A possible role of clomiphene citrate
in the control of pre-ovulatory LH surge
during induction of ovulation

Naoki Terakawa, Ikuya Shimizu, Hirohisa Tsutsumi,
Toshihiro Aono and Keishi Matsumoto

Department of Obstetrics and Gynecology and Department of Pathology,
Osaka University Medical School, Osaka 553, Japan

Abstract. A possible role of clomiphene citrate (clomiphene) in the control of ovulation in anovulatory women was investigated. Since a single ip administration of 5 μg oestradiol-17β (E2) to long-term ovariectomized rats did not induce LH surge, the following studies were designed to determine whether pretreatment with clomiphene followed by administration of E2 could induce LH surge in the ovariectomized rats. Changes in cytoplasmic and nuclear oestrogen receptors (ER) were also examined in the pituitaries of these animals. An ip injection of 200 μg clomiphene suppressed serum LH levels significantly for 72 h. The clomiphene injection rapidly caused an elevation of nuclear ER with a concomitant depletion of cytoplasmic ER level in the pituitary and the ER levels remained almost unchanged for 72 h. An administration of E2 12 or 24 h after the clomiphene injection had no significant effects on either the serum LH levels or the cytoplasmic and nuclear ER levels, compared with those induced by clomiphene alone. However, LH surge and the depletion of nuclear ER in the pituitary occurred 24 h later when E2 was injected 48 h after the clomiphene administration. The E2-induced LH release seems to be induced by a replacement of clomiphene by E2 on the nuclear receptor complex. These results suggest that clomiphene may exert actions directly on the pituitary gland to augment oestrogen-induced LH release.

The administration of clomiphene citrate (clomiphene) to anovulatory women is followed by an increase in the circulating levels of both LH and FSH, leading to follicular maturation and ovulation (Ross et al. 1970; Vaitukaitis et al. 1971). For the explanation of an increase in gonadotrophin levels during clomiphene treatment, it is generally accepted that clomiphene interacts as an anti-oestrogen with hypothalamic-pituitary oestrogen receptor (ER) and consequently blocks the negative feedback effect of ovarian oestrogen on the release of gonadotrophins (Kato et al. 1968; Tsutsumi et al. 1983). Studies with the cultured pituitary cells, however, suggested that clomiphene exerts a direct oestrogenic rather than anti-oestrogenic effect on the pituitary cells by enhancing the gonadotrophin-releasing hormone (GnRH) stimulated release of gonadotrophin (Adashi et al. 1981). In vivo studies have suggested that clomiphene can act as both oestrogen agonist and antagonist, depending on the target tissue, the duration of treatment, and the availability of oestrogen in the system (Clark et al. 1974). The precise mechanism of clomiphene to induce ovulation is still not clear.

An administration of clomiphene in vivo, in contrast to oestadiol-17β (E2), has been shown to result in prolonged retention of nuclear ER and depletion of cytoplasmic ER in the pituitary gland (Tsutsumi et al. 1983), both of which effects last for a week (Adashi et al. 1980). These studies imply...
that clomiphene acts as a long-acting agent in the pituitary gland.

On the other hand, several studies both in vivo (Nakai et al. 1978) and in vitro (Frawley & Neill 1984) have suggested that E₂ exerts both positive and negative feedback actions on gonadotrophin secretion in mammals and the pituitary gland serves as a possible site of these actions. These findings raise questions concerning how clomiphene interacts at the pituitary gland with increased ovarian oestrogen at the time of ovulation and whether clomiphene takes part in pre-ovulatory LH surge caused by E₂. In this paper, therefore, the role of clomiphene in the control of LH release by E₂ was investigated in long-term ovariectomized rats.

Materials and Methods

**Animals and treatment**

Adult Wistar female rats weighing about 200 g were purchased from Shizuoka Laboratory Animal Center. Three weeks after bilateral ovariectomy, either 5 µg E₂ in 0.5 ml saline or 200 µg clomiphene in 0.5 ml sesame oil was injected ip between 4.00 and 6.00 p.m. In the other groups of ovariectomized rats, 5 µg E₂ was administered 12, 24 or 48 h after injection of 200 µg clomiphene. The rats were sacrificed by decapitation 1, 4, 12, 24, 48 or 72 h after a single dose of either E₂ or clomiphene. The clomiphene pretreated rats were killed 1, 4 or 24 h after E₂ injection. The trunk blood was collected in tubes and the pituitary was removed immediately. Eight normal female rats in dioestrous of the oestrous cycle served as the control for LH study. Serum levels of LH were determined by the double antibody radioimmunoassay using the RIA kits supplied by NIADDKD as described previously (Hayashi et al. 1979).

