Effects of clomiphene citrate on ovarian function in hypophysectomized rats

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Summary. On Days 28–30 of age, hypophysectomized rats were treated with oestradiol-17 β (0.1 mg/day) and/or clomiphene citrate (0.1 mg/day). Subsequent treatment with PMSG (10 i.u., on Day 31) and hCG (10 i.u., on Day 33) was identical for all animals. Rats were killed on Day 34. Treatment with oestradiol-17 β alone resulted in ovulations of 45.1 ± 5.5 oocytes/rat (mean $\pm$ s.e.m.). There were no ovulations among animals treated with clomiphene citrate alone but treatment with oestradiol-17 β and clomiphene citrate resulted in a significant ($P < 0.05$) reduction (23.1 ± 7.6 oocytes/rat) in ovulatory response. Similarly, ovarian weights and serum progesterone concentrations were highest in the oestradiol-17 β -treated rats, intermediate in those given oestradiol plus clomiphene citrate and the lowest in rats receiving clomiphene citrate alone. We suggest that clomiphene citrate exerts direct ovarian antiovarulatory and oestrogen-antagonist actions.

Keywords: clomiphene citrate; ovulation; rat; hypophysectomy

Introduction

Clomiphene citrate is a triphenylethylene derivative commonly used in ovulation induction in women (Huppert, 1979; Adashi, 1986). It has been shown to interact with oestrogen-dependent/-responsive tissues including the hypothalamic–pituitary unit, ovary, endometrium and vaginal mucosa. Oestrogen-agonist and -antagonist activities of clomiphene citrate have been reported, depending on the species, the target tissue and the length of exposure (Clark & Markaverich, 1982).

Induction of ovulation by clomiphene citrate is thought to be due to its effects at the hypothalamo–pituitary level by removal of negative feedback of endogenous oestrogens and the consequent increased release of gonadotrophins (Roy *et al.*, 1963; Igarashi *et al.*, 1967; Wu, 1977). It has been proposed that, in addition to the hypothalamo–pituitary effects, direct action(s) of clomiphene citrate at the ovarian level may also affect ovulation (Adashi, 1984).

The effects of clomiphene citrate on the ovary, however, remain poorly understood. Clomiphene citrate can exert inhibitory and stimulatory actions on a variety of ovarian functions, including alteration of steroidogenesis (Laufer *et al.*, 1982; Welsh *et al.*, 1984; Westfahl & Resko, 1983; Sgarlata *et al.*, 1984; Zhuang *et al.*, 1982) and promotion of degeneration of oocytes (Laufer *et al.*, 1983; Yoshimura *et al.*, 1985; Schmidt *et al.*, 1985).

The present study was designed to determine whether, and if so, how, ovarian functions are affected by direct action(s) of clomiphene citrate. The effects of clomiphene citrate on (i) the number of ovulated oocytes, (ii) ovarian weights, and (iii) serum steroid concentrations in hypophysectomized immature rats were therefore investigated.

Materials and Methods

Immature (24-day old) Sprague–Dawley rats were obtained from Charles River (St Constant, Quebec, Canada) after hypophysectomy at 21 days of age. The animals were housed in a temperature-controlled environment (21°C) with

lights on at 07:00 h and off at 19:00 h. Daily subcutaneous injections with oestradiol-17 β (0.1 mg/0.4 ml sesame oil) and/or clomiphene citrate (0.1 mg/0.4 ml sesame oil) were given at 09:00 h for 3 days starting at the age of 28 days. The rats received oestradiol-17 β alone (Group 1, N = 8), clomiphene citrate alone (Group 2, N = 7) or oestradiol-17 β + clomiphene citrate (Group 3, N = 7). On the 31st day of age, all rats received a single subcutaneous dose of PMSG (10 i.u./0.4 ml 0.154 M-NaCl), followed on the 33rd day of age by a single dose of hCG (10 i.u./0.4 ml 0.154 M-NaCl). All animals were killed on the morning of Day 34, approximately 24–26 h after the dose of hCG.

Progesterone, oestradiol-17 β , clomiphene citrate, and testosterone were purchased from Sigma Chemical Company (St Louis, MO, USA). PMSG and hCG were purchased from Ayerst, McKenna and Harrison Incorporated (Vancouver, British Columbia, Canada). [1,2,6,7,16,17- ^3H]Progesterone (sp. act. 112 Ci/mmol), [2,4,6,7,16,17- ^3H]oestradiol-17 β (sp. act. 140 Ci/mmol) and [2,6,7- ^3H]testosterone (sp. act. 80 Ci/mmol) were obtained from Amersham Company (Arlington Heights, IL, USA). Solvents were of analytical grade and were used without further purification, except for ethanol, which was redistilled.

Animals were killed by cervical dislocation. Trunk blood was collected and serum was separated. Ovaries were dissected and weighed as a pair. Ovulation was determined by counting oocytes flushed out from oviducts as described previously (Yun *et al.*, 1987). Oocytes were counted under a dissecting microscope ($\times 40$ magnification).

