As previously reported [1], [^{3}H]thymidine incorporation into follicular cells significantly decreased from Day 4 through Day 20 after HX. With 4-day replacement with FSH or LH alone, the amount of [^{3}H]thymidine incorporation into

TABLE 1. Effects of FSH and LH on incorporation of [^{3}H]thymidine into follicles of HX mice ([^{3}H]thymidine, fmol/follicle; mean ± SE)

	Stages of isolated follicles					
Treatment*	1	2	3	4	5	6
1 Day of injections						
HX	0.039 ± 0.002^a	0.35 ± 0.06^a	1.61 ± 0.30^a	6.2 ± 1.2^a	13.5 ± 1.0^a	None
HX + FSH	0.021 ± 0.003^a	0.29 ± 0.02^a	1.34 ± 0.22^a	5.6 ± 0.9^a	32.6 ± 3.0^b	None
HX + FSH + LH	0.055 ± 0.008^a	0.53 ± .008^a	1.34 ± 0.07^a	8.5 ± 1.3^a	29.0 ± 1.5^b	None
2 Days of injections						
HX	0.027 ± 0.002^a	0.37 ± 0.04^a	1.02 ± 0.16^a	5.6 ± 0.9^a	18.2 ± 1.1^a	None
HX + FSH	0.024 ± 0.004^a	0.36 ± 0.01^a	0.89 ± 0.16^a	4.3 ± 0.4^a	25.6 ± 2.2^b	46.9 ± 5.5^b
HX + FSH + LH	0.085 ± 0.008^b	0.89 ± 0.06^b	2.14 ± 0.17^b	14.2 ± 1.3^b	28.6 ± 2.5^b	47.6 ± 1.0^b
3 Days of injections						
HX	0.025 ± 0.002^a	0.45 ± 0.06^a	1.28 ± 0.25^a	6.5 ± 0.6^a	19.8 ± 3.1^a	None
HX + FSH	0.031 ± 0.003^a	0.58 ± 0.14^a	2.14 ± 0.28^a	10.2 ± 1.3^b	41.7 ± 3.9^b	62.9 ± 8.1^b
HX + FSH + LH	0.083 ± 0.080^b	1.22 ± 0.13^b	3.51 ± 0.19^c	18.0 ± 0.8^c	45.8 ± 1.2^b	68.7 ± 4.3^b
4 Days of injections						
HX	0.035 ± 0.004^a	0.44 ± 0.03^a	1.40 ± 0.35^a	8.5 ± 1.5^a	28.2 ± 2.2^a	None
HX + FSH	0.044 ± 0.002^a	0.41 ± 0.01^a	1.90 ± 0.40^a	9.5 ± 1.6^a	63.6 ± 5.1^b	56.5 ± 7.0^b
HX + FSH + LH	0.065 ± 0.002^b	0.90 ± 0.02^b	3.99 ± 0.30^b	13.7 ± 1.1^b	63.7 ± 3.1^b	70.7 ± 1.0^b

*Hormones were injected s.c. twice a day for 1–4 days beginning at Day 12 after HX and mice were killed at Day 13, Day 14, Day 15, or Day 16. The dose of FSH was 4 μg/day and for LH was 2 μg/day.
$^{a-c}$Values with different superscripts are significantly different ($p < 0.05$) in the same stage of each day of treatment.