**Subcellular fractionations**

All procedures were performed at 4°C. Four pituitaries were collected and homogenized in 2 ml of TEM (10 mM Tris, 1.5 mM EDTA and 2 mM mercaptoethanol, pH 7.4) buffer with a Teflon-glass homogenizer. The homogenate was first centrifuged at 800 × g for 10 min. The supernatant was then centrifuged for 60 min at 105000 × g and clear supernatant (cytosol) obtained was then incubated with 0.2 ml of 2.5% charcoal-0.025% dextran suspension for 15 min to remove unbound steroids. After centrifugation, the supernatant was used for the cytosol exchange assay. The pellet from the initial 800 × g centrifugation was washed three times with 2 ml each of TEM buffer, resuspended in 2 ml of TEM buffer and used without further purification for the nuclear exchange assay.

**Exchange assay**

The methods used were the same as those reported previously (Tsutsumi et al. 1983). For the cytosol exchange assay, duplicate 0.3 ml aliquots of cytosol preparations were incubated with 5 nM [³H]E₂ in the presence or absence of 100-fold excess cold diethylstilbestrol (DES) at 4°C for 2 h and then at 25°C for further 2 h. The [³H]E₂ bound to macromolecules was determined by incubation with 35 µl of the charcoal-dextran suspension for 15 min. For the nuclear exchange assay, duplicate 0.45 ml aliquots of the suspension were incubated with 5 nM [³H]E₂ in the presence or absence of 100-fold excess cold DES at 4°C for 2 h followed by incubation at 35°C for 1 h. At the end of the incubation period, the pellet was washed three times with 2 ml each of TEM buffer and the [³H]E₂ in the final pellet was counted.

The concentrations of ER were expressed as fmol per pituitary gland.
Chemicals and reagents

[2,4,6,7-³H]E₂ (104 Ci/mmol) was purchased from New England Nuclear, Boston, MA. Unlabelled E₂ and DES were obtained from Sigma Chemical Co., St. Louis, MO. Clomiphene was provided by Shionogi Co., Osaka. Acid washed charcoal was obtained from Sigma Chemical Co. and dextran T 70 was a product of Pharmacia Fine Chemicals, Uppsala, Sweden.

Statistics

All values were expressed as mean ± SE. Student's unpaired t-test was used for the statistical analysis.

Results

Serum LH concentration in chronically ovariectomized rats was found to be 287 ± 65 ng/ml (Fig. 1), which is almost ten times higher than that obtained in normal female rats at dioestrus (30 ± 3 ng/ml). A single dose of 5 µg E₂ caused a significant decrease in LH level (123 ± 43 ng/ml)/h after the injection and the level remained low for 24 h.

Thereafter, the LH level increased gradually but did not return to the pre-administration value until 72 h after the injection (data not shown). These results are in agreement with those reported previously (Caligaris et al. 1971; Kalra et al. 1973).

Thus a single ip administration of 5 µg E₂ was found to be ineffective in induction of LH surge in chronically ovariectomized rats. Then, the effect of a single dose of 200 µg clomiphene on LH release was examined. As shown in Fig. 1, clomiphene suppressed serum LH level significantly 12 h after the injection, and the decreased level remained almost unchanged for 60 h.

In order to examine whether E₂ induce LH surge in ovariectomized rats pretreated with 200 µg clomiphene, a single dose of 5 µg E₂ was given after the clomiphene injection. When E₂ was given 12 or 24 h after clomiphene, no difference in serum LH concentrations was found during the experiment, compared with the values obtained in rats injected with clomiphene alone. In contrast, a highly significant increase in serum LH concentration occurred 24 h after the E₂ injection (pre-

Fig. 2.

Effect of a single injection of either E₂ or clomiphene on ER distribution in the pituitary of ovariectomized rats. Ovariectomized rats were injected ip with 5 µg E₂ in saline (A), or 200 µg clomiphene in sesame oil (B). Cytoplasmic (●—●) and nuclear (○—○) ER were measured by the exchange assay. Each point represents the mean ± SE of four to five determinations.
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The dose 24 h (A), or 48 h (B) after the clomiphene injection. Other explanations are the same as shown in Fig. 2. Differences from 0 h (P): *P < 0.05, **P < 0.01 (t-test).

injection: 102 ± 25 ng/ml vs post-injection: 494 ± 105 ng/ml, P < 0.05) if E2 was administered 48 h after clomiphene, indicating that E2 promotes release of LH under these conditions. This value was also significantly higher than the LH level obtained 72 h after a single dose of clomiphene (135 ± 35 ng/ml) (P < 0.05).

The dynamics of pituitary ER following a single dose of 5 µg E2 or 200 µg clomiphene is shown in Fig. 2. The administration of clomiphene (Fig. 2B) translocated cytoplasmic ER to the nuclear compartment to the similar degree as that observed with E2 (Fig. 2A). However, in contrast to E2, the highest level of nuclear ER appeared at 4 h (48 ± 6 fmol) remained almost unchanged for 72 h, and the depleted cytoplasmic ER at 4 h (6 ± 2 fmol) increased only slightly until 72 h. When E2 was given 24 h after clomiphene, no difference in ER levels was found in either fraction, compared with those induced by clomiphene alone (Fig. 3A). E2 administration 12 h after clomiphene also showed similar results. On the other hand, when E2 was administered 48 h after clomiphene (Fig. 3B), a significant reduction in nuclear ER occurred 24 h later (pre-injection: 42 ± 6 fmol vs post-injection: 14 ± 3 fmol, P < 0.01). This value was also significantly (P < 0.05) lower than that obtained 72 h after a single dose of clomiphene (38 ± 8 fmol) (Fig. 2B). Although the E2 injected 48 h after clomiphene in castrated rats resulted in an insufficient replenishment of cytoplasmic ER, the dynamics of nuclear ER was considerably similar to that induced by E2 alone in castrated animals (Fig. 2A), suggesting a replacement of clomiphene by E2 occurs on the nuclear receptor complex.

