THE EFFECTS OF CLOMIPHENE CITRATE ON OVARIAN FUNCTION IN RATS

By

TIAN BIN FENG

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Department of ORS / Gyn

The University of British Columbia
Vancouver, Canada

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ABSTRACT

In the present study, the effects of clomiphene citrate (CC) on ovulation, ovarian growth and ovarian steroidogenesis were examined. Ovulation in rats in response to PMSG was completely blocked by administration of three daily treatments of 1.0 mg CC/rat, but was restored by administration of hCG as a preovulatory LH surge substitute. When the number of treatment days was reduced to two days, 1.0 mg of CC enhanced ovulation in response to PMSG, whereas treatment for one day with the same dose of CC did not affect ovulation. The effects of CC on ovulation appear to be dose-dependent.

The effects of CC on ovarian growth were similar to the effects of CC on ovulation. The ovarian growth induced by PMSG was inhibited by high doses of CC, while a lower dose had no effect. The inhibition of ovarian growth in terms of ovarian weight by a high dose of CC was restored by hCG given as a preovulatory LH surge. Treatment duration with CC appears to have an important influence on ovarian growth. Three daily treatments with high doses of CC significantly inhibited ovarian growth. However, when the number of treatment days was reduced from three to two, the opposite results were obtained in that CC significantly stimulated ovarian growth.

The effects of CC on ovarian steroidogenesis in response to PMSG were dose-dependent. A higher dose of CC significantly stimulated estradiol-17β biosynthesis. Clomiphene citrate did not show any inhibitory effects on progesterone production. Progesterone production was stimulated by hCG in CC

treated rats. Lower doses of CC stimulated progesterone and androgen production. Further studies on this are necessary.

Histological examination of the ovary revealed that CC selectively inhibited the development of nondominant follicles. The dominant follicles were unaffected as for they were able to develop to the mature stage.

These results suggest that the effects of CC on ovulation, ovarian growth and ovarian steroidogenesis are dose-dependent and affected by treatment duration. Clomiphene citrate is assumed to exert its action via a gonadotropic mechanism.

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LIST OF ABBREVIATION

CC	-clomiphene citrate
cpm	-counts per minute (radioactive)
DES	-diethylstilbestrol
DPBS	-Dulbecco's phosphate-buffered saline
FSH	-follicle stimulating hormone
GnRH	-gonadotropin releasing hormone
^{3}H	-tritium, a radioactive isotope of hydrogen
hCG	-human chorionic gonadotropin
hMG	-human menopausal gonadotropin
IU	-international unit
IVF	-in vitro fertilization
LH	-luteinizing hormone
MBH	-median basal hypothalamus
MER-25	-thamoxytriphetol
NSB	-nonspecific binding
PMSG	-pregnant mare's serum gonadotropin
RIA	-radioimmunoassay
rpm	-revolutions per minute
SEM	-standard error of means
TACE	-chlorotrianisene

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INTRODUCTION

I. Objective

The clinical use of clomiphene citrate (CC) was first reported by Greenblatt et al. (1961) who induced ovulation in patients with secondary amenorrhea. Since then, CC has been widely used and has been demonstrated to be the most effective oral agent to induce ovulation even though the mode of its action upon the human reproductive system is still not completely known. Most of the data indicates that CC acts as an antagonist and binds to oestrogen receptors of the hypothalamus and pituitary gland thus preventing the negative feedback of endogenous oestrogen. This results in an exaggerated secretion of gonadotropin releasing hormone (GnRH) and release of FSH and LH (Kistner, 1966; Schultz et al., 1973). Clomiphene citrate has also been shown to sensitize pituitary gonadotrophs to GnRH action both in vivo and in vitro, causing an exaggerated secretion of FSH and LH in response to GnRH (Schultz et al., 1973; Hsueh et al., 1978).

The effects of CC on the reproductive system display species-dependent and dose-dependent differences. The results from different investigations are inconsistent and this complicates the understanding of the mode of CC action. The purpose of this study was to study the effects of CC on ovulation, ovarian function in immature rats induced to ovulate with PMSG, and the mechanism(s) and site(s) at which CC exerts its action on the reproductive system.

II. Historical Background

In 1937, Robson and Schonberg (1937) reported that triphenylethylene and triphenychloroethylene acted as oestrogen agonists of low potency but of long duration of action. These observations received little attention until 1953 when Shelton et al. (1953) demonstrated that the biologic potency of the oestrogen agonist could be augmented by alkoxy substitution. Six years later, Allen (1959) obtained a patent for CC, a triphenylethlene derivative substituted with a chloride anion and an aminoalkoxyl.

Clomiphene citrate and other closely related compounds, such as MER-25 and TACE, were originally intended for use as anti-fertility agents because of their close chemical resemblance to oestrogen. Steroid oestrogens are well-known for their capacity to block fertility, therefore, chemically similar compounds should also have this capacity. In 1960, Holtkamp (1960) reported that CC and its closely-related analogue thamoxytriphetol (MER-25) inhibited gonadotropin secretion in rats and could be considered as likely candidates for an agent which would block fertility. However, contrary to this expectation, when they were first used in women as infertility agents, the opposite effect was observed, instead CC and Mer-25 successfully induced ovulation (Tyler et al., 1962; Kistner and Smith, 1961). Later, Greenblatt et al. (1961) were the first to report the successful induction of ovulation and pregnancy following CC therapy. MER-25 was subsequently, removed from further clinical trials because of its toxicity. Since then, CC has become the major drug used for the induction of ovulation.

In 1979, Trounson (1981; 1982) initiated the use of CC to stimulate ovulation in normal cycling women in in vitro fertilization (IVF) programs. Since then, CC and hMG have been generally accepted as the major pharmacologic agent used to stimulate multiple follicular growth in IVF patients. Other pharmacologic agents used to induce ovulation have been developed, but CC has proven to be the most useful oral agent in helping more infertile women to conceive than any other mode of infertility therapy.

III. Chemistry and Pharmacology

Clomiphene citrate is a triarylethylene compound (1-P-β-diethylaminoethoxyphenil-1, 2-diphehyl-2-chloroethylene citrate) chemically related to chlorotrianisene (TACE), a weak oestrogen. Structurally, CC is related to the potent synthetic oestrogen, diethylstilbestrol. Clomiphene citrate may exist in either the cis- or the trans- configuration; the former being significantly more potent. Such variations on this theme occur in the ovary's synthesis of oestrogens: estradiol-17β is the most potent of the natural oestrogens while the 17α isomer is barely oestrogenic. Commercially available preparations of CC usually combine the two isomers in a 1:1 ratio. At recommended doses, CC does not display significant oestrogenic activity in humans (Wm. S. Merrell Company, 1967).

The metabolism of CC is not well understood. The studies of the metabolism of tamoxifen, which is structurally closely related to CC,reveal that tamoxifen acts in a similar manner in both animals and in humans. The major

metabolite has been identify as 4-hydroxytamoxifen (Blankstein, 1986). Jordan et al. (1977) suggested that 4-hydroxytamoxifen is a potent antiestrogen which contributes to the antiestrogenic effect observed in patients receiving tamoxifen treatment. However, recent experiments indicate that the major metabolite of tamoxifen in the serum of female patients receiving tamoxifen treatment is desmethyltamoxifen instead of 4-hydroxytamoxifen (Blankstein, 1986).

Tracer studies of CC with radioactive carbon labelling have shown that the main route of excretion is through the faeces, although small amounts are also excreted in urine. There is good evidence that CC is concentrated in the bile and carried out into the gut. Reabsorption from the gut causes CC to be partially sequestered to enteroheptic circulation from where it slowly leaks out. On the average, half of the administered radioactivity in a tracer dose of CC is excreted within five days (MacGillivary, 1968). Individual variation occurs, and Kistner (1968) reported that in one subject, 94% of orally administered radiolabelled CC was eliminated in this time period. Swyer (1965) detected CC or its metabolites in faeces six weeks after drug administration had ceased. Mikkelson et al. (1986) demonstrated, with a reversed high pressure liquid chromatography (HPLC) assay with fluorescence detection, that each CC isomer has its own distinct pharmacokinetic behaviour in addition to differing pharmacologic activity. The trans- isomer is absorbed faster and eliminated more completely than the cis- isomer and plasma concentrations of the cis- isomer are detectable for longer than one month. Because of the unexpected long half life of the CC, the cis- isomer can accumulate in the plasma of the patients and it is

suggested that this accumulation of the cis- isomer probably contribute to the ultimate pharmacologic effect exhibited over time after chronic treatment.

In rhesus monkeys, intravenously-administered ^{14}C-labelled CC, at a dose of 200 mg, revealed that the major route of excretion was the gastrointestinal tract (84.4%) (Persson, 1965). The biologic total body half-life was estimated at 48 hours (Borgstrom, 1981, Adam et al., 1979). The liver, gallbladder, and bile had the highest concentrations of ^{14}C remaining in the animals followed by the adrenal gland, eye, colon and pancreas. Low levels of ^{14}C were also observed in the pituitary, whereas the ovaries had levels close to the median value of tissues examined (Adashi, 1986).

Regardless of the exact mechanism(s) of its oestrogen-like action, there is little doubt that CC is capable of interaction with oestrogen receptor-binding proteins in a manner generally similar to that of native oestrogens (Clark et al., 1982). Several lines of evidence would seem to suggest, however, that the nature of the interaction of CC with oestrogen receptor-binding proteins may nevertheless display qualitative differences to that of the naturally occurring ligand (Clark et al., 1976). Most importantly, CC, as well as a series of related triphenylethylene derivatives, is best characterized by the tendency to display prolonged nuclear receptor occupancy (Clark, 1974; 1976; Adashi, 1980). In fact, CC has been shown to occupy nuclear receptor proteins for several weeks, a phenomenon sharply contrasting with the rather brief interaction of native oestrogens which are known to clear the cell within 24 hours (Clark et al., 1974). The relevance of these observations to the ability of CC to affect ovulation, to its

possible progressive "nuclear" accumulation upon repetitive administration, and to its possible lingering blastocytotoxic or teratogenic effect(s) remains uncertain (Blankstein et al., 1986; Adashi, 1984).

