Evidence for a Role of Endogenous Estrogen on Follicular Growth in Immature Female Rats

TAKASHI NAKAYAMA, RYOSUKE NAKANO AND MIYAKO IWAO

Department of Obstetrics and Gynecology, Wakayama Medical College,
1 Shichibancho, Wakayama 640

Abstract

The effect of gonadotropin and endogenous estrogen on the growing follicles (especially in relation to the presence or absence of theca cell layers) was studied morphometrically in intact immature female rats, using a chemical antiestrogen.

When pregnant mare serum gonadotropin (PMSG) was given in combination with clomiphene, the uterine weight decreased significantly as compared with that in the animals treated with PMSG alone (p<0.001). The administration of clomiphene and PMSG did not have a significant effect on the total number of follicles (growing plus atretic). The number of atretic follicles in those treated with PMSG alone tended to decrease as compared with sesame oil-treated animals. However, when PMSG was given in combination with clomiphene, the number of atretic follicles increased significantly as compared with the animals treated with PMSG alone (p<0.05). The number of growing follicles in the animals treated with PMSG alone tended to increase as compared with the animals treated with neither PMSG nor clomiphene. In contrast, when PMSG was given in combination with 5 mg/day of clomiphene, the number of growing follicles tended to decrease. The administration of PMSG alone and PMSG combined with 1 and 5 mg/day of clomiphene inhibited the number of growing follicles without theca cell layers, as compared with sesame oil-treated animals (p<0.05, p<0.01 and p<0.001, respectively). On the other hand, the number of growing follicles with theca cell layers was increased markedly by the administration of PMSG alone (p<0.05).

It is suggested that the early stage of follicular growth in the absence of theca cell layers may be promoted by endogenous estrogen. In the presence of theca cell layers, in addition to being mediated by estrogen, gonadotropin-induced follicular growth may be promoted by the maturative effect of gonadotropin itself.

Previous investigators have reported that a chemical estrogen antagonist, clomiphene citrate, fails to block the effect of exogenous gonadotropin on mean ovarian weight gain (Holtkamp et al., 1960; Callantine et al., 1966). Recently, Harman et al. (1975a) have shown that failure of previous workers to see an inhibitory effect of clomiphene directly on the ovary is due to their use of lower doses, and higher doses of clomiphene inhibit ovarian weight gain response to human chorionic gonadotropin (HCG) plus follicle-stimulating hormone. In addition, they have suggested that endogenous estrogen might play an important role in the follicular growth. However, little is known about the precise mechanism whereby gonadotropin and estrogen may act to stimulate the follicular growth of the ovary.

The purpose of the present investigation is to further elucidate morphometrically the
effect of gonadotropin and endogenous estrogen on ovarian follicular growth (especially in relation to the presence or absence of theca cell layers) in intact immature rats using a chemical antiestrogen, clomiphene.

**Materials and Methods**

Twenty-four day-old intact female Sprague-Dawley rats were used in the present experiment. The rats were kept at 25°C with intervals of 12 h light and 12 h darkness, and given food and water *ad libitum*.

Commercial pregnant mare serum gonadotropin (Primantron, Schering AG) and commercial human chorionic gonadotropin (Primogonyl, Schering AG) were dissolved at a concentration of 10 IU in 0.4 ml of a 0.15 M NaCl solution containing 1% bovine serum albumin. The chemical antiestrogen, clomiphene citrate, was supplied by Merrell Corporation. One and 5 mg of clomiphene were suspended in 0.4 ml of sesame oil.

The rats were divided into six groups for the following treatments: sesame oil; 1 and 5 mg/day of clomiphene alone; PMSG alone and with 1 and 5 mg/day of clomiphene. Each group consisted of eight animals except for two groups of nine animals treated with PMSG alone and PMSG combined with 1 mg/day of clomiphene. Intact rats, 24 days of age, were treated with 10 IU of PMSG by subcutaneous injection. Also on the same day, at a separate site 1 or 5 mg of clomiphene was given subcutaneously. All animals were sacrificed by decapitation 48 h after the first injection.