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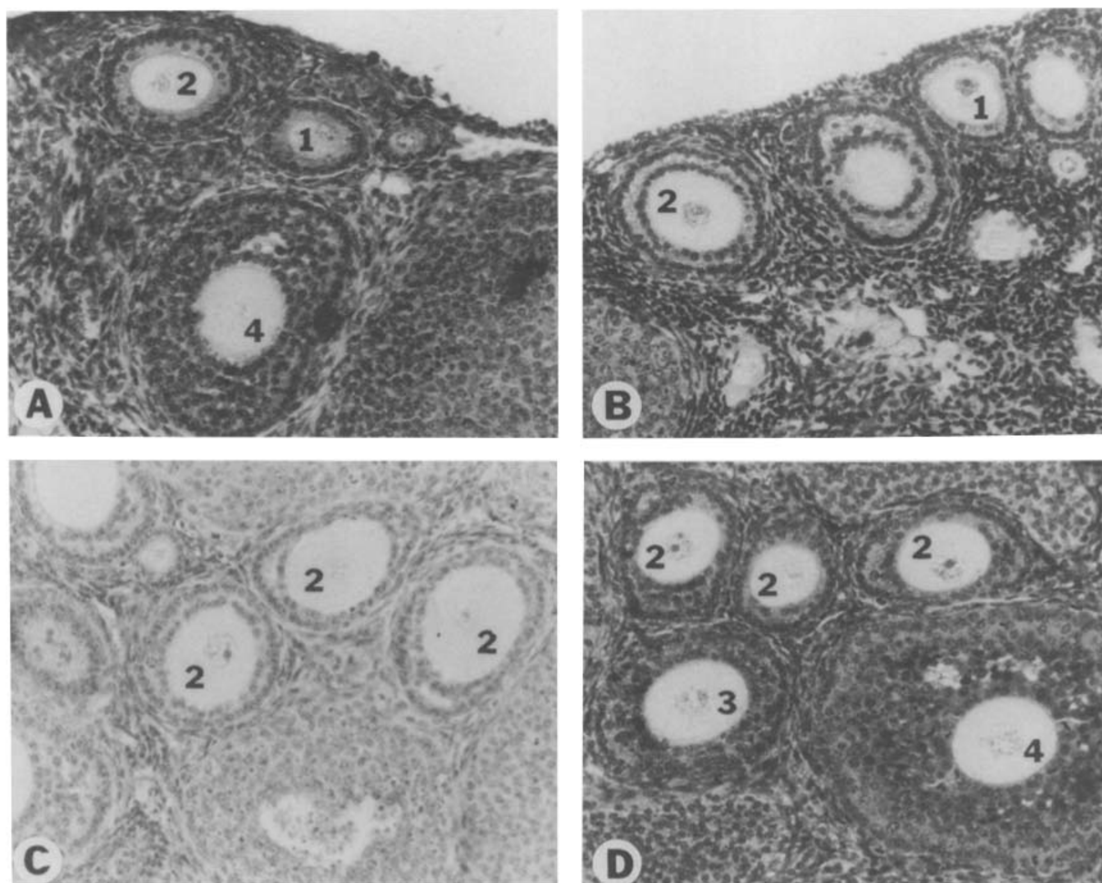

FIG. 3. Effects of FSH and LH on the growth of follicles and oocytes in HX mice. Panels show ovarian sections from A) control mouse at random stages of the estrous cycle; B) HX + LH (2 μg/day); C) HX + FSH (4 μg/day); D) HX + FSH + LH. The numbers in oocytes represent follicular stages of development (e.g., 2 in oocyte represents that the follicle is a stage 2 follicle). ×288.

follicular cells at stages 1 to 4 did not increase (Table 1; data for LH alone not shown). Incorporation of [^{3}H]thymidine into stage 5 follicles significantly increased after only 1 day of FSH. However, injection of FSH + LH for 2 to 4 days significantly increased [^{3}H]thymidine incorporation into follicular cells for stages 1–6 (Table 1).

Morphology of Follicles after FSH and/or LH Replacement in HX Mice

When HX mice were treated with FSH or LH alone for 1–4 days, the size of oocytes in preantral follicles was similar to that in the HX group, which had significantly larger oocytes than did the cyclic mice (Fig. 3, A-C); the photomicrograph for ovarian sections of HX mice was included in the previous paper [1]. Thecal cells and interstitial tissue were poorly developed in the HX + FSH group; the layer of thecal cells was thin and the interstitial cells were condensed (Fig. 3C).

When HX mice were injected with both FSH and LH for 4 days, the growth of oocytes and follicles was coordinated; i.e., the proliferation of granulosa cells matched the growth of the oocyte (Fig. 3D), in a manner comparable to that in cyclic control animals (Fig. 3A). In this group, the follicles were morphologically similar to those in cyclic animals. There were many layers of healthy-looking granulosa cells with a large antral cavity in stage 5 and 6 follicles, and well-developed thecal layers and interstitial tissue. Some ovaries contained histologically normal corpora lutea (CL); this was also observed in the HX + LH group. Whether these were new CL or CL atretica was not determined.