IV. The Mechanism(s) and Site(s) of Action

It has now been over thirty years since the synthesis of CC (Allen et al., 1959; Palopoli et al., 1965), however, despite the intensive basic and clinical investigations, the mechanism(s) and site(s) of action of CC on ovulation induction remain largely unknown. It is thought that the ability of CC to induce ovulation may be primarily due to its ability to be recognized by and to interact with oestrogen receptors in the hypothalamus. By doing this, CC displaces endogenous oestrogens thereby alleviating the putative negative feedback effect exerted by endogenous oestrogens, and therefore causes an exaggerated secretion of GnRH, FSH and LH. This stimulates follicular recruitment, selection, assertion of dominance and ultimately follicular rupture (Sasson et al., 1982; Vanderberg et al., 1973; Adashi, 1984). According to this description, the hypothalamus is the primary site of CC action. As the mechanism which controls normal ovulation involves many feedback loops of the entire hypothalamus-pituitary-ovarian axis, the pituitary and ovary may also be actively involved in the process (Sasson et al., 1982). Clomiphene citrate has been proposed to affect the whole axis to induce ovulation (Adashi, 1984).

A. Action at the Level of Hypothalamus

Evidence supports the hypothesis that the hypothalamus is one of the sites at which CC exerts its action. It has been documented that the hypothalamus possesses oestrogen receptors in various species (Stumpf, 1968; Pfaff et al., 1976). Moreover, studies in rodents have demonstrated the ability of CC to bind to hypothalamic oestrogen receptors and to affect their translocation to the nucleus (Eisenfeld et al., 1967; Kahwanago et al., 1970; Maurer, 1971; Morries, 1976). Igarashi et al. (1967) showed that implantation of CC directly into the anterior hypothalamus or median eminence caused increased gonadotropin levels in the circulation of rats. Docke (1971) demonstrated that CC caused ovulation when implanted in the hypothalamus in rats or by systemic administration; CC has been shown to increase the hypothalamic content of bioactive GnRH, and gonadotropin release. In women, Masala (1978) and Miyake (1980) reported an increase in the peripheral circulating levels of immunoreactive GnRH during CC therapy. The most convincing evidence in support of a hypothalamic site of action of CC was shown by Miyake et al. (1983). In this study, the releasing effects of CC on GnRH and LH were examined in a sequential double-chamber perfusion system using medial basal hypothalamus (MBH) and/or pituitary excised from normal female rats at dioestrus. When the MBH and pituitary were perfused with CC plus oestradiol in sequence, a significant increase in GnRH release was observed. The authors suggested the mechanism of action of CC to be as follows: CC competes with oestradiol for binding sites and therefore causes the release of GnRH from the hypothalamus and subsequent LH release

from the pituitary.

There is little doubt that the hypothalamus is one site at which CC exerts its action to induce ovulation. However, whether CC interacts at the level of the hypothalamus in its capacity as an oestrogen or antiestrogen remains uncertain.

B. Action at the Level of Pituitary

In addition to the effects of CC at the hypothalamic level, the pituitary also appears to be a primary target for this compound. The pituitary has been shown to be more sensitive to CC than the hypothalamus (Kato et al., 1968; Kurl and Morries, 1978; Adashi et al., 1980). Clomiphene citrate was able to induce corpus luteum formation in rats rendered anovulatory by electrolytic lesioning of the medial preoptic-suprachiasmatic regions of the hypothalamus, by postnatal androgenization or continuous light exposure (Docke, 1969). Treatment of pituitary cells from adult ovariectomized female rats with oestradiol or CC results in 3-fold and 3.3-fold increases in pituitary sensitivity to GnRH, respectively. These findings suggest that oestradiol, as well as CC, is able to enhance the GnRH-stimulated release of FSH from the pituitary. Similar observations were made with respect to LH (Adashi, 1981). Clomiphene citrate, like oestradiol-17β, increases the responsiveness of pituitary gonadotrophs to GnRH in vitro (Hsueh, 1978). These observations support the theory of the direct effect of CC on the pituitary with CC acting as an oestrogen rather than an antiestrogen.

Using a sequential double-chamber perfusion system involving the MBH and/or pituitary of normal female rats at dioestrus, Miyake et al. (1983) also

addressed the possibility of a direct pituitary effect of CC. Treatment with CC alone resulted in an increase in pituitary LH release, but this increase was not accompanied by a concomitant increment in hypothalamic GnRH release. These findings give strong support to the possibility that CC, by itself, may exert a direct stimulating effect on pituitary LH release without apparent involvement of the hypothalamus.

C. Action at the Level of Ovary

The administration of CC is followed by an increase in oestrogen output early in the course of therapy that can not be correlated with any preceding or accompanying increase in urinary gonadotropins. The ovary was therefore suspected to be the primary site of action of CC (Smith, 1962). After over thirty years of clinical and basic studies, there is little doubt that CC can and probably does exert a direct effect on ovarian function.

It appears that both ovarian folliculogenesis and steroidogenesis are affected by CC. Clomiphene citrate was found to inhibit an increase in the ovarian weight in response to hCG and FSH in hypophysectomized immature female rats (Harman et al., 1974). Using normally cycling rhesus and cynomolgus monkeys, CC was shown to attenuate ovarian folliculogenesis (Marut and Hodgen, 1982). Laufer et al. (1982) examined the direct effect of CC on follicular function in rats and showed that CC inhibited both basal and LH-stimulated steroid accumulation. Follicles incubated for 24 hours with CC exhibited dose-dependent atretic-like changes. More recently, using the isolated perfused rabbit ovary, the direct

ovarian effect of CC was examined. Clomiphene citrate increased the rate of follicular degeneration which was reversed by oestradiol treatment, suggesting CC directly affects the ovary (Yoshimura et al., 1985). In wild rats, CC inhibited granulosa cell and thecal cell mitosis and increased follicular atresia. Clomiphene citrate may induce partial inhibition of ovulation through its action on follicular growth and atresia (Sahu, 1987). These observations provide strong evidence in support of a direct inhibitory effect of CC at the level of the ovary.

The effects of CC on ovarian steroidogenesis are controversial. Both inhibiting and stimulating effects have been reported. Laufer et al. (1982) reported an inhibition of oestrogen production by CC in vitro in rat preovulatory follicles. In contrast, Zhuang et al. (1982) and Welsh et al. (1984) reported that CC pretreatment stimulated oestrogen production by rat granulosa cells in vitro. Because CC is usually given to oestrogen-primed subjects in vivo, Zhuang et al. (1982) examined the effects of concomitant treatment with CC and oestradiol on FSH stimulated granulosa cell aromatase activity. The results indicated that CC may act independently of the hypothalamus and pituitary to enhance the stimulation of ovarian oestrogen production by FSH and LH. Clomiphene citrate, like diethylstilbestrol (DES), enhances aromatase activity and is unable to antagonize the augmenting effect of DES on the FSH-stimulated aromatase activity. This suggests that CC exerts a direct oestrogenic rather than antiestrogenic effect on aromatase induction. A conclusion was also reached by Engels, et al. (1968) showing an increase in oestrogen production by canine ovaries perfused with CC. In an in vivo study using immature rats treated with

PMSG to induce ovulation as the model, CC significantly stimulated the oestrogen production (Feng, et al., 1989). There are also conflicting reports concerning the effects of CC treatment on oestrogen production in vivo. Marut and Hodgen (1982) reported that plasma oestrogens decreased during high dose CC treatment in normally cycling monkeys despite concurrent elevations of gonadotropins. Decreased oestrogen concentrations in follicular fluid have also been reported in women treated with CC as compared to women treated with human menopausal gonadotropin (hMG) (diZerega et al., 1983; Dlugi et al., 1985). However, other reports have described an increase in oestrogen values during CC treatment in monkeys (Littman and Hodgen, 1985), in women (Wu, 1977; Maxson et al., 1984), in hypophysectomized rats (Moon et al., 1989), and in immature rats (Feng et al., 1989).

Inhibitory effects of CC on in vitro progesterone biosynthesis have been reported for rat granulosa cells (Welsh et al., 1984), monkey luteal cells (Westfhal and Resko, 1983), rat preovulatory follicles (Laufer, 1982), and human granulosa cells (Ho Yuen et al., 1988). In addition, CC has been found to inhibit progesterone biosynthesis by affecting cholesterol side-chain cleavage enzyme, but not the 3β-steroid dehydrogenase enzyme activity in hen granulosa cells (Sgarlata et al., 1984).

The effects of CC on androgen synthesis have not been reported yet. Although the previous studies suggest a direct effect of CC on ovarian function, the exact nature of the interaction of CC at the level of ovary needs to be further investigated.

V. The Detrimental Effects of CC

Clinical studies have demonstrated that CC induces ovulation in 80% of anovulatory women, but results in only a 40% pregnancy rate (Speroff et al., 1983; Seegar-Jones et al., 1970). The discrepancy between the rates of ovulation and pregnancy has led investigators to examine the negative effects of CC more intensively. One of the negative effects of CC is alteration of ovarian steroidogenesis. Clomiphene citrate inhibits progesterone synthesis in rat follicles (Laufer et al., 1982), rat granulosa cells (Westfahl and Resko, 1983) and hen granulosa cells (Sgarlata et al., 1984). Both inhibitory (Laufer et al., 1982) and stimulatory effects (Zhuang et al., 1982; Welsh et al., 1984; Feng et al., 1989; Moon et al., 1989) on oestrogen biosynthesis in vivo and in vitro have been reported.

Using the isolated perfused rabbit ovary, the effects of CC on ovum maturation was studied (Yoshimura et al., 1985). Results showed that CC causes a high percentage of degenerating follicular oocytes. Its action was reversed by oestradiol treatment, suggesting an antiestrogenic action of CC.

At the gamete level, CC was shown to exert a direct degenerating effect on post ovulatory rat and rabbit ova and blastocysts when administered after mating or at the time of ovum passage through the oviducts (Chang, 1964; Prasad 1965; Davidson et al., 1965; Andrade et al., 1972). Clomiphene citrate also caused a dose-dependent decrease in the rate of ovulation, embryo development and blastocyst formation (Laufer et al., 1983). Additionally, high doses of CC have

been reported to exert a direct toxic effect on developing embryos when administered during the preimplantation stage of pregnant rats and rabbits (Prasad et al., 1965; Davidson, 1965).