The concentrations of progesterone, oestradiol-17 β and androgens in serum were measured by radioimmunoassay after extraction of serum twice with 5 volumes of diethyl ether. Specific antisera were kindly donated by Dr David T. Armstrong from the University of Western Ontario (London, Ontario). The cross-reactivity of the progesterone antiserum was progesterone, 100%; 5 β -pregnane-3,20-dione, 35.5%; 5 α -pregnane-3,20-dione, 15.7%; 3 α -hydroxy-5 β -pregnan-20-one, 2.0%; 20 β -hydroxy-4-pregnen-3-one, 1.3%; 17-hydroxyprogesterone, 1.2%; other major steroids known to be secreted by the follicle, less than 0.2%. The cross-reactivity of the oestradiol-17 β antiserum was: oestradiol-17 β , 100%; oestrone, 2.9%; oestriol, 0.5%; other major steroids known to be secreted by the follicle, <0.2%. The cross-reactivity of the testosterone antiserum was: testosterone, 100%; 5 α -dihydrotestosterone, 75%; 5 α -androstane-3 α ,17 β -diol, 13.5%; 5 α -androstane-3 β ,17 β -diol, 10.9%; 19-hydroxytestosterone, 4.7%; other major steroids known to be secreted by the follicle, <1%. Intra-assay and inter-assay coefficients of variation were less than 10% and 15%, respectively. The cross-reactivity with other androgens was substantial and therefore steroids measured using this antiserum are referred to as androgens rather than testosterone.

Experimental data were evaluated statistically by analysis of variance followed by Scheffé's F-test. Comparisons at $P < 0.05$ were considered significant.

Results

Ovulation occurred in all 8 rats in Group 1, none in Group 2 and 5 of 7 rats in Group 3 (Fig. 1a).

The average weight of a pair of ovaries for rats in Group 1 was greater than the corresponding value for Group 2 rats and ovarian weights in Group 3 were intermediate (Fig. 1b).

Significant effects were observed for only progesterone with its highest levels observed in Group 1 and the lowest levels in Group 2 (Fig. 2).

Fig. 1. Effect of oestradiol-17 β (0.1 mg/day) and/or clomiphene citrate (0.1 mg/day) administered on Days 28–30 of age on (a) total number of released oocytes and (b) ovarian weight. Group 1, 8 rats oestradiol-17 β alone; Group 2, 7 rats, clomiphene citrate alone; Group 3, 7 rats, oestradiol-17 β + clomiphene citrate. Values are means $\pm$ s.e.m. The means with no superscripts in common are significantly different ($P < 0.05$).

Fig. 2. Effect of oestradiol-17 β (0.1 mg/day) and/or clomiphene citrate (0.1 mg/day) administered on Days 28–30 of age on serum concentrations of progesterone, androgens and oestradiol-17 β . Group 1, 8 rats, oestradiol-17 β alone; Group 2, 7 rats, clomiphene citrate alone; Group 3, 7 rats, oestradiol-17 β + clomiphene citrate. Values are means $\pm$ s.e.m. The means with no superscripts in common are significantly different ($P < 0.05$).

Discussion

In the present study we have induced ovulation in immature hypophysectomized rats by a sequential treatment with oestradiol-17 β , PMSG and hCG. In such animals, the effects of sex steroids and their agonists and/or antagonists are presumed to be due to direct ovarian effects rather than those exerted via the hypothalamo–pituitary axis.

The administration of clomiphene citrate resulted in inhibition of the ovulatory response with an associated decrease of ovarian weight and serum progesterone concentrations. It appears that the early phase of follicular development ensuring gonadotrophin-induced ovulation is oestrogen-dependent, and that administration of clomiphene citrate has detrimental effect(s) on this process. Oestrogens have been shown to stimulate ovarian function directly and to promote follicular growth (Pencharz, 1940; Williams, 1940; Richards *et al.*, 1976) and to prevent follicular atresia (Reiter *et al.*, 1972; Louvet *et al.*, 1975). The antiovarian and ovarian weight-limiting actions of clomiphene citrate observed in the present study may therefore be viewed as oestrogen-antagonistic. Indeed, while clomiphene citrate has been described as a mixed oestrogen agonist/antagonist, most of its documented actions are of an oestrogen-antagonistic nature (Adashi, 1984). It is believed that clomiphene citrate binds to oestrogen receptors and translocates these receptors to the nucleus. Clomiphene citrate–oestrogen receptor complexes appear to remain in the nucleus

for a prolonged period of time with a resultant long-term depletion of the cytoplasmic oestrogen receptor pool (Sutherland & Murphy, 1982; Jordan, 1984; Clark *et al.*, 1985). Consequently, it is postulated that oestrogen-agonistic effects of clomiphene citrate are observed within the first day of its administration, while oestrogen-antagonistic effects are apparent only later.

The results of the present study, in which clomiphene citrate was administered over 3 days and its effects were observed even later, seem representative for the proposed delayed antioestrogenic action.

Previous studies with women and monkeys have also indicated that, while clomiphene citrate induced ovulation via the hypothalamic–pituitary axis, it also exerted inhibitory effects on ovarian follicular maturation and steroidogenesis (Marut & Hodgen, 1982; Dlugi *et al.*, 1985). Those observations, however, were carried out in the presence of an intact hypothalamus and pituitary and consequently any interferences regarding direct ovarian effects of clomiphene citrate were hypothetical.

Our observation of decreased serum progesterone concentrations in clomiphene citrate-treated animals is probably a secondary effect of a decreased number of ovulations and thus decreased number/mass of corpus luteum tissue. However, an additional direct inhibitory effect of clomiphene citrate on progesterone production may also be contributory. Such an effect has been observed previously in rat (Welsh *et al.*, 1984) and human (Ho Yuen *et al.*, 1988) granulosa cells.

In the present experiment we did not include animals treated with PMSG and hCG without clomiphene citrate and/or oestradiol. Our experience indicated that such animals ovulated erratically or not at all. Consequently, the most meaningful effects of clomiphene citrate observed in the present study represent its interactions with oestradiol-17 β .

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