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# Prolonged Stimulation by Follicle-Stimulating Hormone Is Required for the Induction of Ovarian Follicular Cysts by Human Chorionic Gonadotropin in Hypophysectomized Rats

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**Combined stimulation by follicule-stimulating hormone (FSH) and subovulatory doses of human chorionic gonadotropin (hCG, luteinizing hormone [LH]-like activity) produces large ovarian follicular cysts in hypophysectomized (HYPOXD) immature rats. To obtain a better understanding of the extent to which stimulation by FSH is required in order for hCG to induce these ovarian cysts, immature HYPOXD rats were given subcutaneous (sc) injections of 1 IU hCG twice daily for 9 d, either alone or with daily injections of 2 μg of highly purified ovine FSH on**

1. **Day one of hCG treatment;**
2. **Days one and two of hCG treatment;**
3. **Days one through five of hCG treatment; or**
4. **All 9 d of hCG treatment.**

**Ovaries and serum samples were collected on the morning of d 10 of treatment. Animals that were treated for 9 d with hCG, but that received either no FSH or only 1 or 2 d of FSH treatment, did not display antral follicles on day 10 of treatment. The largest cross-sectional areas for the ovaries from animals that received 1 or 2 d of FSH treatments ranged between 6.84 ± 0.51 $mm^2$ and 8.94 ± 0.89 $mm^2$. The diameters of the largest preantral follicles in the ovaries of these two groups ranged between 0.278 ± 0.011 and 0.320 ± 0.028 mm, respectively. In contrast, ovaries from hCG-treated HYPOXD rats that received FSH treatments for either 5 or 9 d displayed follicular cysts by the morning of day 10. The largest cross-sectional areas for the ovaries from these two treatment groups were similar (15.68 ± 1.61 and 18.7 ± 5.13 $mm^2$, respectively), as were the mean diameters of the cystic follicles in these two groups (0.929 ± 0.096 and 0.830 ± 0.063 mm, respectively). Although serum androstenedione and testosterone concentrations were greater for HYPOXD rats that received combined FSH + hCG treatments than for animals that received hCG treatments alone, these concentrations did not increase with increasing numbers of days of FSH treatment. As with serum androstenedione and testosterone concentrations, serum estradiol and estrone concentrations for HYPOXD rats treated with hCG alone were limited (0.002 ± 0.001 and 0.004 ± 0.002 ng/mL, respectively), but had increased by day 10 after a single injection of FSH on day one of treatment. In contrast to serum androgen concentrations, serum estradiol and estrone concentrations continued to increase as the number of days of combined FSH + hCG treatment increased. These observations indicate that, in the rat, a significant period of exposure to tonic stimulation by both FSH and LH-like activity is required for the development of large ovarian cysts. Further, this period of exposure to FSH appears to be linked to increased peripheral serum estrogen concentrations, rather than to increased androgen concentrations. Therefore, the data provide indirect support for the concept that estrogens play a direct role, at the level of the ovary, in the induction of large ovarian cysts in the rat.**

**Key Words:** Ovarian cysts; PCO; FSH; androgens; estrogens.

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## Introduction

Although serum hormone profiles associated with the established cystic ovary state have been characterized extensively for both women and other mammals expressing ovarian cysts (Roberts, 1955; Stein, 1975; Peluso et al., 1979, 1981; Yen, 1980; Kesler and Garverick, 1982; Coney, 1984; Futterweit, 1984), the mechanisms involved in the etiology of this state and the hormonal interactions required at the level of the ovary for the induction of cystic follicles remain to be elucidated. It has been shown, however, that prolonged stimulation of progesterone-synchronized immature rats and pregnant rats (Bogovich 1989, 1991) by subovulatory doses of human chorionic gonad-

otropin (hCG), or prolonged stimulation of hypophysectomized (HYPOXD) immature rats by both follicle-stimulating hormone (FSH) and hCG (Bogovich 1992), results in the development of large ovarian follicular cysts that possess preovulatory amounts of aromatase activity. These observations suggest that tonic stimulation by FSH plays at least a permissive role at the level of the ovary in the development of large ovarian cysts.