The detrimental effects of CC are species-dependent. In humans, there is little doubt that CC stimulates or induces ovulation, but it blocks the ovulation in response to PMSG in immature rats (Feng et al., 1989).

The estimated half-life of CC is 5 days in humans (Schreiber et al., 1966) and 24 hours in rats (Clark et al., 1982; Fromson et al., 1973). Clomiphene citrate is accumulated in tissues with high levels of oestrogen receptors. Therefore, pretreatment with CC may exert a long-term effect on fertilization and embryo development in early pregnancy. At present, it is not clear whether the discrepancy between the rates of ovulation and pregnancy is due to a direct cytotoxic effect on oocytes/embryos or whether it is the result of an unsuitable uterine environment related to the prevailing ovarian dysfunction.

VI. Immature Rats Treated with PMSG

In the present study, immature female rats were treated with PMSG to induce ovulation. This model has been studied extensively and is now widely used. Pregnant mare serum gonadotropin (PMSG) is a glycoprotein, possessing both FSH- and LH-like activities (Passerson, 1976). The FSH:LH activity ratio has been estimated to be at 0.87-1.92 (Allen and Stewart, 1978). Like other pituitary glycoprotein hormones, PMSG consists of a hormonally non-specific subunit (PMSG-α) and a hormone specific subunit (PMSG-β) which is similar in amino

acid composition to LH-β (Papkoff, 1978). The high sialic acid content (45%) accounts for the long half life (60 hours) and the slow clearance rate of PMSG and may be responsible for the potency of a single dose (Schamma et al., 1978; Papkoff, 1981).

Results of several studies using immature rats indicate that low doses (4-8 IU) of PMSG induce a synchronized ovulation within 72 hours by eliciting an endogenous LH surge between 54 and 57 hours after injection of the gonadotropin (Sorrentino et al., 1972; Kostyk et al., 1975). The patterns of circulating steroid hormones (oestradiol-17β and progesterone), LH during the 24 hours preceding ovulation (Meyer et al., 1971; Wilson et al., 1974, Nuti, et al., 1975) and the temporal relationship between LH levels and oocyte maturation (Hillensjo et al., 1974) in this regimen are comparable to those observed on the days of proestrus and oestrous in normal cycling adult rats (Barraclough et al., 1971; Linkie and Niswender, 1972). The PMSG-treated immature rat is a model well suited for studying the effects of CC on ovulation and ovarian functions.

MATERIALS AND METHODS

I. Animals and Materials

Immature female Sprague Dawley rats were purchased from Charles River Canada Inc. (St. Constant, Quebec) at 21 days of age and housed under 12 hours light/12 hours dark cycle at 20-25^{o}C. Standard rat chow and water were available ad libitum throughout the experiments.

Progesterone, oestradiol-17β, CC, testosterone and hyaluronidase (ovine type II) were purchased from Sigma Chemical Company (St. Louis, MO, USA). Pregnant mare serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) were purchased from Ayerst, Mckenna and Harrison Incorporated (Vancouver, BC, Canada). [1, 2, 6, 7, 16, 17-^{3}H] progesterone (sp. act. 112 Ci/mmol), [2, 4, 6, 7, 16, 17-^{3}H] oestradiol-17β (sp. act. 140Ci/mmol) and [2, 6, 7-^{3}H] testosterone (sp. act. 80 Ci/mmol) were purchased from Amersham Company (Arlington Heights, IL, USA). Haematoxylin (C.I. 75290) and eosin (C.I. 45380) were purchased from BDH Chemicals Ltd. (St. Louis, MO, USA). Solvents were of analytical grade and were used without further purification, except for ethanol, which was redistilled.

II. Treatment Schedules

All treatments were administered at 9:00 a.m. Control animals in all experiments received sesame oil vehicle only.

The treatment schedule is summarized below:

Treatment Schedule

Age (days)	25	26	27	28	29	30	31	32	33
Experiments									
Experiment I									
Control (N=11)	V	V	V	PMSG		S	X		
CC (N=13)	CC	CC	CC	PMSG		S	X		
CC + hCG (N=7)	CC	CC	CC	PMSG		hCG	X		
Experiment II									
Control (N=6)	V	V	V	PMSG			X		
CC (N=15)	CC	CC	CC	PMSG		X(5)	X(5)	X(5)	
Experiment III									
Control (N=7)	V	V	V	PMSG			X		
CC 0.05 mg	CC	CC	CC	PMSG			X		
0.1 mg	CC	CC	CC	PMSG			X		
1.0 mg	CC	CC	CC	PMSG			X		
Experiment IV									
Control (N=19)		V	V	PMSG			X		
1-day CC (N=16)		V	CC	PMSG			X		
2-day CC (N=16)		CC	CC	PMSG			X		

X represents sacrifice; S represents saline; V represents vehicle.

(a) Experiment I. A total of 31 animals were divided into three groups. Begining at the age of 25 days, three daily injections of 1.0 mg of CC/rat were given to animals in CC Group (N=13), and HCG Group (N=7). Fifty-four hours after PMSG injections 10 IU hCG/rat was given to animals in HCG Group. Animals in the other two groups received saline as vehicle.

(b) Experiment II. A total of 15 rats treated with three daily injections of 1.0 mg of CC/rat were killed at 72, 96, and 120 hours after PMSG injection.

(c) Experiment III. Three groups of seven animals, Group I-III, were given daily injections of 1.0 mg, 0.1 mg and 0.05 mg of CC/rat respectively.

(d) Experiments IV. Starting at the age of 26 days, one or two daily injections of 1.0 mg of CC/rat were given to 16 rats in each group.

III. Sample Collection

Trunk blood of 3-4 mls was collected from inferior vena cava from the animals in Experiments I-III. The blood was allowed to stand at room temperature for 0.5-1 hour to clot and then centrifuged at 2,000 rpm for 10 min to separate serum. Sera of 1 ml were stored at -20^{o}C for subsequent assays of oestradiol-17β, progesterone and androgens.

Ovaries were dissected free from oviducts, cleaned of bursae, connective tissue and fat and weighed.

IV. Assessment of Ovulation

Ovulation was determined by counting oocytes flushed out from the oviducts

as described previously (Yun et al., 1987).

Oviduct and uterine horns were separated at the uterotubal junction. The oviducts obtained were placed in a few drops of DPBS in a 10 X 35 mm petri dish (Falcon Plastics, Los Angeles, CA). Under a stereo dissecting microscope (10 X magnification, Nikon SMZ-10), the oocyte mass was readily accessible in the enlarged and translucent ampulla region of the oviduct. This distended segment was then punctured and oocytes were expelled. In order to facilitate the oocyte counting, the extracoronal cumulus cells surrounding the oocytes were dispersed by being exposed to a few drops of DPBS containing 0.1% of hyaluronidase for 5-10 min.

The recovered oocytes were counted under the stereo dissecting microscope (40 X magnification). Fragmentation and other degenerative changes were assessed as described elsewhere (Elsden et al., 1978; Miller and Armstrong, 1981).

V. Histological Examination

Ovaries obtained at 72 hours after PMSG injections from the three doses of 1.0 mg CC/rat treated rats and control animals were immediately fixed in Bouin's solution (saturated picric acid-75%, buffered formalin-20%, glacial acetic acid-5%; all reagents obtained from BDH Chemicals Ltd., Toronto, Ontario) for approx. 4 hours and washed for 2 to 3 hours in running water to remove excess fixative. The ovarian tissue was subsequently dehydrated in sequential concentrations of ethanol (70, 80, 90 and 100%), cleared, and embedded in paraffin wax. Serial

sections (10 um thick) were stained with haematoxylin (CIN 7529, BDH Chemicals Ltd., Toronto, Ontario) and eosin (CIN 45380, BDH Chemicals Ltd., Toronto, Ontario). All sections were examined for evidence of the development of follicles, and representative sections were taken for photomicroscopy.

VI. Determination of Steroid Hormones

Aliquots of 1.0 ml sera were extracted twice with 5.0 ml diethyl ether (anhydrous, BDH Chemicals Ltd., Toronto, Ontario) by vortexing for 2 min; the extracts were evaporated under nitrogen flow at 35 ^{o}C and reconstituted in 1.0 ml absolute ethanol. Aliquots of 20-100 ul of the extracts, if necessary after 10 or 100 times of dilution with absolute ethanol, were dried down and assayed in duplicate for oestradiol-17β, progesterone and androgens by specific radioimmunoassay (RIA) using the antisera donated by Dr. D. T. Armstrong, University of Western Ontario, London, Ontario. Non-radiolabelled steroids, oestradiol-17β, progesterone and testosterone purchased from Sigma Chemical Co. (St. Louis, MO), and radiolabelled steroids [2, 4, 6, 7, 16, 17-^{3}H] oestradiol-17β (sp. act. 140 Ci/mmol), [1, 2, 6, 16, 17-^{3}H] progesterone (sp. act. 112 Ci/mmol) and [2, 6, 7-^{3}H] testosterone (sp. act. 80 Ci/mmol) from Amersham Co. (Arlington Heights, IL) were used as standard and tracers, respectively. Solvents for RIA were of analytical grade and were used without further purification, except for ethanol, which was redistilled.