Binding Activity of [^{125}I]-rFSH and [^{125}I]-hCG

In intact controls, binding sites for hCG were normally found in the mural granulosa layers of stage 6 follicles, the thecal layers, and the interstitial tissue and CL (Fig. 4A). After HX, hCG-binding sites precipitously declined in granulosa cells, theca, and corpora lutea; only trace amounts of hCG binding were visible in the interstitium (Fig. 4B). Replacement with LH (2 μg/day) alone for 4 days increased [^{125}I]-labeled hCG-binding sites in theca and interstitial tissue in comparison to the HX control group (Fig. 4, B and

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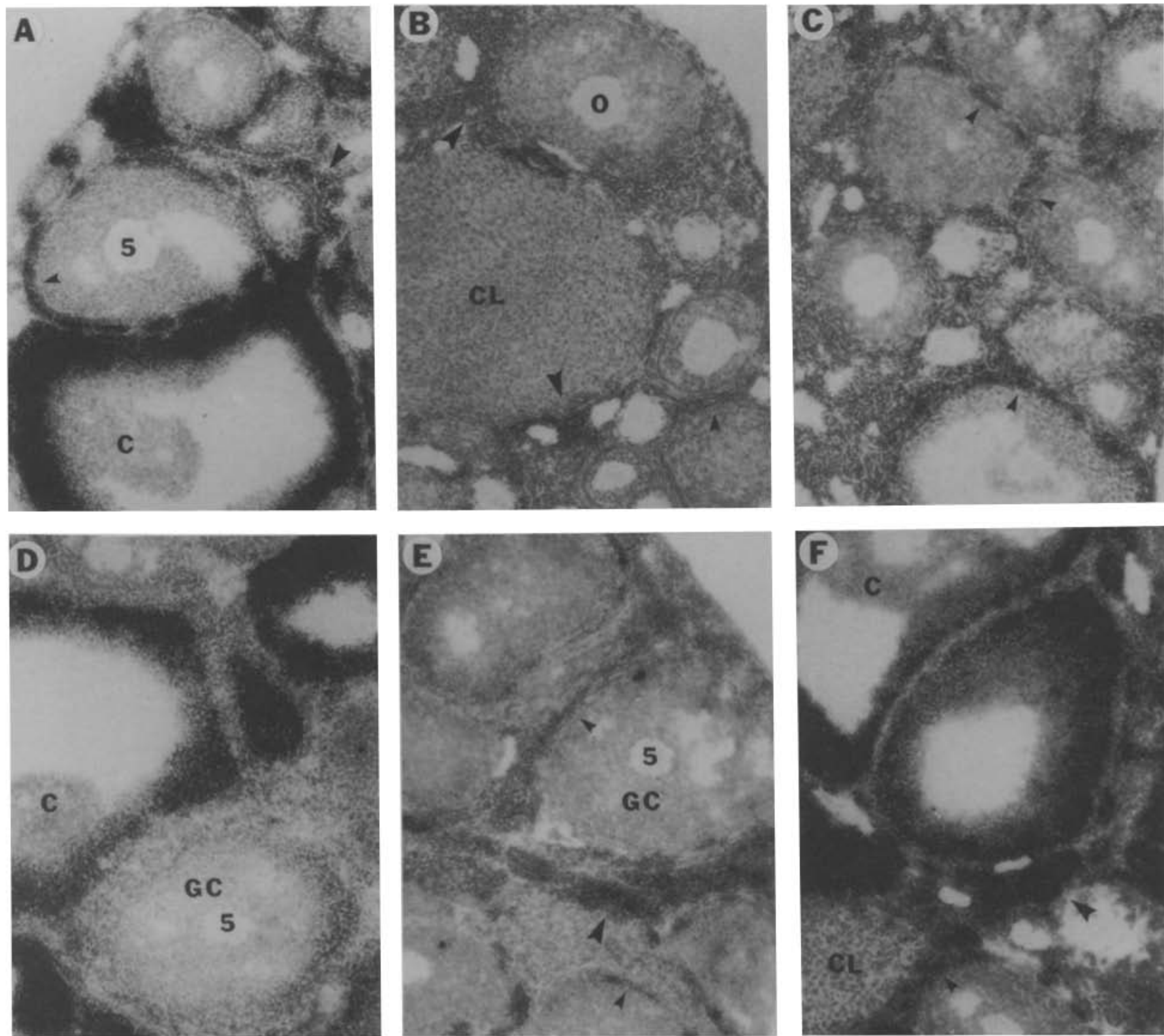

FIG. 4. Effects of FSH and LH replacement in HX mice on intraovarian distribution of binding sites for [^{125}I]-hCG by topical autoradiography. The panels represent ovarian sections from A) normal proestrous mouse; B) Day 16 after HX; C) HX + LH treatment for 4 days; D) HX + FSH injection for 4 days; E) HX + both FSH and LH injection for 1 day; F) HX D16 with both FSH and LH treatment for 4 days. GC, granulosa cells; CL, corpus lutea; O, oocyte; C, cumulus area. The larger arrows point to the binding sites in interstitial tissue and the smaller arrows to the binding sites over thecal layers of follicles. ×144.