The obligatory role of FSH in the transition of preantral follicles to the small antral stage as well as in the induction of several granulosa cell functions that are required for preovulatory follicular development are well documented (Goldenberg et al., 1972; Richards et al., 1976, 1987; Richards, 1980). Luteinizing hormone [LH]-like activity, as well as FSH, stimulates many FSH- induced granulosa cell functions during normal preovulatory follicular development (Richards et al., 1976, 1987; Richards 1980). Indeed, 2 d of treatment with low doses of hCG results in the development of preovulatory follicles in both progesterone-synchronized immature rats and pregnant rats in which serum FSH concentrations are maintained at tonic follicular-phase values (Bogovich et al., 1981; Richards and Bogovich, 1982). In addition, 2 d of treatment with 2 μg of highly purified FSH plus 3 d of treatment with estradiol in the HYPOXD rat are sufficient for the development of preovulatory follicles (Goldenberg et al., 1972; Richards et al., 1976). Together, these observations indicate that the obligatory role for FSH during normal preovulatory follicular development in the rat may be limited to that interval in which FSH induces granulosa cell functions that can be maintained/stimulated by LH-like activity or by estradiol.

HYPOXD rats produce large ovarian follicular cysts in response to 9 d of combined treatment with daily injections of 2 μg FSH and twice daily injections of 0.5 IU hCG (Bogovich, 1992). Further, pretreatment with hCG has no effect on the number of days of combined FSH + hCG treatments needed for the development of cystic follicles in this model. This observation indicates that a certain critical period of stimulation by FSH is required for the induction of ovarian cysts in the rat. If the effects of stimulation by FSH during cyst development are analogous to those observed during normal follicular development in the rat, prolonged stimulation by LH/hCG alone may be sufficient to drive the development of large ovarian cysts in these animals once FSH has induced the antrum and, perhaps, other granulosa cell factors that are required for the development of ovarian cysts. Conversely, if tonic stimulation by FSH plays more than a permissive role in the induction of large ovarian cysts, more than 1 or 2 d of stimulation by FSH would be required for the induction of large cystic follicles by subovulatory doses of hCG in the HYPOXD rat. The present series of experiments were undertaken to determine the extent to which tonic stimulation by FSH is needed for the induction of large ovarian cysts in the rat.

## Results

### *Effects of Prolonged Stimulation by FSH + hCG In Vivo on Ovarian Morphology*

Figure 1 illustrates that HYPOXD rats that received twice daily treatments with 1 IU hCG alone for 9 d (Fig. 1A) or hCG treatments with FSH injections only on days one or two of the in vivo treatment regimen (Fig. 1B and C, respectively) displayed only preantral follicles by the morning of day 10. However, HYPOXD rats that received 2 d of combined FSH + hCG treatments did display antral follicles on the morning of day three of treatment (Fig. 1D). Similar follicles have been shown to possess preovulatory amounts of steroidogenic activity on this day of treatment (Bogovich, 1992).

In contrast to the animals that received only 1 or 2 d of FSH treatment, hCG- treated rats that received either 5 or 9 d of FSH injections (Fig. 2A and B) displayed large cystic follicles by the morning of day 10. That is, the ovaries possessed multiple large follicular cysts with stimulated thecal shells and just a remnant of granulosa cells. In addition, the stromal-interstitial tissue in these ovaries appeared stimulated.

The diameters of the largest follicles in the ovaries of HYPOXD rats treated for 9 d with hCG alone and in the ovaries of HYPOXD rats given hCG plus 1 or 2 d of FSH treatment were similar by the morning of the 10th d of treatment (Fig. 3): 0.320 ± 0.024, 0.289 ± 0.008, and 0.330 ± 0.037 mm, respectively. In marked contrast, the diameters of the largest follicles/cysts in the ovaries of hCG-treated HYPOXD rats that received either 5 or 9 d of FSH treatment were 0.929 ± 0.096 and 0.830 ± 0.063 mm, respectively ($p \leq 0.05$ when compared with follicles in ovaries from HYPOXD rats treated for 9 d with hCG ± 1 or 2 d of FSH treatment).

The effects of the in vivo treatments on the largest ovarian cross-sectional areas are illustrated in Fig. 4. The mean of the average largest ovarian cross-sectional area for HYPOXD rats treated for 9 d with hCG alone was 6.84 ± 0.51 $mm^2$. Ovarian cross-sectional areas for HYPOXD rats given hCG plus 1 or 2 d of FSH treatment were 4.93 ± 0.49 and 5.02 ± 0.44 $mm^2$, respectively. In contrast, the ovarian cross-sectional areas for HYPOXD rats given hCG plus 5 or 9 d of FSH treatment were markedly greater than those of the preceding treatment groups: 12.48 ± 1.71 and 12.39 ± 1.55 $mm^2$, respectively ($p \leq 0.05$ when compared with follicles in ovaries from HYPOXD rats treated for 9 d with hCG ± 1 or 2 d of FSH treatment).