In the assay procedures, approximately 10,000 cpm of tracer (^{3}H) was added to each tube. The unbound hormone was removed by the dextran-coated

charcoal adsorption method, and the bound steroid was counted in a LKB 1217 Rackbeta liquid scintillation counter. The assays were linear between 25-300 pg/tube. The binding efficiency of the steroid antibodies was 40 -60% with non-specific binding (NSB) less than 5%. The intra- and inter-assay coeffecient variation (CV) for the steroid assays were as follows: 7% and 5% for oestradiol-17β, 8% and 9% for progesterone, 5% and 6% for androgens, respectively. The respective cross-reactivities of the antisera (from Dr. D.T. Armstrong) were as follows:

Oestradiol-17β Antibody

oestradiol-17β	100.0%
oestrone	2.9%
oestriol	0.5%
other major steroids	<0.2%

Progesterone Antibody

progesterone	100.0%
5β-pregnane-3, 20-dione	35.5%
5α-pregnane-3, 20-dione	15.7%
3α-hydroxy-5β-pregnan-20-one	2.0%
20β-hydroxy-4-pregnene-3-one	1.3%
17α-hydroxyprogesterone	1.2%
other major steroids	<0.2%

Testosterone Antibody

testosterone	100.0%
5α-dihydrotestosterone	75.0%
5α-androstane-3α, 17β-diol	13.5%
5α-androstane-3β, 17β-diol	10.9%
19-hydroxytestosterone	4.7%
other major steroids	<0.1%

Based on the above cross-reactivity data, the testosterone antibody was relatively nonspecific, thus the steroids measured using this antiserum were

referred to as androgens rather than testosterone. Hormone levels were expressed as ng/ml for serum.

VII. Statistical Analysis

Experimental data were evaluated statistically by Analysis of Variance followed by a Student t-test where appropriate. Comparisons of $P<0.05$ were considered significant.

RESULTS

I. Effects of CC on Ovulation

Treatment with three doses of 1.0 mg CC/rat blocked ovulation in response to PMSG in immature rats. Rats treated with three doses of 1.0 mg of CC exhibited 0.2 $\pm$ 0.2 oocytes per rat (Table I), 0.86 $\pm$ 0.86 oocytes per rat (Table III) which was significantly ($P<0.01$) less than that from controls (9.6 $\pm$ 1.2 and 7.3 $\pm$ 1.97 oocytes per rat respectively). In Experiment II, no oocytes were recovered from the three doses of 1.0 mg CC/rat treated rats at any of the three time intervals.

The ovulation was completely restored by hCG (Table I); all rats treated with CC and hCG ovulated with a mean number of 7.0 $\pm$ 1.4 oocytes per rat which was comparable to that in controls.

Treatment with three doses of 0.1 mg CC/rat appeared slightly, but not significantly, to inhibit the ovulatory response (Table III). In rats treated with three doses of 0.05 mg CC/rat both the percentage of rats ovulating (100%) and the mean number of oocytes were comparable to that from control animals.

Two daily treatments with 1.0 mg CC/rat resulted in an increase in the mean number of oocytes (9.4 $\pm$ 0.5 per rat) which was significantly greater ($P<0.01$) than that of either the control animals (6.7 $\pm$ 0.7 oocytes per rat) or the rats treated with one dose of 1.0 mg CC/rat (6.9 $\pm$ 0.8 oocytes per rat) (Table IV).

TABLE I. Inhibitory effects of CC on ovulation and ovarian weight and the effects of hCG on overcoming this inhibition.

Treatment	Proportion of rats ovulating	No. of oocytes per rat	Ovarian weight (mg) per rat
Vehicle	10/11 (90.9%)	9.6 ± 1.2^{a}	40.2 ± 2.0^{d}
CC	2/13 (15.4%)	0.2 ± 0.2^{b}	28.0 ± 2.9^{c}
CC + hCG	7/7 (100 %)	7.0 ± 1.4^{a}	47.6 ± 3.3^{d}

Values represent means ± SEM.
a $P<0.001$ vs b; c $P<0.01$ vs d.

TABLE II. The effect of CC on delaying ovulation and ovarian weight.

Time after PMSG treatment	Proportion of rats ovulating	No. of oocytes per rat	Ovarian weight (mg/rat)
72 hr	0/5	~	26.3±2.9*
96 hr	0/5	~	26.5±2.5*
120 hr	0/5	~	27.5±2.6*
Control (72 hr)	6/6	6.5±1.8	42.2±2.0

Values represent the mean ± SEM.
*$P < 0.01$ vs control.

TABLE III. Dose-dependent effects of CC on ovulation and ovarian weight.

Treatment	Proportion of rats ovulating	No. of oocytes per rat	Ovarian weight (mg/rat)
CC (1.0 mg)	1/7	0.86±0.86[a]	29.86±1.68[a]
CC (0.1 mg)	5/7	5.71±1.96[b]	48.96±2.98[b]
CC (0.05 mg)	7/7	8.60±1.62[b]	47.57±2.29[b]
Control	7/7	7.30±1.97[b]	48.00±0.00[b]

Values represent the mean ± SEM.

[a] $P<0.01$ vs [b].

TABLE IV.Treatment time dependent effects of CC on ovulation and ovarian weight.

Treatment Duration	Proportion of rats ovulating	No. of oocytes per rat	Ovarian weight (mg) per rat
2 X 1.0 mg CC	16/16 (100%)	9.4 ± 0.5^{a}	44.6 ± 1.9^{a}
1 X 1.0 mg CC	15/16 (94%)	6.9 ± 0.8^{b}	39.0 ± 1.6^{b}
Control	18/19 (95%)	6.7 ± 0.7^{b}	38.8 ± 1.1^{b}

Values represent mean ± SEM.
[a] $P<0.01$ vs [b].

II. Effects of CC on Ovarian Weight

Treatment with three daily doses of 1.0 mg CC/rat significantly reduced the ovarian weight. As shown in Tables I, II and III, the mean weight of a pair of ovaries from Experiment I (28.0 $\pm$ 2.9 mg), from Experiment II at 72 hours (26.3 $\pm$ 2.9 mg), and from Experiment III (29.8 $\pm$ 1.08 mg), were significantly ($P<0.01$) lower than that from controls (40.2 $\pm$ 2.0 mg, 42.2 $\pm$ 2.0 mg and 48.0 $\pm$ 0.0 mg respectively).

The inhibition of ovarian weight was overcome by the administration of hCG. The mean weight of a pair of ovaries from rats treated with CC and hCG was comparable to that from control (Table I).

The inhibition of ovarian weight by the three daily doses of 1.0 mg CC/rat lasted for at least three days after PMSG injection as shown in Table II.

As shown in Table IV, ovarian weight was enhanced by two daily treatments with 1.0 mg CC/rat. The mean ovarian weight (44.6 $\pm$ 1.1 mg/rat) was significantly greater than that from either control rats (38.8 $\pm$ 1.1 mg/rat) ($P<0.05$) or rats treated with one dose of 1.0 mg CC/rat (39.0 $\pm$ 1.6 mg/rat) ($P<0.05$).

III. Effects of CC on Ovarian Steroidogenesis

The values of serum levels of oestradiol-17β, androgens and progesterone are presented in Figures 1-3.

Treatment with three doses of 1.0 mg CC/rat significantly stimulated the estrogen biosynthesis in rats (Figure 1). The serum levels of oestradiol-17β from

Experiment I (0.13 ± 0.01 ng/ml), from Experiment II at 72 hours (0.125 ± 0.01 ng/ml), and from Experiment III (0.098 ± 0.01 ng/ml) were significantly higher than that from controls (0.07 ± 0.01 ng/ml). The elevation of serum level of oestradiol-17β by CC was prevented by hCG (Figure 1).

Treatment with three doses of 1.0 mg CC/rat did not significantly affect the androgen and progesterone biosynthesis in rats. The serum levels of both androgens and progesterone from Experiment I, Experiment II at 72 hours and Experiment III were comparable to that from controls respectively. Human chorionic gonadotropin (hCG) significantly stimulated progesterone production in CC-treated rats (Figure 1).

The serum levels of oestradiol-17β and androgens in rats treated with three doses of 1.0 mg CC/rat declined at all time intervals observed (Figure 2). The serum level of oestradiol-17β (0.058 ± 0.01 ng/ml) at 120 hours was significantly lower than that at 96 hours (0.099 ± 0.02 ng/ml) ($P<0.05$) and at 72 hours ($P<0.01$). The serum levels of androgens declined to undetectable levels at 96 hours and 120 hours. The changes in the serum levels of progesterone at all the time intervals were comparable to each other.

Treatment with three doses of 0.05 mg CC/rat resulted in the elevation of serum levels of progesterone (28.24 ± 2.62 ng/ml) and androgens (0.53 ± 0.08 ng/ml) (Figure 3) which was significantly ($P<0.05$) higher than that from controls (13.62 ± 1.92 ng/ml and 0.32 ± 0.04 ng/ml respectively). The level of oestradiol-17β was not significantly affected in this treatment regimen (Figure 3). Treatment with three doses of 0.1 mg CC/rat resulted in no significant difference of the serum levels of oestradiol-17β, androgens and progesterone (Figure 3).