C). Treatment with FSH for 1 to 4 days increased hCG-binding sites progressively on mural granulosa cells of stage 6 follicles but not in the theca or interstitium (Fig. 4D). As seen in Figure 4D, there was no clear-cut thecal hCG-binding activity in stage 5 follicles compared to similar-sized follicles in Figure 4, A and E. Treatment with FSH and LH for only 1 day induced hCG receptors in thecal and interstitial tissues as compared to HX controls (Fig. 4, E and B). After 4 days of injections of FSH and LH, hCG-binding activity was very intense in mural granulosa layers in large follicles, in thecal layers for middle-sized follicles, and in the interstitial tissue (Fig. 4F). The number of hCG receptors in mural granulosa cells was greater than in the HX group treated with FSH alone (Fig. 4, D and F).

[^{125}I]-FSH receptors were exclusively localized in granulosa layers of follicles in the normal cyclic mouse (Fig. 5A). HX reduced the number of FSH receptors to baseline levels (Fig. 5B), which were barely detectable by topical autoradiography. Replacement with FSH (4 μg/day) for 1 to 4 days resulted in a gradual increase in the intensity of [^{125}I]-FSH binding in granulosa layers of growing follicles; this was observed in stage 2 and larger follicles as well as in the oocytes (Fig. 5C). Combined FSH and LH treatment resulted in [^{125}I]-FSH binding greater than with FSH alone (Fig. 5D). It is noteworthy that injection of LH (2 μg/day) into the HX animals induced discernible FSH binding to granulosa cells of small and medium-sized follicles (stages 1 to 5; Fig. 5, E-G).

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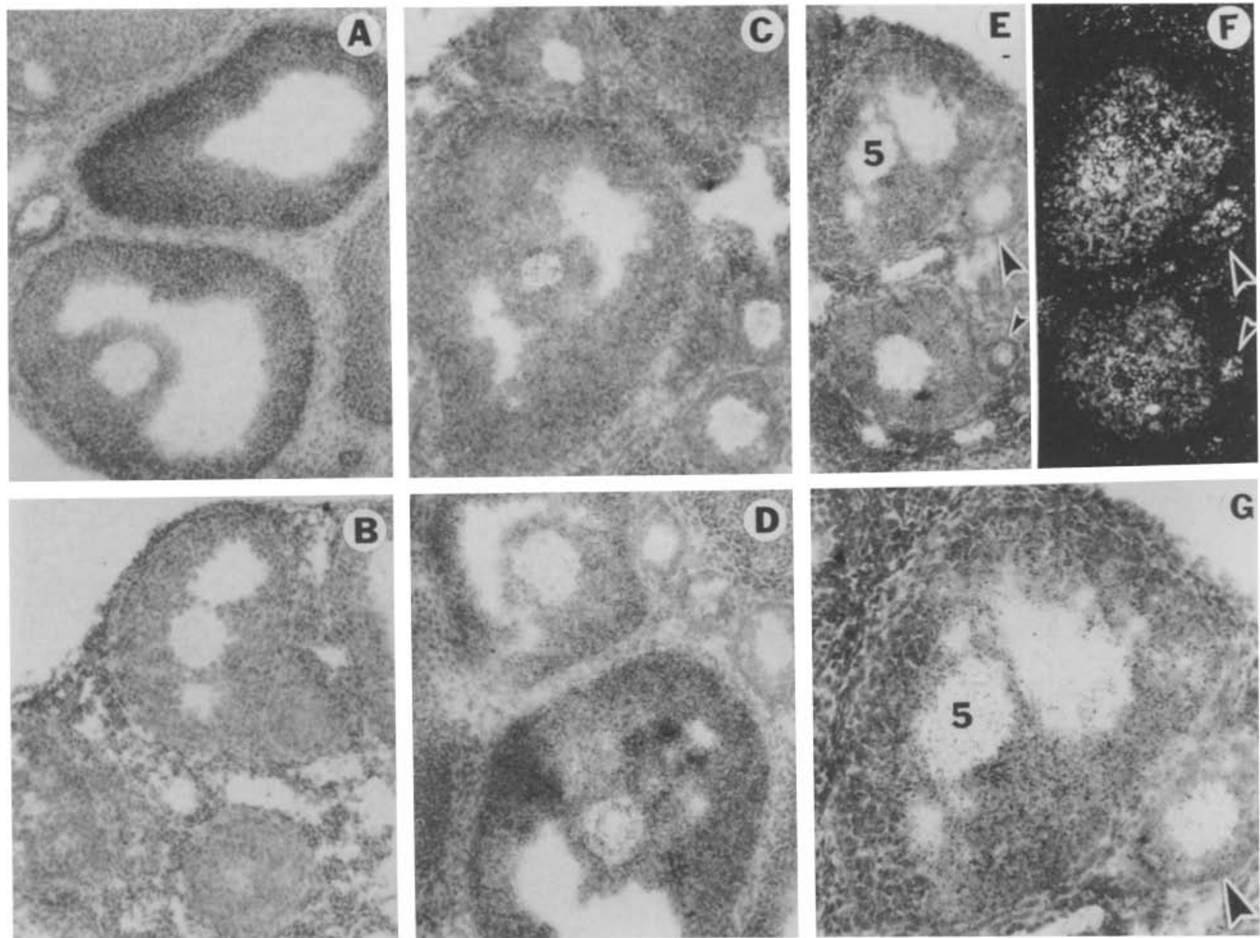