### *Serum Hormone Concentrations*

The effects of the individual in vivo treatment regimens on serum androstenedione, testosterone, estradiol, and estrone concentrations are illustrated in Fig. 5. Serum androstenedione values for HYPOXD rats treated for 9 d with hCG alone (Fig. 5A) were 0.47 ± 0.10 ng/mL on the

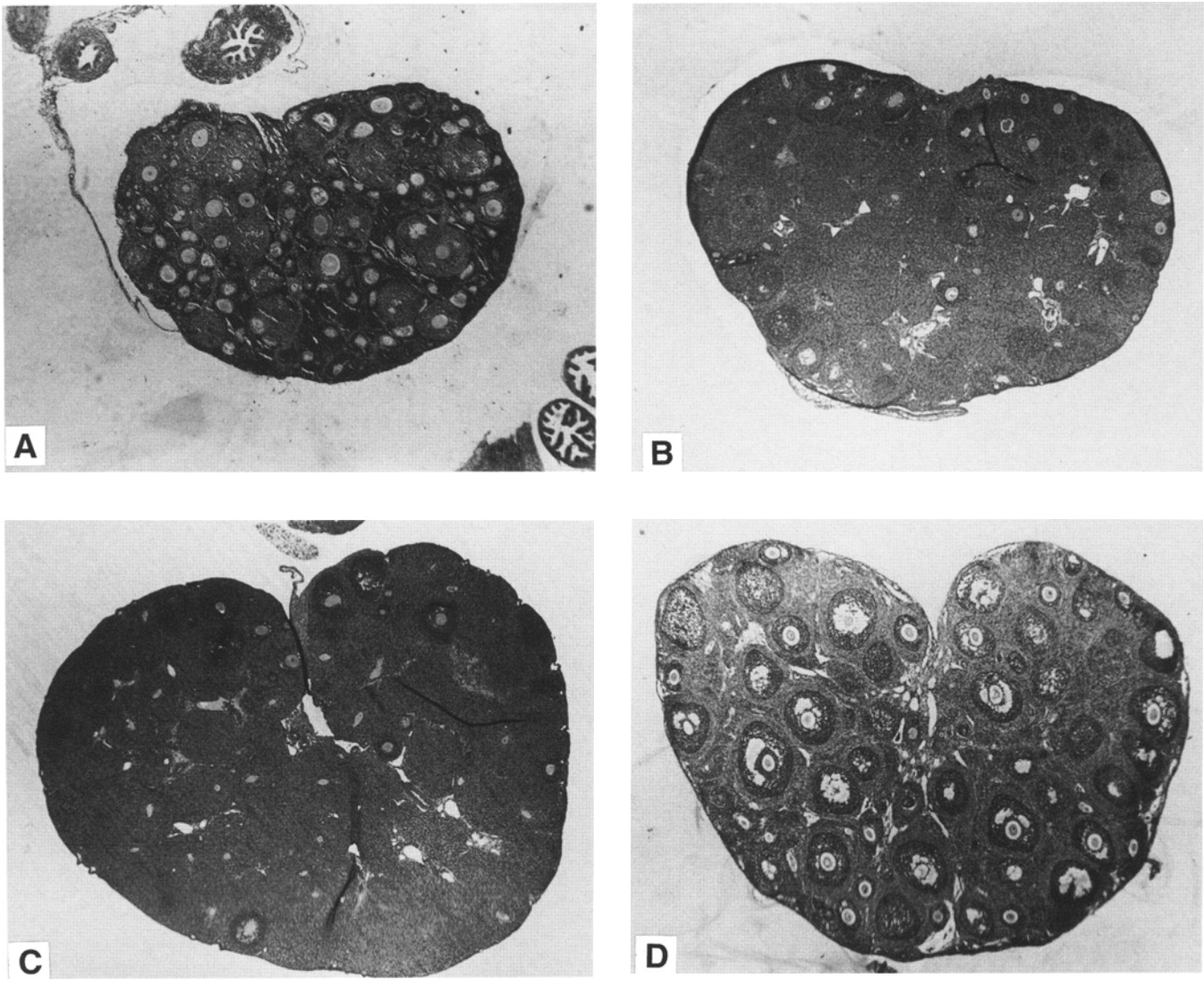


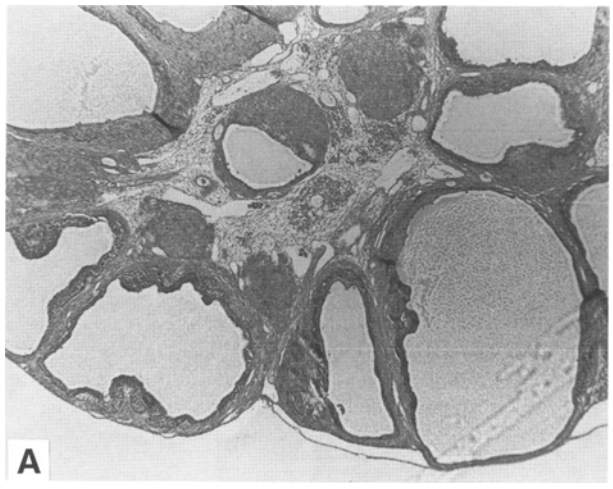

**Fig. 1.** The effect of hormonal stimulation on ovarian morphology. Panels **(A)**, **(B)**, and **(C)** depict typical ovarian morphology after 9 d of twice-daily injections with 1 IU hCG plus either (A) no FSH, (B) 1 d of FSH treatment (2 μg sc, once a day), or (C) 2 d of FSH treatment. Panel **(D)** illustrates typical ovarian morphology on the morning of day three of treatment in response to 2 d of combined FSH + hCG treatments. Photomicrographs were obtained on a Zeiss IM35 microscope using a 2.5× objective.

morning of day 10. In contrast to the histological data, hCG-treated animals that received as little as one injection of FSH displayed a significant increase in serum androstenedione (3.08 ± 1.01 ng/mL, $p \leq 0.05$) compared to values observed for animals treated with hCG alone. No further increases in serum androstenedione concentrations were observed in response to increasing numbers of days of hGC ± FSH treatment in vivo. Serum testosterone concentrations (Fig. 5B) followed a pattern similar to that observed