Fig. 3.
Effect of a single injection of E2 on ER distribution in the pituitary of ovariectomized rats pretreated with clomiphene. Ovariectomized rats were first injected ip with 200 µg clomiphene in sesame oil; a single ip injection of 5 µg E2 in saline was given 24 h (A), or 48 h (B) after the clomiphene injection. Other explanations are the same as shown in Fig. 2. Differences from 0 h (P): *P < 0.05, **P < 0.01 (t-test).

Discussion
The present study demonstrates that E2 induces a LH surge in long-term ovariectomized rats pretreated with clomiphene. The biological effects of E2 on LH surge occurred when nuclear ER in the pituitary decreased significantly. To our knowledge, the present study demonstrates for the first time that an anti-oestrogen, clomiphene, shows a facilitatory effect on E2-induced LH surge. It has
been well established that E₂ is the primary hormone involved in triggering the pre-ovulatory LH surge, but a single dose of E₂ failed to elicit LH release in long-term ovariec tomized rats. These findings of the present study agree with previous results indicating that E₂ injection stimulated LH release in intact or acutely ovariec tomized rats but not in long-term ovariec tomized animals. In these animals, pretreatment with oestrogen or progesterone was required to induce LH release (Caligaris et al. 1971; Mann & Barraclough 1973; McGinnis et al. 1981). On the other hand, an anti-oestrogen, nafoxidine, inhibited LH surges induced by silastic E₂ implants in the immature female rats and had no stimulatory effect on gonadotrophin secretion by itself (Attardi & Palumbo 1981). In cultured ovine pituitary cells, tamoxifen, CI-680 and nafoxidine showed only antagonistic effects on LH and FSH secretions (Miller & Huang 1981). These findings suggest that the non-steroidal anti-oestrogen possess only antagonistic effect on gonadotrophin secretion. However, Adashi et al. (1981) reported that clomiphene, unlike tamoxifen, sensitized the gonadotroph to GnRH action resulting in an enhancement of the release of LH by rat pituitary cells in vitro. The non-steroidal anti-oestrogens are the group of compounds which inhibits the effects of oestrogen but show various degrees of gonistic activity in the same tissue. Recently, it has been reported that enclomiphene, a cis-isomer, acts as an E₂ antagonist but zucloclomiphene, a trans-isomer, acts as an agonist, in terms of the LH response to GnRH in ovine pituitary cells in culture (Huang & Miller 1983).

In clomiphene-pretreated ovariec tomized rats, the administration of E₂ induced LH surge and nuclear ER reduction. These findings seem to suggest that clomiphene was substituted by E₂ on the nuclear hormone receptor complex in the pituitary. In anti-oestrogen-pretreated rat uterus, however, the administration of E₂ showed no significant biological effects (Clark et al. 1974; Katzenellenbogen & Ferguson 1975). In MCF-7 cells, on the other hand, the growth of human breast cancer cells was inhibited by nafoxidine but the inhibited growth could be restored by E₂. This restoration was characterized by nuclear ER reduction, namely, so-called processing (Horwitz et al. 1981).

Previous findings in rat uterus (Gardner et al. 1978) that pretreatment with nafoxidine blocked E₂-induced late uterine response but did not block early uterine response may explain the present findings that LH release was induced by E₂ in clomiphene pretreated rats. At present, the reason why LH release and nuclear ER reduction in the pituitary did not occur when E₂ was administered 12 or 24 h after clomiphene can not be explained. Since the levels of nuclear ER did not decrease significantly following E₂ injection given 12 or 24 h after clomiphene, the degree of association of clomiphene-ER complex with nuclear chromatin sites may be changed with time after clomiphene administration.

The results of the present study do not rule out the anti-oestrogenic effects of clomiphene on the hypothalamo-pituitary system. In our recent studies, clomiphene reduced the pituitary weight significantly in E₂-implanted rats (Tsutsumi et al. 1983). As previously mentioned, clinical studies suggest that clomiphene acts as an anti-oestrogen during follicular phase, and consequently blocks the negative feedback effect of ovarian oestrogen on the release of gonadotrophin and increases the levels of circulating gonadotrophin. The present findings seem to show that clomiphene also acts directly on the pituitary gland to augment ovarian E₂-induced LH release at the pre-ovulatory stage.

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References


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