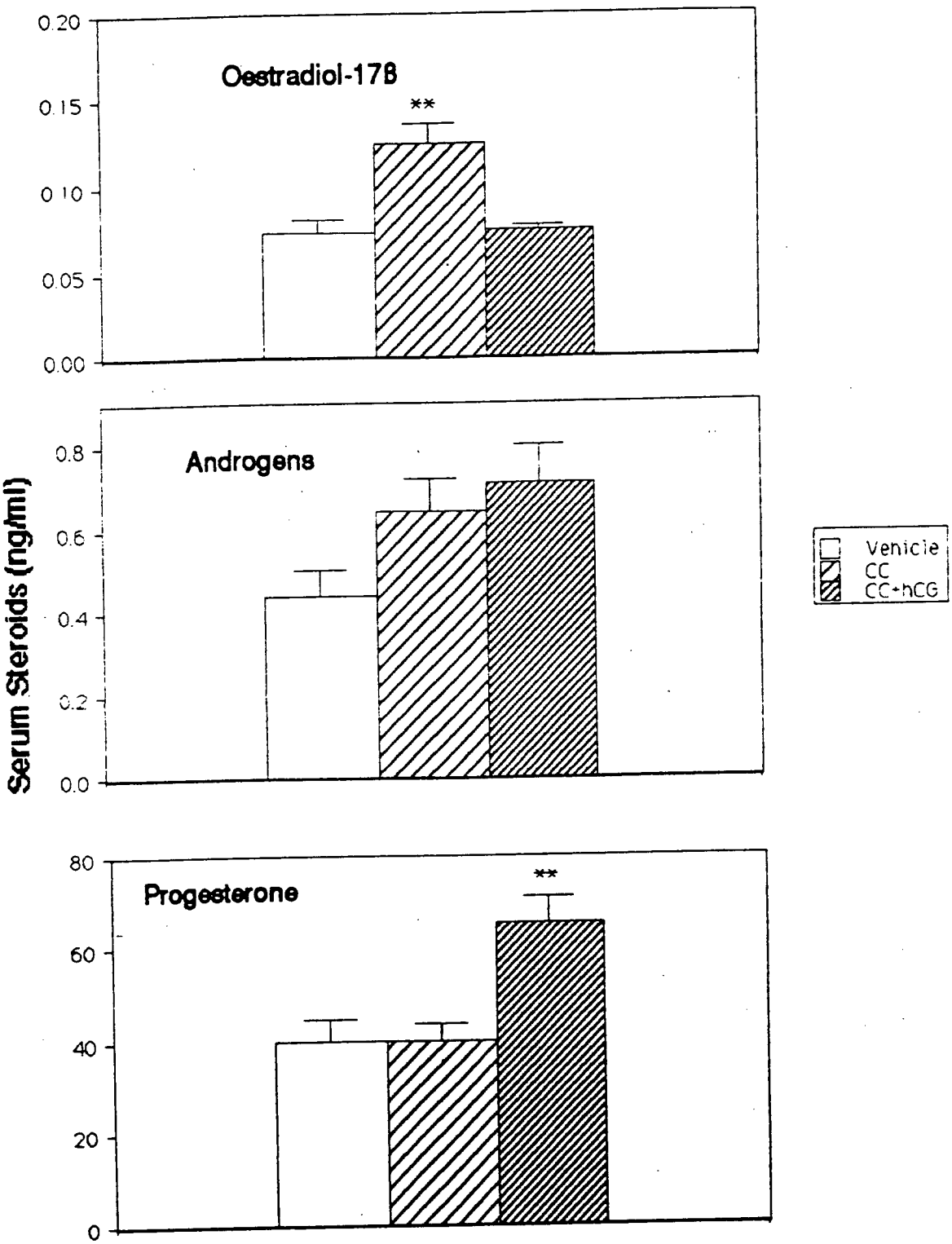


Figure 1: Serum levels of oestradiol-17β, androgens and progesterone from rats treated with CC; CC + hCG; and control rats at 72 hours after PMSG injection. Values are given as mean ± SEM. ** $P < 0.01$ compared to columns without **.

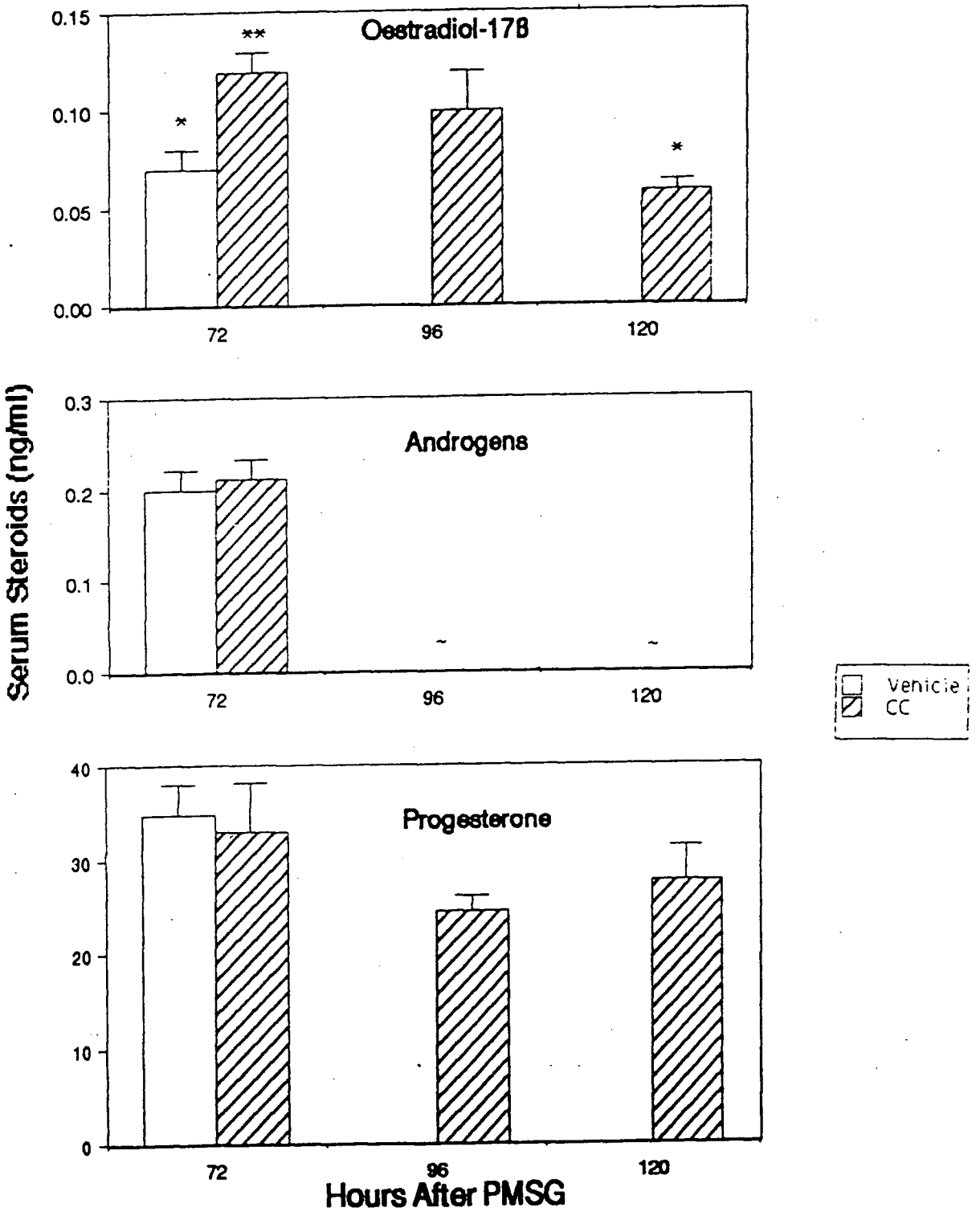


Figure 2 Serum levels of oestradiol-17β, androgens and progesterone from rats treated with CC and control rats at different time intervals after PMSG injection. Values are given as mean ± SEM. • $P < 0.01$ vs ••. ~ represents undetectable.

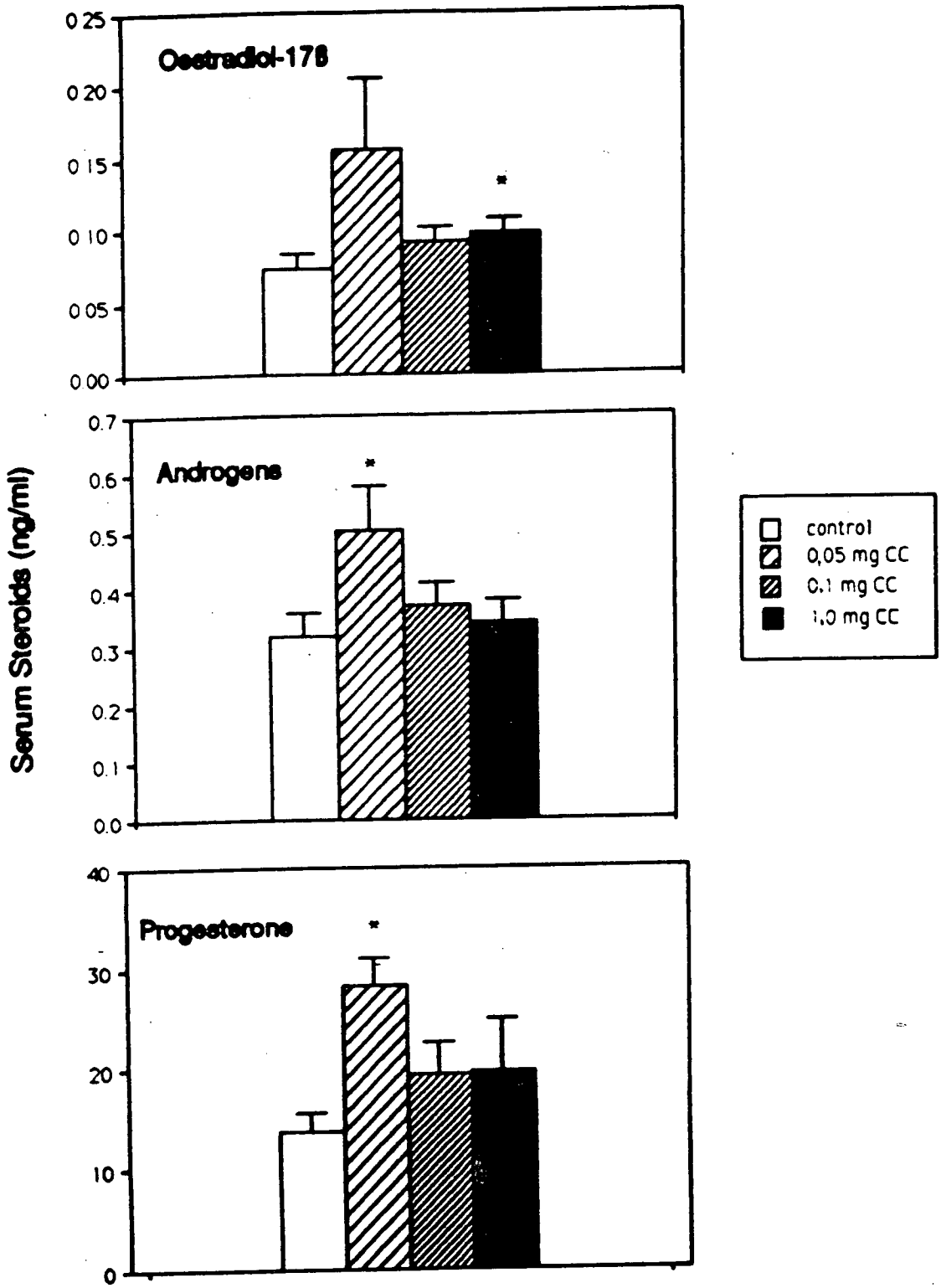


Figure 3: Serum levels of oestradiol-17β, androgens and progesterone from rats treated with different doses of CC and control rats at 72 hours after PMSG injection. Values are given as mean ± SEM. * $P < 0.05$ compared to control.