for androstenedione. HYPOXD rats treated for 9 d with hCG alone displayed serum testosterone values of 0.14 ± 0.04 ng/mL on the morning of day 10. All groups that received FSH treatments displayed serum testosterone values that were greater than those observed for animals treated with hCG alone ($p \leq 0.05$), but were similar to each other (between 1.24 ± 0.62 and 1.96 ± 0.76 ng/mL).

In contrast to the observed changes in serum androgen concentrations, serum estrogen values (Fig. 5C and D) increased as the number of days of FSH treatments increased. Serum estrone concentrations for HYPOXD rats treated with hCG only (Fig. 5C) were limited to 0.004 ± 0.002 ng/mL on day 10 of treatment, but underwent a gradual increase to 0.133 ± 0.032 ng/mL as FSH treatments were increased to 5–9 d. Similarly, estradiol concentrations for HYPOXD rats treated for 9 d with hCG alone (Fig. 5D) were limited to 0.002 ± 0.001 ng/mL on day 10, but increased in response to increasing days of FSH treatment: attaining maximal values (0.257 ± 0.078 ng/mL) after 5–9 d of combined treatment with hCG + FSH.

## Discussion

The data from these experiments demonstrate that:



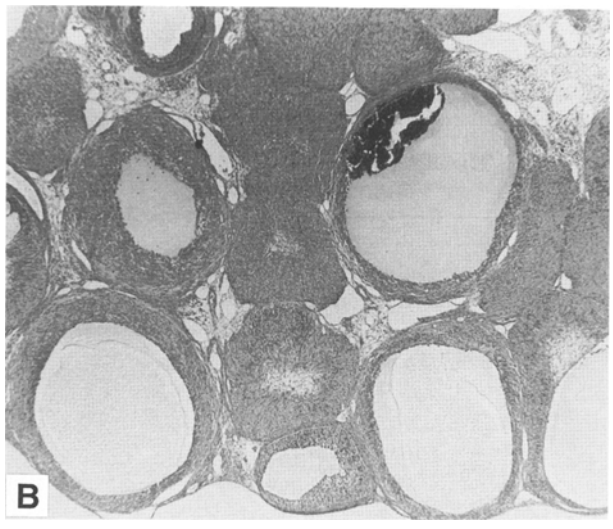




**Fig. 2.** Ovarian morphology after 9 d of twice-daily injections of 1 IU hCG plus **(A)** 5 d of FSH treatment, and (B) 9 d of FSH treatment. Photomicrographs were obtained on a Zeiss IM35 microscope using a 2.5× objective.