Superovulation was induced in the immature rats by PMSG followed by HCG. The animals were divided into three groups treated with sesame oil (n=11), 1 mg/day of clomiphene (n=10) and 5 mg/day of clomiphene (n=10). Ten IU of PMSG, and 1 and 5 mg/day of clomiphene were given by the same method as above. In addition, 10 IU of HCG was given subcutaneously in a single volume of 0.4 ml at 48 h following an initial injection of PMSG. All animals were sacrificed by decapitation 20 h following the administration of HCG.

Uterine and ovarian weights were measured on a torsion balance. For histological studies, the ovaries were fixed in Bouin's fluid, embedded in paraffin, serially sectioned at 5 µm and stained with hematoxylin and eosin. The growing follicles were classified into primary and secondary follicles without theca cell layers, and tertiary and Graafian follicles with theca cell layers. Atretic follicles were identified according to the criteria of stage I and II in the classification of atretic follicles (Himelstein-Braw et al., 1976). Every fifth section of one ovary was examined and the number of growing and atretic follicles showing the ovum nucleus were counted. Ova in tube and ovarian bursa were counted under the microscope.

Blood samples were collected in small glass tubes and stored at -20°C until assayed. The concentration of serum estradiol was measured by radioimmunoassay according to the modified method of Wu and Lundy (1971).

Values were given as mean ± SEM. Student's t-test for paired data was used to determine the significance of the observed differences.

**Results**

**Effect of PMSG and clomiphene on uterine and ovarian weights**

As indicated in Fig. 1, the uterine weight of rats treated with PMSG alone (120.1 ± 5.9 mg) showed a significant increase (p < 0.001) compared to that of the animals treated with sesame oil alone (47.4 ± 2.6 mg). When PMSG was given in combination with 1 and 5 mg/day of clomiphene, the uterine weight (73.2 ± 1.5 and 71.1 ± 2.0 mg) decreased significantly compared to the animals treated with PMSG alone (p < 0.001). In addition, the uterine weight of the animals treated with 1 and 5 mg/day of...
clomiphene alone (66.4 ± 2.6 and 72.3 ± 3.2 mg, respectively) was significantly higher than that of the rats treated with sesame oil alone (p<0.01).

Fig. 2 shows the effect of PMSG and clomiphene on ovarian weight in the intact immature female rats. The ovarian weight of the animals treated with PMSG alone increased significantly compared with that of the animals receiving sesame oil alone (p<0.001). However, when PMSG was given in combination with 1 and 5 mg/day of clomiphene, the ovarian weight decreased significantly (p<0.01).

**Effect of PMSG and clomiphene on the number of growing and atretic follicles**

The administration of clomiphene and PMSG did not have a significant effect on the total number of follicles (growing plus atretic) (Fig. 3).

The number of growing follicles tended to increase upon administration of PMSG alone. In contrast, the number of growing follicles showed a tendency to decrease if the rats were treated with clomiphene in addition to PMSG (Fig. 4).

As shown in Fig. 5, the number of primary follicles in the rats treated with either PMSG alone or PMSG combined with clomiphene decreased significantly compared with sesame oil-treated animals (p<0.01 and p<0.001, respectively). The administration of PMSG alone tended to cause a decrease in the number of secondary follicles. In addition, the number of secondary follicles in the animals treated with PMSG combined with clomiphene showed a significant decrease (p<0.05). The number of tertiary follicles also showed a tendency to decrease in the animals treated with PMSG combined with clomiphene.
The growing follicles were further classified into those with and without theca cell layers (Fig. 6). Compared with sesame oil-treated animals, the administration of PMSG alone and PMSG combined with 1 and 5 mg/day of clomiphene caused a significant decrease in the number of growing follicles without theca cell layers (p<0.05, p<0.01 and p<0.001, respectively). The number of growing follicles with theca cell layers was increased significantly by the administration of PMSG alone (p<0.05). However, there was no difference in the number of growing follicles with theca cell layers between the animals treated with PMSG combined with clomiphene and sesame oil-treated animals.
Fig. 7. Sections of the ovaries from intact immature female rats sectioned at 5 μm and stained with hematoxylin and eosin (×20 magnification)

(A) Typical appearance of whole ovary treated with sesame oil. All stages of follicles are present.