FIG. 5. Effects of FSH and LH replacement in HX mice on intraovarian distribution of binding sites for [^{125}I]-FSH by topical autoradiography. The panels represent ovarian sections from A) normal proestrous mouse; B) Day 16 after HX; C) HX + FSH (4 μg/day) for 4 days; D) HX + both FSH (4 μg/day) and LH (2 μg/day) for 4 days; E) HX + LH (2 μg/day) for 4 days (in which there were 4 follicles at stages 1, 2, 4, and 5 with specific [^{125}I]-FSH binding). F) dark field of section E. G) Twofold enlargement of the upper part of photo E. The larger arrows point to the same stage 2 follicle in E, F, and G; the smaller arrows to the same stage 1 follicle in E and F. The number 5 in oocytes in E and G indicates the same stage 5 follicle. ×144 for A-F; ×288 for G.

Steroid Levels in Serum and Incubation Medium

No preovulatory follicles (stage 6) were present in HX control mice, and no detectable steroids accumulated in the spent incubation medium of follicles smaller than stage 5 (data not shown). P_4 and A accumulated in the medium of the isolated stage 6 follicles from the HX + FSH and the HX + FSH + LH groups; amounts of accumulation were higher for the latter group but were lower than for stage 6 follicles from proestrous mice (Fig. 6). Serum levels of P_4 and A in the HX and HX + FSH groups were undetectable by our RIA method. However, when HX mice were given both FSH and LH, the serum levels of P_4 and A increased. Serum levels of P_4 and A at proestrus were unavailable.

Although there were minimal detectable serum levels of E_2 from HX mice and HX + FSH mice, E_2 was undetectable in the medium from incubated stage 6 follicles of the latter group. However, combined FSH and LH resulted in E_2 accumulation in vitro and E_2 serum levels in the same range as proestrous values (Fig. 6). The effects of E_2 were also reflected in uterine weight (Fig. 2b). The weight of the uterus increased significantly ($p < 0.05$) after 3 days of treatment with FSH in HX mice, but this was minor compared to the effects of combined FSH and LH. Uterine weight increased significantly after only 2 days of FSH + LH, then continued to increase until it reached the average uterine weight of cyclic animals (120 ± 30 mg with a range from 48 to 217 mg).

Replacement with LH (2 μg/day) for 4 days led to the same serum P_4 values as in the HX + FSH + LH group; in the LH group, serum A was undetectable and serum E_2 was at the same baseline level as in the HX group (data not shown). These effects were similar to those reported for LH alone in the immature mouse [14] and in the HX immature rat [15].

DISCUSSION

Four days after HX, the growth of follicles is reduced to baseline levels as in the juvenile stage, and follicular go-