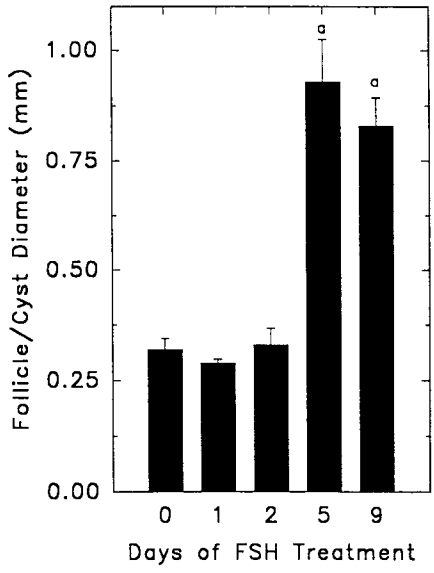



**Fig. 3.** Mean diameters of the largest follicles/cysts present in each pair of ovaries from HYPOXD rats treated twice daily with 1 IU hCG without or with daily FSH injections for up to 9 d. The average diameter of the largest follicles/cysts was calculated for each pair of ovaries from each animal. The means ± SEM of these average diameters were calculated for each treatment group and are depicted by the bars in this figure ($n = 3$ for animals receiving 0 d of FSH treatment; $n = 10, 6, 13$, and 9 for hCG-treated animals receiving 1, 2, 5, and 9 d of FSH treatment, respectively). a = significantly different from values obtained for rats treated for 0 d with FSH ($p < 0.05$).

1. In addition to stimulation by LH like activity, a prolonged period of stimulation by FSH may play a role in the induction of large ovarian cysts in HYPOXD rats;
2. FSH-stimulated increases in serum aromatizable androgen concentrations in hCG-treated HYPOXD rats are not sufficient to induce the development of large ovarian cysts; and
3. Serum estrogen concentrations increase in response to increasing number of days of combined stimulation by hCG + FSH, and are associated with the induction of large ovarian cysts in this model.

These latter observations indirectly support the concept that estrogens may play a direct role at the level of the ovary in the induction of follicular cysts.

Our previous observations regarding the effects of combined FSH + hCG stimulation on follicular steroidogenesis (Bogovich, 1992) and our present histological observations indicate that preovulatory follicles are present in the ovaries of HYPOXD rats that have been treated for 2 d with FSH + hCG. The present data clearly demonstrate, however, that subsequent treatments with hCG alone are not sufficient to maintain these follicles for a prolonged period of time or to drive the development of these follicles into large ovarian cysts. Instead, the data indicate that up to three more days of combined stimulation by FSH + hCG are needed to lay the foundation for the eventual induction of large ovarian cysts by LH-like activity in this model. One can only speculate at this time whether this requirement for stimulation by FSH involves the expression of (1) granulosa cell factors that may enhance the ability of LH-like activity to stimulate/maintain thecal cell functions at a critical time in the development of large ovarian cysts, and/or (2) granulosa cell steroidogenic enzymes that affect the production of estadiol by these follicles. The observation of a marked increase in serum estradiol concentrations, in association with increasing number of days of combined FSH + hCG treatment and the development of large ovarian cysts indicates that prolonged, combined stimulation by these hormones promotes ovarian estradiol production. Our previous work in this model indicates that at least part of the increased estradiol serum concentrations observed in these animals is derived from the follicles that are destined to become large ovarian cysts in response to these treatments (Bogovich, 1992). Further, the correlation between mark-

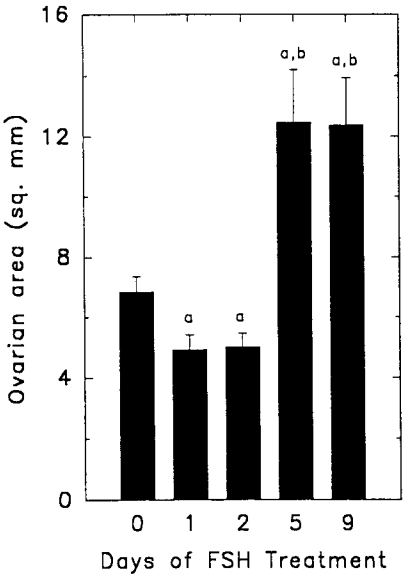



**Fig. 4.** Largest average ovarian cross-sectional area per rat in response to twice-daily injections of 1 IU hCG without or with daily FSH injections for up to 9 d. Each bar represents the mean ± SEM for each treatment group. a = significantly different from values obtained for rats treated for 0 d with FSH ($p < 0.05$); b = significantly different from values obtained for rats treated for 1 or 2 d with FSH ($p < 0.05$).