IV. Effects of CC on Ovarian Histology

Ovaries from the control animals were pink and spherical. They contained numerous corpora lutea (Figure 4) and many follicles in different stages of development (Figure 5) but lacked follicles with mature appearance.

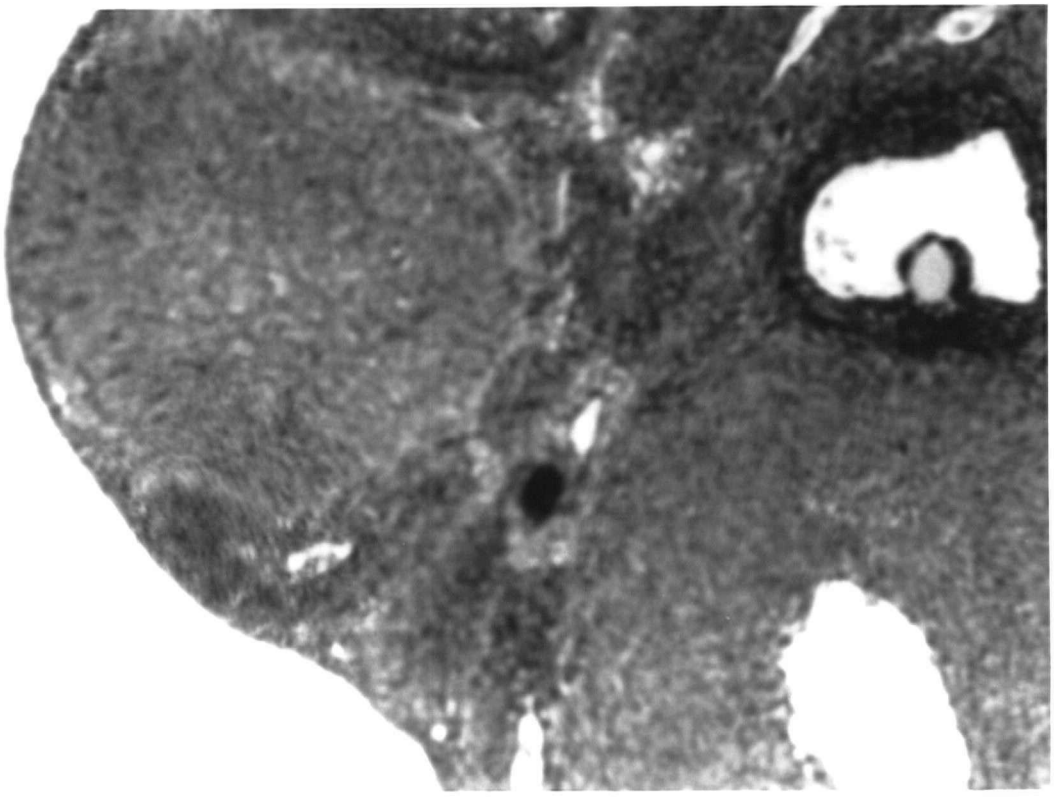
In contrast to control, the ovaries from the CC-treated rats were markedly reduced in size and appeared pale and immature. They had large preovulatory follicles (Figure 6) with healthy appearance and many small follicles (Figure 7) probably undergoing degeneration (Figure 8), but exhibited no follicles of medium size.

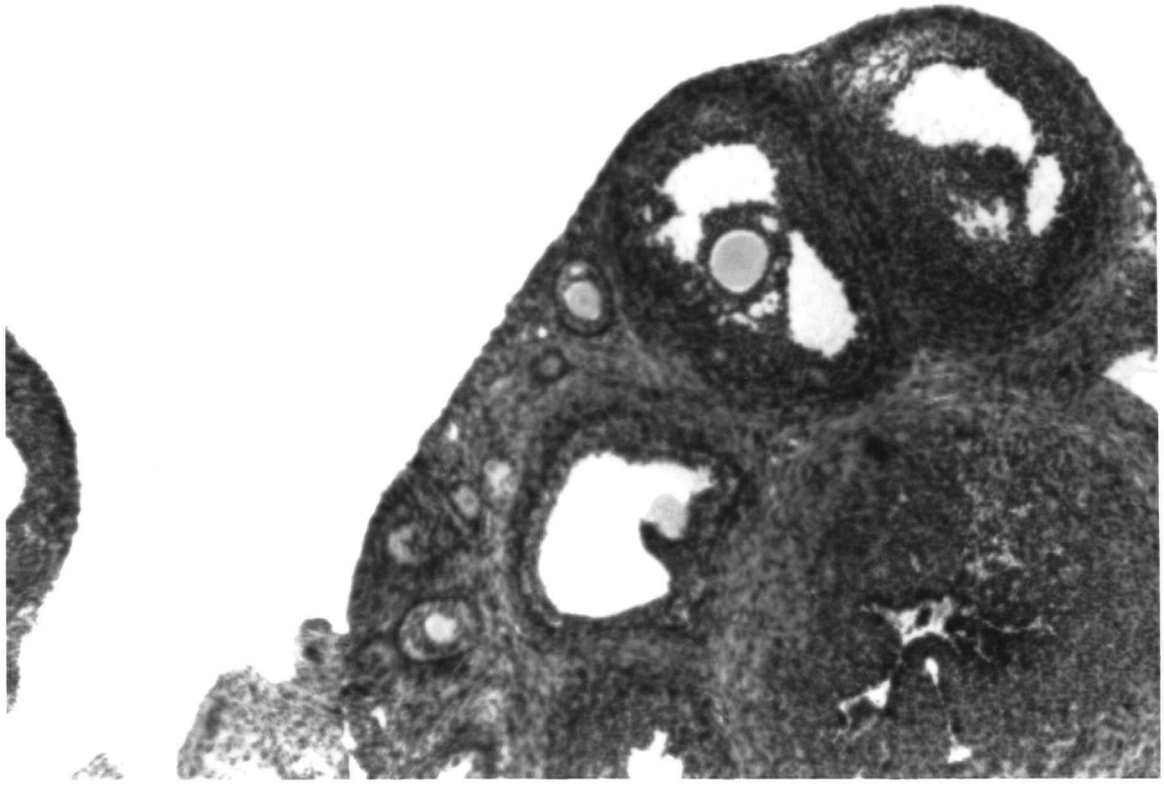
Figures 4-5

Light microscopical appearance of a representative section of ovary from a control animal (stained by haematoxylin and eosin, magnified by 40 X).

Figure 4 note the corpora lutea (upper).

Figure 5 note the follicles in different stage of development and the surrounding corpus luteum (bottom).



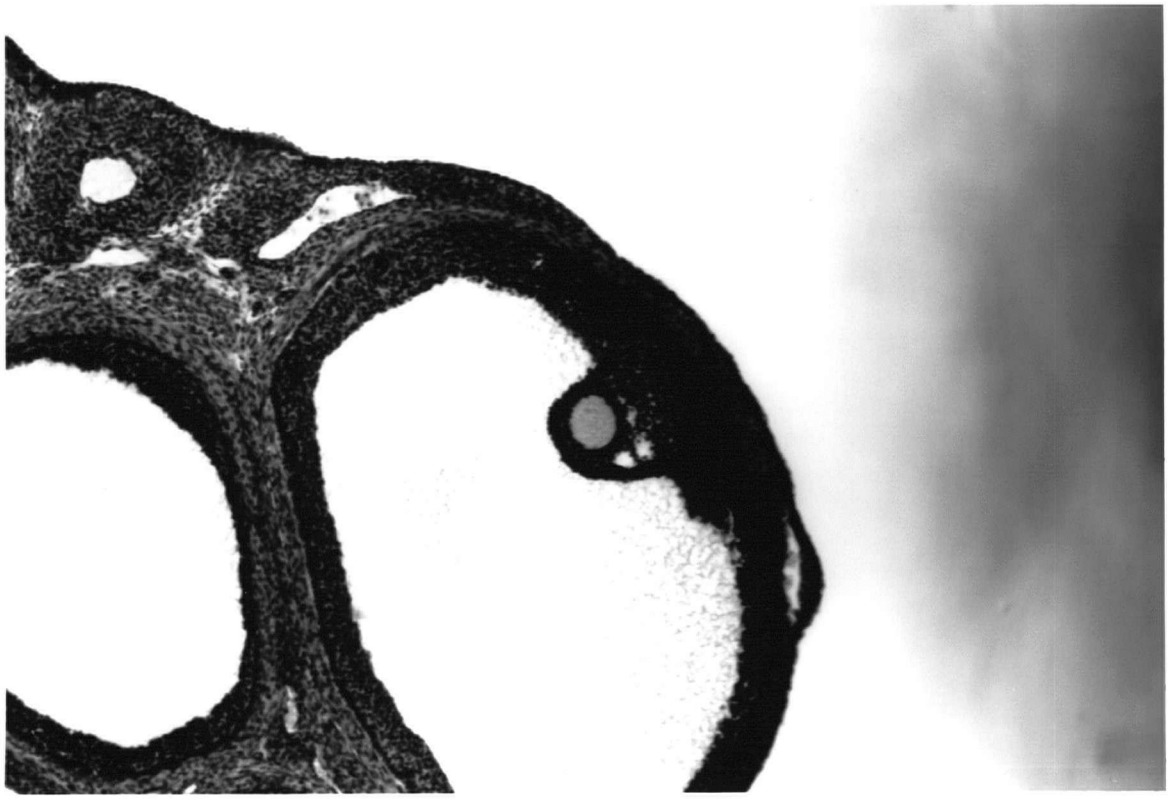


Figures 6-7

Light microscopical appearance of a representative section of ovary from rats treated with three doses of 1.0 mg CC/rat (stained by haematoxylin and eosin, magnified by 40 X).

Figure 6 note the large antral follicle with healthy appearance of granulosa and theca cell layers and oocyte (upper).

Figure 7 note the small follicles and the absence of mid-size follicles (bottom).