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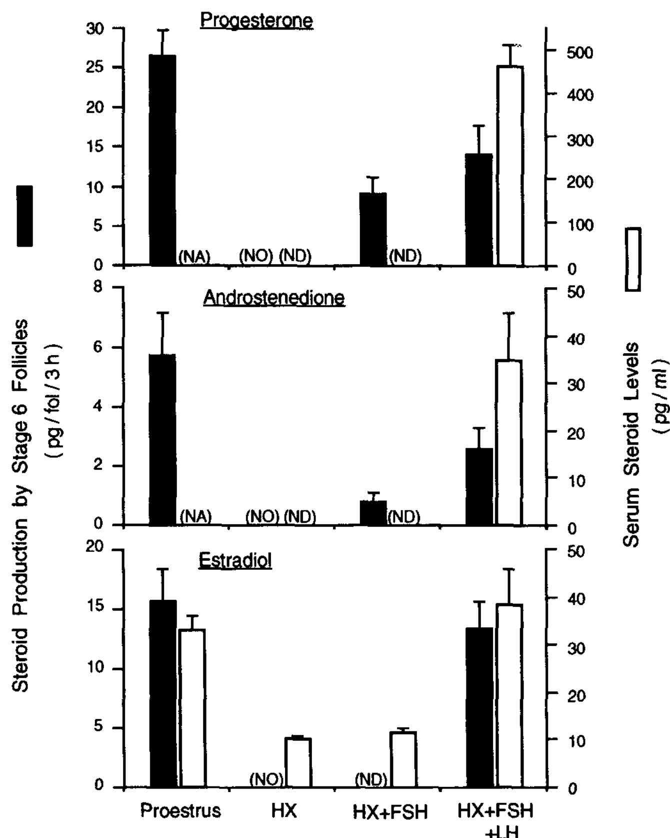

FIG. 6. Effects of FSH and LH on steroid levels in serum (open bars) and in incubation medium of isolated preovulatory follicles (stage 6) (solid bars) in HX mice. FSH (4 μg/day) or an equal dose of FSH and LH (2 μg/day) were injected s.c. twice a day for 4 days beginning at Day 12 after HX, and animals were killed at Day 16 of HX. (NA) indicates not applicable, because the groups were not assayed; (NO), no preovulatory follicles in HX D16 group; (ND), not detectable. The sensitivities of steroid RIAs used in the present studies are given in *Materials and Methods*.

nadotropin receptors decrease to levels undetectable by autoradiography [1]. The ovary in HX adult mice therefore constitutes a good starting point with all parameters reduced to the baseline. The present studies provide convincing evidence that in the adult HX mouse, FSH alone or FSH + LH significantly increases the number of antral follicles and their DNA synthesis as well as FSH or LH receptor binding after only 1 day of treatment; FSH is essential for stimulating the first steps of follicular development and continuous growth until the preovulatory stages as well as for inducing its own receptors and LH/hCG receptors in granulosa cells (but not in other ovarian compartments). The effects of a low dose of oLH (0.2 μg/day) are not additive with FSH, and therefore these data are not included in the present paper. However, LH is needed in a higher dose (2 μg/day) to synergize with FSH for normal development of the follicles and interstitial tissue in HX animals as well as for the maintenance of healthy growing follicles and apparently normal steroidogenesis.

Our results suggest that FSH administered alone increases the number of preantral follicles (stages 1–3) after 4 days of treatment in HX mice, but without a significant increase in the rate of follicular DNA synthesis. For antral follicles (stages 4–5), FSH alone at first decreased the number of follicles lost to atresia and consequently increased the number of healthy follicles without increasing the total number of follicles. Then, after 4 days of FSH treatment, the growth of follicles accelerated, with the rate of DNA synthesis at stages 4–5 increasing significantly along with an increase in the total number of follicles compared to that in the HX controls (Fig. 1 and Table 1). Thus, the immediate effect of FSH is to prevent antral follicles from undergoing atresia; subsequently FSH stimulates preantral follicle growth. However, it is noteworthy that 4 days of FSH replacement increased the number of atretic follicles at stages 4 and 5 to the number in HX control groups; about two thirds of the total number of stage 6 follicles are atretic. Therefore, a superfollicular response did not occur with the dose of FSH (4 μg/day) used in this study. Halpin and Charlton [5] performed a similar experiment in the hypogonadal mouse, using different preparations of FSH in different doses (0.6–12 μg/day) for 4 or 5 days. They also demonstrated a saturation response to FSH; i.e., doses of 6 μg/day and above produced approximately the same number of antral follicles as found in normal adult females. Since Halpin and Charlton [5] did not classify follicles as healthy or atretic, the factors preventing superfollicular development were not clarified in their paper.