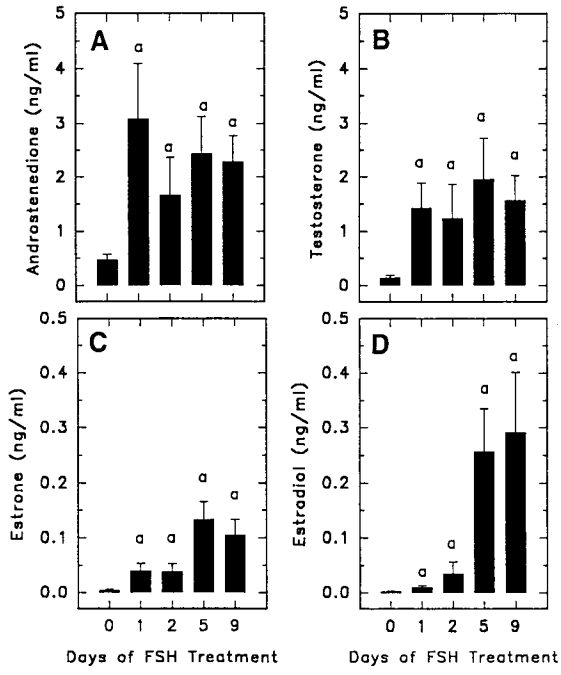



**Fig. 5.** Changes in serum steroid concentrations for HYPOXD rats in response to twice-daily treatments with 1 IU hCG without or with daily injections of FSH for up to 9 d. Each bar represents the mean ± SEM for the respective in vivo treatment group. a = significantly different from values obtained for rats treated for 0 d with FSH ($p < 0.05$).

edly increased serum estradiol concentrations and the development of large ovarian cysts in this model indirectly supports the concept that estradiol may play a role at the level of the ovary in the induction of these cysts.

The observation that a single injection of FSH can increase serum concentrations of aromatizable androgens in the hCG-treated HYPOXD rat complements our previous observations that FSH alone can increase serum testosterone concentrations but that hCG is required to maintain these concentrations (Bogovich, 1992). The present study extends these observations by demonstrating that stimulation by LH-like activity is sufficient to sustain the FSH-induced increases in serum aromatizable androgen concentrations. Together, these in vivo observations support the in vitro observation that FSH can stimulate thecal-interstitial androgen production through a paracrine interaction (Smyth et al., 1993). However, the mechanisms by which FSH affects serum aromatizable androgen concentrations in hCG-treated HYPOXD rats remain to be determined.

It has been suggested that inhibin and insulin-like growth factor-I (IGF-I) may have roles in the paracrine effects of FSH on thecal androgen synthesis (Smyth et al., 1993). FSH stimulates the production of both of these factors by granulosa cells (Adashi et al., 1985; Bicsak et al., 1986); and both factors stimulate thecal-interstitial tissue androgen production in vitro (Hsueh et al., 1987; Magoffin et al., 1990). Further studies are needed to determine if these FSH-induced factors affect serum androgen concentrations in this model. Such an observation, however, may argue against inhibin and IGF-I being the FSH-induced factor(s) required for the induction of large ovarian cysts in the HYPOXD rat, since FSH-induced increases in serum androgen concentrations were not associated with the induction of large ovarian cysts in the present study. However, this series of experiments cannot rule out a potential synergy, at the level of the ovary, between elevated serum androgens, inhibin, IGF-I, and increasing serum estrogen concentrations that results in the development of large ovarian cysts in the FSH + hCG-treated HYPOXD rat. Indeed, hyperinsulinemia acts synergistically with prolonged stimulation by subovulatory doses of hCG to increase the size of ovarian cysts in intact adult rats (Poretsky et al., 1992). Further, the association of normal-to-low FSH serum concentrations and insulin resistance with the established polycystic ovary state in women has led to the hypothesis that inhibin and IGF-I may play a role in the etiology of this syndrome (Futterweit, 1984; Givens and Wild, 1992). Further experiments are required in the present model to determine the effects of the in vivo treatments on follicular inhibin and IGF-I expression.

Part of the early effect of FSH on serum testosterone concentrations in this study, and in our previous study in the HYPOXD rat (Bogovich, 1992), may be owing to increased expression of granulosa cell type 1-like 17β-

hydroxysteroid oxidoreductase (17HSOR; Ghersevich et al., 1994; Blomquist et al., 1994), which catalyzes the reversible conversion of androstenedione to testosterone and estrone to estradiol. Ovarian follicular 17-ketosteroid reductase (17KSR), which catalyzes the forward metabolism of androstenedione and estrone by the 17HSOR isozymes, is extremely active during prolonged exposure to tonic, basal FSH serum concentrations and twice daily injection of 1 IU hCG in the pregnant rat (Bogovich, 1995). 17KSR activity decreases before follicular aromatase activity during the development of large ovarian cysts in the pregnant rat (Bogovich, 1995). However, this loss in follicular 17KSR activity does appear to coincide with the loss of granulosa cells as these large ovarian cysts develop. These observations indirectly support the concept that prolonged, tonic stimulation by gonadotropins maintains and stimulates the thecal shells of these follicles, in part by promoting the production of estradiol until the granulosa cells are no longer viable.