(B) and (C) Typical appearance of whole ovaries treated with 1 (B) and 5 mg/day (C) of clomiphene alone. There is no difference between these groups and sesame oil-treated group.

(D) Typical appearance of whole ovary treated with PMSG alone. Many Graafian follicles are present, and the interstitial glands are well-developed.

(E) and (F) Typical appearance of whole ovaries treated with PMSG combined with 1 (E) and 5 mg/day (F) of clomiphene. The number of Graafian follicles is lower than in the group treated with PMSG alone. However, there is no difference between the interstitial glands in these groups and the group treated with PMSG alone.
The number of atretic follicles in the rats treated with either 5 mg/day of clomiphene alone or PMSG combined with 5 mg/day of clomiphene tended to increase compared with sesame oil-treated animals. In addition, the administration of PMSG alone tended to decrease the number of atretic follicles. However, when PMSG was given in combination with 5 mg/day of clomiphene, the number of atretic follicles increased significantly compared to the animals treated with PMSG alone (p<0.05) (Fig. 5).

Photomicrographs of sections of the various whole ovaries are shown in Fig. 7. There was no difference between the appearance of ovarian sections treated with 1 and 5 mg/day of clomiphene and sesame oil. In the sections of PMSG alone treated animals, many Graafian follicles were present and the interstitial glands were well-developed. When PMSG was given in combination with 1 and 5 mg/day of clomiphene, the number of Graafian follicles decreased. However, there was no difference between the interstitial glands in the groups given PMSG with clomiphene and those without clomiphene.

Effect of PMSG and clomiphene on the concentration of serum estradiol

The levels of serum estradiol rose markedly following the administration of PMSG (p<0.001) and the administration of 1 and 5 mg/day of clomiphene did not affect the levels of serum estradiol (Fig. 8).

Effect of clomiphene on superovulation in intact immature rats following treatment with PMSG and HCG

The administration of 1 and 5 mg/day of clomiphene resulted in a significant decrease in the number of ova in the rats treated with PMSG and HCG (p<0.001) (Fig. 9).

Fig. 8. Effect of PMSG and clomiphene on the levels of serum estradiol in intact immature female rats.
Discussion

In immature female rats, LH release is initiated between 55 and 56 h after the injection of PMSG (Zarrow and Quinn, 1963; Quinn and Zarrow, 1964). Similar results have also been obtained by Ferrin et al. (1969). Wilson et al. (1974) have used radioimmunoassay to measure the changes in plasma levels of LH in immature rats treated with PMSG. They report that plasma LH levels started to rise 52 h after the administration of PMSG, reached a peak at 54 h and then fell to undetectable levels by 58 h. The animals used in the present study were sacrificed 48 h after the injection of PMSG because there was no need to allow for the effect of endogenous LH.

The changes in plasma estradiol levels after PMSG treatment have been reported as follows: the levels begin to rise about 28 h after the injection and reach a plateau between 42 and 52 h (Wilson et al., 1974). It was demonstrated in this study that uterine weight increased significantly after the administration of PMSG as a result of the significant serum estradiol increase. However, there was no significant difference in serum estradiol levels between the groups given PMSG with and without clomiphene. This result agrees with the finding by Stähler et al. (1975) that the conversion of androstenedione into estradiol showed no significant difference with and without high doses of clomiphene. Clomiphene did not inhibit the effect of LH in morphologically repairing the interstitial glands. In addition, no systemic toxicity in the animals in terms of decreased vigor, decreased body weight or increased mortality was noted with doses of 1 and 5 mg/day. Judging from the above results, it is concluded that clomiphene acts directly on the ovary as an estrogen antagonist.