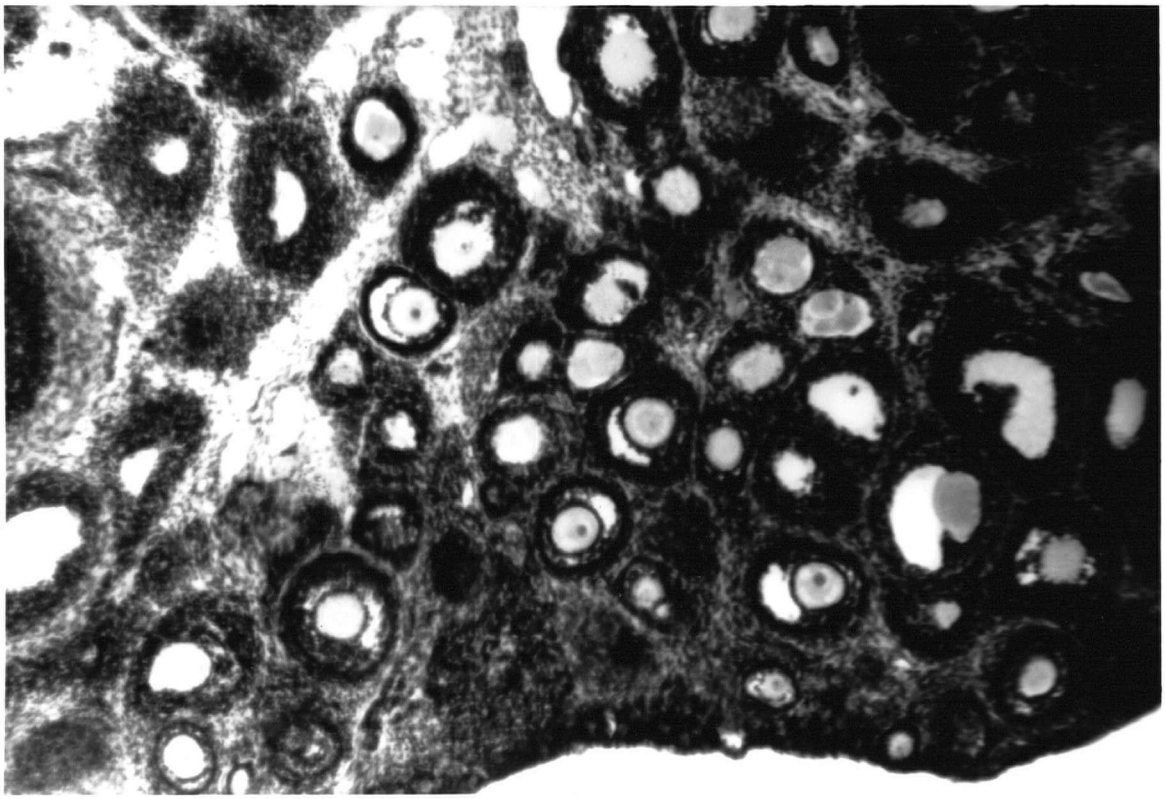
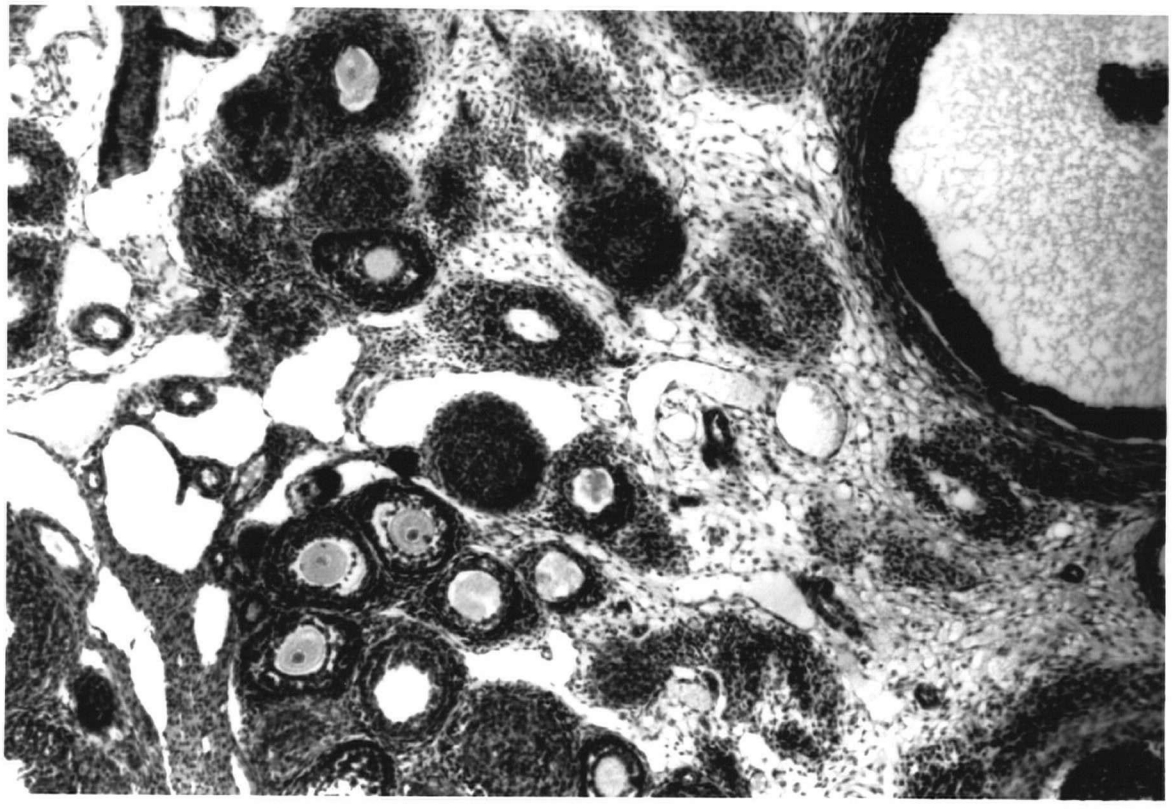


Figure 8

Light microscopical appearance of a representative section of ovary from rats treated with three doses of 1.0 mg CC/rat (stained by haematoxylin and eosin, magnified by 40 X). Note the small atretic follicles with fragmented oocytes and loss of granulosa cell continuity.



DISCUSSION

I. Effects of CC on Ovulation

The results of the present study show that three daily treatments with 1.0 mg CC/rat inhibited ovulation in response to PMSG in immature rats. This study supports the earlier reports which indicated the inhibitory effect of CC on ovulation in rats (Holtkamp et al., 1960; Greenblatt et al., 1962; Shirley et al., 1968; Labhsetwar, 1970). More convincingly, in this study ovulation was assessed by counting the oocytes flushed from the oviduct instead of other techniques including counting the corpora lutea (Docke, 1969; Mayer, 1973; Koch et al., 1971) and the gonadotropin changes in the serum as the index of ovulation (Schally et al., 1970; Nagel et al., 1970) since the gonadotropin change may not be sufficient enough to reflect ovulation events.

In order to determine whether CC delays ovulation or blocks ovulation in rats, ovulation of rats treated with three doses of 1.0 mg CC were examined at different time intervals after PMSG. The results show that CC blocked the ovulation for at least three days (Table II). This indicates that the mode of effect of CC on ovulation in rats is different from that in primates in which CC was shown to delay ovulation (Marut et al., 1982). Oestrogens have been shown to stimulate ovarian function and promote follicular growth (Pencharz, 1940; Willams, 1940; Richards et al., 1976) and antibodies to estradiol-17β blocked the ovulation in PMSG-treated immature rats (Ferin et al., 1968) just as CC did in the present study. The antiovulatory actions of CC observed in the present studies may be

therefore viewed as an oestrogen antagonistic effect.

Clomiphene citrate shows dose-dependent effects on the gonadotropin secretion in rats. Low doses of CC (0.1-0.5 mg/kg) in immature male rats caused some increases in the weight of the ventral prostate and seminal vesicles most probably by stimulating gonadotropin secretion (Roy et al., 1964). It has also been demonstrated that low doses of CC (0.06-1.0 mg/kg) in immature female rats caused precocious puberty as judged by vaginal opening and the formation of corpora lutea, while high doses (10 mg/kg) did not stimulate early follicular maturation and corpora lutea formation (Meyer, 1973). The stimulating effects of lower doses of CC on ovulation was not observed in the present study. Treatment with three doses of 0.1 and 0.05 mg of CC did not significantly affect ovulatory response. Both the proportion of ovulating rats and the mean number of oocytes were comparable to that of the control animals (Table III). The most suitable doses of CC may not have been selected in the present study.

The effect of treatment duration of CC on ovulation was observed in the present study. When the number of treatment days was reduced from three days to two days, 1.0 mg CC/rat significantly enhanced ovulation in rats while administration with a single dose of 1.0 mg CC did not show any effect on ovulation (Table IV). This supports the earlier report that decreasing the number of treatment days from the usual 10-20 to 5 in rats CC increased preovulatory gonadotropin secretion (Clark et al., 1982).

II. The Effects of CC on Ovarian Functions

That CC may, in fact, exert a direct ovarian effect has been suspected for more than 20 years, however, sufficient evidence to prove this is lacking. In the present study, the ovarian weight, ovarian steroidogenesis and the ovarian morphology were affected by CC. This is supported by the demonstration that CC directly inhibited ovarian, weight ovulation and ovarian steroidogenesis in hypophysectomized rats (Moon et al. (1989). This suggests that there may be a direct effect of CC on the ovarian functions.

A. Effect On Ovarian Weight

Results of the present study showed that CC had dose-dependent effects on ovarian weight in rats. Three daily treatments with 1.0 mg CC/rat significantly decreased the ovarian weight which was restored by the administration of hCG and this inhibition of ovarian weight persisted at least for three days. Treatment with three doses of 0.5 or 0.1 mg CC/rat did not affect the ovarian weight.

In addition to its dose-dependent effects, CC also has temporal effects on ovarian weight. Two daily treatments with 1.0 mg CC/rat significantly increased the ovarian weight unlike the results from three daily treatments with 1.0 mg CC/rat which significantly decreased ovarian weight in rats. One dose of CC did not significantly affect the ovarian weight (Table IV).