Many, but not all, intact rats and guinea pigs that receive estrogen treatment display ovarian cysts (Barraclough, 1966; Brawer et al., 1978; Campion et al., 1993). However, since estrogens affect central mechanisms as well as ovarian functions, it is not possible to determine the degree to which the treatments in these earlier studies affect cyst development directly at the level of the ovary. Indeed, the variability of the results in these earlier models suggests that the estrogenic effects are not primarily at the level of the ovary. Nevertheless, these earlier observations and our present observations in the FSH + hCG-treated HYPOXD rat, provide strong indirect support for the concept that estrogens, of peripheral or ovarian origin, may play a key role at the level of the ovary in the induction of follicular cysts. Indeed, prolonged stimulation by FSH + estradiol is sufficient for the development of **small** ovarian cysts in the HYPOXD rat (Bogovich, 1993). Together, these observations support the concept that estrogens also may play a direct role in the induction of **large** ovarian cysts in response to prolonged stimulation by hCG + FSH in this model.

The observation that stimulation by FSH is not required throughout the in vivo treatment period for the induction of large cystic follicles may reflect the onset of the loss of granulosa cells, which is an integral aspect in the development of ovarian cysts. The loss of granulosa cells begins in HYPOXD rats treated with FSH + 0.5 IU hCG between days five and seven of combined hormonal treatment (Bogovich, 1992). Thus, the timing of the initial loss of granulosa cells in response to the hormonal treatments used in this series of experiments agrees well with the extent to which daily treatments with FSH are required for the induction of large ovarian cysts by 1 IU of hCG.

The possibility that fewer days of more frequent injections of the same dose of ovine FSH (oFSH), or fewer days of daily treatments with higher doses of oFSH, may result in the induction of large ovarian cysts in response to twice daily injections of 1 IU hCG cannot be completely ruled out at this time. The HYPOXD rat model for the induction of large ovarian cysts was established originally (Bogovich, 1992) by trying to mimic the timing of the development of large ovarian cysts in response to twice daily injections of 0.1–1.5 IU hCG in progesterone- synchronized, immature rats (Bogovich 1989). During our initial experiments in the HYPOXD rat, daily injections of 1 μg oFSH together with twice daily injections of 0.5 IU hCG failed to induce cystic follicles (unpublished observation). In contrast, treating HYPOXD rats once daily with 2 μg oFSH and twice daily with 0.5 IU hCG induced similar changes in follicular steroidogenic activity as well as the development of large ovarian cysts in a period of time that was similar to that observed for the induction of large follicular cysts in intact, progesterone- synchronized, immature rats (Bogovich, 1989). These observations indicate that administration of 2 μg of oFSH to HYPOXD rats and exposure of progesterone-synchronized immature rats to endogenous basal amounts of rat FSH (Bogovich, 1989) have similar effects on the ability of subovulatory doses of hCG to induce large ovarian cysts in this species. Studies regarding the effects of increasing doses of oFSH on the ability of LH-like activity, as well as other steroid and peptide hormones, to induce ovarian follicular cysts will help to delineate the mechanisms by which tonic stimulation by FSH contributes to the induction of ovarian cysts.

In conclusion, the results of the present series of experiments indicate that prolonged stimulation by FSH may play an important role in the induction of large ovarian cysts by subovulatory doses of LH-like activity in the HYPOXD rat. In addition, increased serum estradiol, rather than increased serum androgen concentrations, is associated with the induction of large ovarian cysts in response to prolonged, combined stimulation by FSH + hCG in the HYPOXD rat. Together, these observations support the concept that estrogens of ovarian or peripheral origin are an important factor, directly at the level of the ovary, in the induction of ovarian follicular cysts.

## Materials and Methods

### *Animals and Histology*

The following procedures were performed according to a protocol approved by the University of South Carolina Institutional Animal Care and Use Committee. Immature female rats, hypophysectomized at 21 d of age, were obtained from Charles River (Boston, MA) and housed in groups of 5–6/container in a temperature-controlled room with a light:dark cycle of 12:12 with lights on at 0600 h. To help maintain viability, these animals were fed oranges in addition to the rat chow provided by the USC Animal Resource Facility and were provided with water containing 10% sucrose. There were a total of three animals in the hCG-only-treated group, since the results obtained with

this group were virtually identical to those observed in our previous study with this model (Bogovich, 1992). The number of animals in the four groups that received both hCG + FSH treatments ranged between 6 and 14.