Regarding the mechanism of clomiphene antagonism, it has been reported by Roy et al. (1964) that this agent competes with the natural estrogen for the receptor sites in the uterus and pituitary gland, preventing the entry of estrogen to, and probably displacing it from, the receptor sites. Moreover, Katzenellenbogen and Ferguson (1975) and Baudendistel et al. (1978) have postulated that this compound acts by binding to estrogen receptors in the target organs, thus preventing estrogen from becoming active at its sites of efficacy. Furthermore, it has been reported by Stumpf (1969) and Richards (1975) that the nuclei of the granulosa cells in rats possess an estrogen receptor. Based on these reports, clomiphene was utilized as a chemical antiestrogen in order to study the role of endogenous estrogen on ovarian folliculogenesis.

Peters (1979) supported the theory that
the administration of clomiphene and PMSG did not have a significant effect on the total number of follicles (growing plus atretic). However, the number of atretic follicles in the rats treated with PMSG combined with clomiphene increased compared those treated with PMSG alone, whereas the administration of PMSG reduced the number of atretic follicles compared with sesame oil-treated animals. This result is supported indirectly by the report that estrogen may have an anti-atretic effect (Harman et al., 1975b). Judging from the above results, a decrease in the number of growing follicles results from inhibition of the effect of endogenous estrogen using clomiphene.

The growing follicles were classified into four types: primary and secondary follicles without theca cell layers, and tertiary and Graafian follicles with theca cell layers. The number of primary and secondary follicles decreased while the number of tertiary and Graafian follicles was increased by the administration of PMSG, compared with the animals given sesame oil. In addition, these increases were inhibited significantly by the administration of 1 and 5 mg/day of clomiphene. However, the number of Graafian follicles in the animals given PMSG combined with 1 and 5 mg/day of clomiphene still showed a significant increase compared with the clomiphene group without PMSG treatment. An increase in the number of tertiary and Graafian follicles, caused by treatment with PMSG alone, can be attributed to the effects of PMSG-produced estrogen and PMSG itself on the growing follicles. The number of growing follicles without theca cell layers in all hormone treated groups decreased compared with sesame oil-treated animals. In addition, the number of growing follicles with theca cell layers increased significantly following the administration of PMSG alone. The following data confirm the abovementioned results. Namely, the ovarian weight of the animals treated with PSMG was decreased significantly by the administration of clomiphene, despite the fact that there was no difference between the interstitial glands in the groups given PMSG with and without clomiphene. This result is agreement with the previous experiment that gonadotropin-induced ovarian weight gain is inhibited by the administration of antiestradiol serum in hypophysectomized immature female rats (Reiter et al., 1972). In addition, the number of ovulated ova in tube and ovarian bursa following treatment with PMSG and HCG is decreased markedly by the administration of clomiphene. Furthermore, the findings made in this study are also supported indirectly by the reports of Williams (1944), de Wit (1953), Payne and Hellbaum (1955), Bradbury (1961), Goldenberg et al. (1972) and Nakano et al. (1977), all of which showed a positive effect of exogenous estrogen on follicular growth.

In this study, the effect of gonadotropin and endogenous estrogen on the growing ovarian follicles (especially in relation to the presence or absence of theca cell layers) was investigated morphometrically in intact immature female rats using a chemical antiestrogen, clomiphene citrate. The morphometric findings suggest that the effect of gonadotropin on the growing follicles may differ according to whether the growing follicles possess theca cell layers. Therefore the early stage of follicular growth in the absence of theca cell layers may be promoted by gonadotropin-produced endogenous estrogen. On the other hand, in growing follicles with theca cell layers, in addition to being mediated by estrogen, the gonadotropin-induced follicular growth may be promoted by the maturative effect of gonadotropin itself.
References