Our results indicate, however, that using FSH (4 μg/day) + LH (2 μg/day) results in an increased number of preantral follicles after 3 days instead of the 4 days required with FSH alone; it also reduces the number of atretic follicles to zero by 2 days of treatment and causes superfollicular development (Fig. 1). These outcomes correspond to the effects of eCG in hypogonadal mice [16] and to the effects of estrogen and recombinant FSH (recFSH) in HX immature rats [17]. Furthermore, the fact that [^{3}H]thymidine incorporation into all stages of follicles started to increase significantly by 2 days of FSH + LH treatment (Table 1) indicates that FSH and LH not only reduce follicular atresia, but also accelerate proliferation of follicular cells at all stages, speeding up the transit from one stage to the next. The unanswered question is how LH acts on small follicles to synergize with the effects of FSH, as there are no detectable LH receptors in granulosa cells of preantral follicles. There are three possibilities. 1) In mice, thecal formation occurs at very early stages of follicular development; follicles contain 1–2 layers of granulosa cells surrounded by a clearly defined theca [3, 10, 14]. Thus, LH may act directly on thecal cells to enhance the growth of preantral follicles by stimulating the growth and differentiation of thecal cells. 2) LH (2 μg/day) replacement for 4 days induces FSH receptors in granulosa cells at stages 1 to 5 (Fig. 5, E-G) to synergize with FSH. 3) LH may stimulate estrogen production; the latter, in turn, stimulates granulosa cell proliferation and prevents atresia in follicles of HX rats [17, 18]. The action of

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LH on ovarian aromatase is known to be mediated via testosterone and 5-reduced androgens, causing amplification of cAMP-mediated FSH responses [19].

The interaction between ovarian interstitial cells and follicles is essential for normal development and function. In the group of mice receiving FSH alone, follicles could grow to the preovulatory stage but had a thin layer of theca and undeveloped interstitial tissue. After treatment with combined FSH and LH, the antral follicles possessed a healthy theca interna surrounded by well-developed interstitial tissue. In addition to the concurrent development of granulosa cells and interstitial cells, another important correlative event for normal follicular development and function is the interaction between granulosa cells and their contained oocytes. In HX, HX + LH, and HX + FSH groups, the ratio of the oocyte diameter to the size of the surrounding granulosa cells was increased (Fig. 3). This resulted mainly from a reduction in proliferation of granulosa cells while the oocytes appeared to be relatively little affected by the absence of hormones or the abnormal hormone environment. With replacement of both FSH and LH for 3 and 4 days, the growth of granulosa cells matched the growth of their oocyte (Fig. 3D) because the proliferation of granulosa cells was restored to normal levels. This is also demonstrated by the increase in [^{3}H]thymidine incorporation into follicles in the HX + FSH + LH group as opposed to the HX group given FSH alone (Table 1).

It is believed, on the basis of isolated granulosa cell models, that FSH induces its own receptors and LH receptors at both transcriptional and translational levels, but this sheds no light on the effects of FSH on thecal cells [20, 21]. The present experiments provide additional insight into the manner in which FSH and LH affect their ovarian receptor levels. Treatment with FSH alone or FSH plus a low dose of LH to HX mice induced LH/hCG receptors in only the granulosa layers of large antral follicles; it failed to induce hCG receptors in the theca and interstitium (Fig. 4D). The reasons may be that 1) FSH does not directly act on thecal and interstitial cells since these contain no FSH receptors; 2) the low dose of LH (0.2 μg/day) used in this study plus the 1.73% LH contained in oFSH (4 μg/day) was not enough to induce LH receptors; 3) both FSH and the low dose of LH were insufficient to induce other factors to stimulate thecal cells to produce LH/hCG receptors. However, a higher dose of LH (2 μg/day) alone increased LH receptor in theca and interstitium (Fig. 4C); and when LH + FSH was administered, LH receptors were observed in both mural granulosa cells of large antral follicles and thecal and interstitial tissues (Fig. 4, E and F). This finding is consistent with the report that eCG, which contains approximately equal amounts of FSH and LH activity, enhances the expression of LH receptor mRNA in granulosa cells as well as in the thecal and interstitial cells [22].

The combination of FSH and LH was seen to have more effects than FSH alone on induction of FSH receptors on granulosa cells of preantral and antral follicles (Fig. 5, C and D). This indicates that FSH and LH in HX mice act synergistically to up-regulate the production of FSH receptors during follicular growth as previously reported in rats, in which FSH or eCG increases the levels of two FSH receptor mRNA transcripts mediated by cAMP [23–26].