Beginning on day 27 of age, and continuing for 9 d, all HYPOXD rats were given twice-daily sc injections of 1 IU of hCG (4000 IU/mg, Sigma Chemical Co., St. Louis, MO) in 0.2 mL phosphate-buffered saline, pH 7.0, containing 0.09% pig skin gelatin (gel-PBS) plus one of five in vivo treatments with single, daily injections of 2 μg ovine FSH (20 U/mg; NIADDK-oFSH-17; <0.04 times NIH-LH-S1) in 0.2 ml gel-PBS between 0700 and 0800 h:

1. No FSH (hCG only controls);
2. FSH on day one of hCG treatment;
3. oFSH on days one and two of hCG treatment;
4. oFSH on days one through five of hCG treatment; and
5. oFSH on all 9 d of hCG treatment.

Ovaries and trunk blood were collected from all treatments groups between 0730 and 0830 h on day 10 of treatment. Additional animals, treated for 2 d with oFSH + hCG, were sacrificed on the morning of day three of treatment to determine if the ovaries possessed follicles similar in appearance to the preovulatory follicles observed on this day of treatment in our previous work with this model (Bogovich, 1992). Ovaries were excised and fixed in formalin for sectioning and staining (hematoxylin-eosin) by the University of South Carolina School of Medicine Histology Core Facility. Photomicrographs were obtained using a 2.5× planachromat objective on a Zeiss 35IM microscope.

### *Serum*

Serum samples were harvested by centrifugation and stored in individual polypropylene tubes at –20°C. Each serum sample was extracted with high-pressure liquid chromatography (HPLC)-grade diethyl ether and chromatographed as previously described (Bogovich, 1992) using HPLC-grade acetonitrile (AcN, Fisher Scientific Co, Pittsburgh, PA) and Nylon-66 filtered Milli-Q-grade water. Briefly, each serum sample was extracted individually in the presence of 1500 cpm of tritiated estradiol, testosterone, and estrone (DuPont New England Nuclear, Boston, MA) to monitor procedural losses. The ether phases were dried, and the residues were dissolved in HPLC-grade AcN with vortexing and warming. Each sample was brought to a final AcN:$H_2O$ ratio of 40:60 with filtered, Milli-Q-grade water just prior to loading onto the C-18 column.

The reconstituted samples were loaded onto a 25-cm Rainin C-18 microsorb, end-capped column and chromatographed at a flow rate of 1.5 mL/min using a 30-min isocratic AcN:$H_2O$ (37:67) gradient followed by a 1-min linear gradient to 100% AcN. Samples were collected at 0.4-min intervals using a Gilson FC-80 fraction collector. Pooled peak fractions for each steroid of interest were dried, reconstituted with 1 mL gel-PBS, warmed in a 45°C water bath for 30 min with vortexing, and stored at –20°C until analyzed for steroid content by radioimmunoassay (RIA). Procedural recoveries were assessed by determining the residual amount of tritiated steroid present in a precise volume (0.2 mL) of each of the reconstituted steroid samples.

### *Radioimmunoassays (RIAs)*

Antisera against estradiol was the generous gift of Gordon Niswender (Fort Collins, CO). Antisera against androstenedione was generously provided by Gerry Nordblom and Barry England (Ann Arbor, MI). G. Nordblom also provided the antisera against testosterone. Iodinated E2 and A4 were obtained from Diagnostic Systems Laboratories (Webster, TX). Iodinated testosterone was obtained from Diagnostic Products Corporation (Los Angeles, CA). Iodinated estrone and its antisera were obtained from Pantex (Santa Monica, CA).

All RIAs were performed using previously described procedures (England et al., 1974; Nordblom et al., 1981; Bogovich, 1992). The standard curves for the testosterone, androstenedione, estradiol, and estrone RIAs ranged from 4 to 500, 4 to 1000, 0.2 to 50, and 0.4 to 100 pg, respectively. The lowest value of the standard curves was greater than the limit of detection (reference binding minus 2 SD) for each of the steroid RIAs. In addition, these assays were counted and analyzed on a Packard Cobra 5000 γ-counter, which does not extrapolate values outside the range of the standard curve. Intra- and interassay coefficients of variation were: 6 and 18% for androstenedione; 4 and 10% for testosterone; 7 and 12% for estrone; and 6 and 10% for estradiol.

### *Statistics*

Statistical differences between and among treatment groups were determined using the SigmaStat multiple-comparison analysis of variance and Student-Newman Keuls test. Differences were considered significant when $p \leq 0.05$.

## Acknowledgment

A preliminary report of these data was presented at the Xth Ovarian Workshop: Frontiers in Ovarian Research, Ann Arbor, MI, July 21–23, 1994.

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