Unexpectedly, injection of LH (2 μg/day) alone for 4 days increased FSH receptors in granulosa cells of preantral and antral follicles, as shown in light- and dark-field photomicrographs (Fig. 5, E-G), in the absence of FSH and estrogens. Numerous investigators have studied the down-regulatory effects of an ovulatory dose of LH on FSH receptor at the periovulatory stage [24, 25, 27]. However, little is known about the effects of LH up-regulation on granulosa FSH receptors and follicular differentiation. In the present study, treatment with LH alone in the HX mouse increased the number of preantral follicles (stages 2–3). This agrees with previous studies in the immature rat [23, 28], the pregnant rat [29], and the infantile mouse [14, 30], in which hCG, but not FSH, can act on preantral follicles to stimulate the growth of preovulatory follicles and to induce the appearance of gonadotropin receptors and steroidogenesis. It is noteworthy that even stage 1 follicles have attached thecal cells in mice [10]. Thus, the effects of LH on follicular growth and differentiation may be mediated by LH acting on thecal and interstitial tissues to induce the production of cAMP, progesterone, androgens, and some protein factors such as transforming growth factor α, epidermal growth factor, and insulin-like growth factor-I; these products, in turn, stimulate granulosa cells to synthesize FSH receptors [31–34]. In addition, although ovarian responsiveness to exogenous gonadotropins depends on genetic differences in different strains of mice [35], the sensitivity of follicles to FSH may be very high after HX. If this is the case, the very low level of FSH (about 0.01 μg contamination with each LH injection) may induce the synthesis of its own receptors.

Replacement therapy with FSH alone or with FSH plus a low dose of LH (0.2 μg/day) was inadequate to induce the synthesis of estradiol in this study. In contrast, Halpin et al. [5] reported that antral follicles in hypogonadal mice appear capable of secreting physiologically significant amounts of estrogen under the influence of FSH alone, since FSH produced a significant increase in uterine weight and stimulated vaginal opening. However, steroid levels were not measured in that experiment. Our findings do not agree with the conclusions of Halpin et al. First, although there was a statistically significant increase in uterine weight as of 3 and 4 days of FSH injections (Fig. 2B), the uterine weight was considerably lower than the minimum weight of cyclic animals. Second, serum P_4 and A levels were undetectable by our RIA methods, and only stage 6 follicles in vitro (not stage 4 or 5 follicles) secreted one third to one fourth the amounts of P_4 and A of proestrous follicles (Fig. 6). A minimal amount of serum estradiol was detectable in the HX

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and HX + FSH groups, but this may have been due to cross-reaction of the E_2 antibody with other products having a ring structure similar to that of E_2 (such as equol [36]), since no E_2 was detectable in the incubation medium of preovulatory follicles. One of the most likely explanations for the lack of E_2 production by stage 6 follicles is the poor development of thecal and interstitial tissues and the failure to localize hCG receptors in these tissues (Fig. 4D). In rodents, both FSH and LH are required for steroidogenesis, with LH stimulating the production of androgens by the thecal cells and FSH stimulating their aromatization by granulosa cells. Many in vivo and in vitro studies support this theory [4, 7, 14]. In the present study, with replacement of both FSH and LH, serum E_2 and in vitro accumulation of E_2 from incubated follicles were restored to the same levels as in proestrous animals. This theory is further supported by experiments using recombinant human FSH (recFSH), in which recFSH alone administered to HX immature rats increased ovarian weight and aromatase activity, but not plasma E_2; when recFSH was supplemented with hCG, both ovarian weight and aromatase activity were further augmented, resulting in a large increase in plasma E_2 [15]. However, in the current study, poor development of thecal and interstitial tissues in the HX + FSH group may not have been the sole reason for deficient production of E_2. After 4 days of FSH replacement, most of the preovulatory follicles were atretic and the levels of P_4 and A were also much lower than in the proestrous control group and the HX + FSH + LH group. The impaired E_2 production for stage 6 follicles may thus have resulted from degeneration of granulosa cells as well as from a shortage of steroid precursors for estrogen synthesis.

In conclusion, the current experiments show that FSH regulates folliculogenesis in HX adult mice at different stages. This regulation involves 1) initiation and maintenance of follicular growth, 2) slowing down the rate of atresia, and 3) induction of FSH and LH receptors only on granulosa cells. But FSH alone does not stimulate development of theca and interstitium, does not induce LH receptors on thecal and interstitial tissue, and does not stimulate E_2 production. With combined FSH and LH, both follicular structure and function are restored to normal proestrous status in terms of number of growing follicles at all stages of development, rate of proliferation of granulosa cells and FSH and LH receptors, and steroidogenesis. Thus, both FSH and LH are required for the entire gamut of folliculogenesis encompassing initiation, proliferation, and differentiation and culminating in ovulation.

ACKNOWLEDGMENTS

We appreciate the generous gifts of purified hormones from the National Hormone and Pituitary Program; the National Institute of Diabetes and Digestive and Kidney Diseases, University of Maryland School of Medicine, Baltimore, MD; and Pituitary Hormones and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA. We are also grateful to Ms. Darlene Limback for her excellent assistance.

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