It is unknown whether the inhibition of ovarian weight by CC is via exerting an antiestrogenic action or interfering with the gonadotropin release. During follicular development, oestradiol-17βacts locally to stimulate granulosa cell

proliferation, increasing the number of follicles capable of forming an antrum in response to gonadotropin. This increase in the number of antral follicles results in an increase in ovarian weight. Thus, the inhibitory effects of CC on ovarian weight observed in the present study could be viewed as a direct anti-oestrogenic action. It is also possible that CC inhibited ovarian weight by suppressing gonadotropin release as hCG could restore the ovarian weight. However, CC also showed a stimulatory effect on follicular development by the different treatment regimen, indicating that the action of CC on the reproductive system is influenced by the dose and the treatment time.

B. Effect On Ovarian Histology

The effect of CC on the ovarian histology of rats has not previously been reported. In normally cycling monkeys, Marut and Hodgen (1984) reported a direct inhibitory effect of CC on ovarian folliculogenesis. Nakano et al. (1986) also reported an inhibition of FSH induced ovarian follicular growth by cis- CC in hypophysectomized immature female rats. The results of the present study indicate that treatment with high doses (1.0 mg) of CC caused atresia in the less developed follicles while the dominant follicles were unaffected. These appeared healthy and ready to ovulate (Figures 6), indicating that CC selectively interfered with follicular development. The development of the dominant follicles in response to PMSG was unaffected as follicles continued to develop to the mature preovulatory stage. The mechanism of selective interference with follicular development by CC requires further investigation.

C. Effect On Ovarian Steroidogenesis

Another aspect of this study which supports the theory of a direct ovarian effect by CC is that CC significantly affected ovarian steroidogenesis. The effects of CC on oestrogen biosynthesis in various studies have been inconsistent. Some investigators reported a stimulatory (Zhuang et al., 1982; Welsh et al., 1984), and others an inhibitory (Laufer et al.,1982) effect in steroidogenesis. The present study supports the theory that CC has stimulatory effects on oestrogen biosynthesis. Results from this study consistently show that administration of three doses of 1.0 mg CC considered to be a "high dose" significantly elevates the serum level of oestradiol-17β(Figures 1-3). This elevation was diminished by hCG (Figure 1) when administered as an LH substitute suggesting that the direct effects of CC on ovarian steroidogenesis are modulated by gonadotropins. This strongly supports the suggestion by Ho Yuen et al. (1988) that hCG abolishes the effect of CC on ovarian steroidogenesis.

Clinical studies have demonstrated that CC induces ovulation in 80% of anovulatory women, but results in only a 40% pregnancy rate (Speroff et al., 1983). The discrepancy between the rates of ovulation and pregnancy was attributed to luteal insufficiency as in vivo and in vitro investigations have shown that CC inhibits progesterone synthesis in rat follicles (Laufer et al., 1982), rat granulosa cells (Welsh et al., 1984), monkey luteal cells (Westfahl and Resko, 1983), hen granulosa cells (Sgarlata et al., 1984) and human granulosa cells (Ho Yuen et al., 1988). However, the results from this study do not support these

investigations. Clomiphene citrate did not affect the progesterone level of serum in response to PMSG but hCG did stimulate the progesterone production in CC-treated rats (Feng et al., 1989). This further supports the suggestion that hCG given to patients with corpus luteum insufficiency following CC-induced ovulation may enhance their progesterone production (Seegar-Jones et al., 1970; Murray et al., 1971).

It is generally accepted that CC inhibits progesterone biosynthesis in several species including rats (Adashi, 1984). However, stimulating effects of CC on progesterone production in rats was observed in the present study. Administration of three doses of 0.05 mg CC/rat significantly elevated the progesterone levels of serum (Figure 3). Treatment with three doses of 0.1 mg CC/rats did not affect progesterone production indicating the effects of CC on steroidogenesis is dose-dependent.

The effects of CC on ovarian androgen biosynthesis in rats has not previously been reported. In the this study, serum levels of androgens were elevated by administration of three doses of CC (0.05 mg) (Figure 3). Further studies are necessary to confirm this.

In rats treated with three doses of 1.0 mg CC/rat, both oestradiol-17β and androgens showed decreasing trends during the time intervals of 120 hours after PMSG. It is presumed that in this experimental model, treatment with PMSG initiates follicular development and steroidogenesis, therefore stimulating the endogenous gonadotropin secretion. As endogenous gonadotropin is suppressed by CC and the exogenous gonadotropin degenerates (PMSG 1/2 life

is 60 hours), the follicles lose the gonadotropin support and ovarian steroid biosynthesis is decreased. However, this does not explain why progesterone remains unchanged during the entire experimental period (Figure 2).

III. Mechanism(s) by which CC Exerts Its Action

Ovulation is a process involving the hypothalamus, pituitary and ovary and is dependent upon complicated but coordinated events that take place within these organs which are often referred to as the hypothalamic-pituitary-ovary axis. Functional changes from any part of the axis will influence the whole axis and consequently will affect ovulation. Thus, the mechanism by which CC affects ovulation might be by changing any or all parts of the hypothalamic-pituitary-ovarian axis.

There is evidence that CC may exert its action as either an oestrogen agonist or as an antagonist (Adashi, 1984; Clark 1982) over the whole hypothalamic-pituitary-ovarian axis. It has been observed that the administration of CC results in an increase in the release of pituitary gonadotropin in humans (Adashi, 1984; Kerin, 1985) and increases the frequency of LH pulse released by the pituitary gland, suggesting that the effects of the drug may be mediated via the GnRH mechanism (Adashi, 1986).

The inhibition of ovulation by CC observed in the present study is believed to be a result of the suppression of the endogenous preovulatory LH surge by CC either at the hypothalamic or at the pituitary level. This is supported by the fact that ovulation in response to PMSG when completely blocked by treatment with

CC was restored when 10 IU hCG was administered as a substitute for the endogenous LH surge (Sugawara et al., 1969; 1970) to CC-treated animals at the expected time of endogenous LH surge (Sorrentino et al., 1972; Costoff et al., 1974). In immature rats, treatment with 10 IU hCG did not initiate ovulation until 72 hours after the treatment (Sugawara, 1969) indicating that the ovulatory follicles restored by hCG could not be originally recruited by hCG but had been developing under the influence of PMSG. The hCG injected merely played the role of a preovulatory LH surge; therefore, the LH deprivation is a plausible explanation for the ovulatory block by CC in this treatment regimen.

It may be possible that CC blocks ovulation by interfering with follicular development. Clomiphene citrate treatment did appear to cause follicular atresia as indicated by reduced ovarian weight (Table I-III). Ovaries from CC-treated rats weighed significantly less than those of the control group. It is assumed that CC did not affect the dominant follicles but selectively caused atresia in the less developed follicles. This explanation is supported by the observation of the ovarian histology as ovaries from CC-treated rats possessed many mature healthy follicles which appeared ready to ovulate (Figures 6) and had a marked absence of developing follicles (Figures 7-8) when compared to those from the control ovaries (Figure 5).

Oestradiol plays a crucial role in follicular development, reduction of atresia (Richards et al., 1976), follicular cell responsiveness to FSH and LH, enhancement of FSH ability to stimulate adenylate cyclase, FSH-induced appearance of granulosa cell LH receptors (Richards, 1980) and regulation of

gonadotropin release by paracrine, autocrine and endocrine mechanisms. Lower than normal oestradiol levels could interfere with any of the above actions (Richards et al., 1976). At doses higher than physiological levels, oestradiol exerts a different effect on the secretion of FSH and LH (Goh et al., 1980). Either lower or higher serum levels of oestradiol than normal could interfere with ovulation. The high doses (1.0 mg) of CC consistently blocked ovulation and at the same time significantly enhanced oestradiol-17β production. The inhibition of ovulation by CC, which appeared to be a consequence of preovulatory LH suppression could be a result of CC stimulation of the oestradiol-17β production which in turn suppresses the preovulatory LH surge and subsequently blocks ovulation.

It is generally accepted that CC inhibits ovulation in rats. However, stimulatory effects of CC on ovulation were induced in the rats rendered anovulatory by either androgen-sterilized or light-induced persistent oestrus (Docke, 1969). In these rats, CC increased the corpora lutea formation significantly. The results of the present study support this finding (Table IV). As the number of treatment days was reduced from three to two with the same CC dose (1.0 mg/rat), ovulation in response to PMSG was enhanced. The three daily treatments with the same dose of CC consistently blocked ovulation (Feng et al., 1989). An explanation for these temporal effects of CC on ovulation in this study may be the following: acting in its capacity as an antiestrogen, CC displaces endogenous oestrogen secreted from follicles (under the influence of PMSG) from hypothalamus and pituitary oestrogen receptor sites. This results in

the alleviation of the negative feedback effect exerted by endogenous oestrogens and subsequently enhances the gonadotropin release. This, in turn, enhances the follicular development initiated by PMSG and CC is metabolized. The high level of endogenous oestrogen secretion from follicles under the influence of endogenous gonadotropin and PMSG exerts a positive feedback effect at the hypothalamus and pituitary and results in the preovulatory LH release which ultimately causes the rupture of chosen follicles.

Considering the long half-life of CC in rats (24 hours) (Clark, 1982), the three daily CC treatments prior to PMSG treatment which will initiate the oestrous cycle, are believed to persist into the later follicular phase, thereby alleviating the positive feedback effect of CC on the preovulatory LH surge and subsequently blocking the ovulation. The two daily CC treatments only alleviated the negative feedback, stimulating endogenous gonadotropin, follicular development and endogenous oestrogen biosynthesis, but did not affect the positive feedback effect, thus, stimulating the preovulatory LH surge and resulting in the enhancement of ovulation.

SUMMARY AND CONCLUSION

Immature female rats stimulated with PMSG to induce follicular development and ovulation was the animal model used in this study to determine the effects of CC on ovulation and ovarian function. The effects of CC on ovulation were shown to be dose-dependent. Ovulation in response to PMSG was completely blocked by administration of high doses (1.0 mg) of CC for three days, and in this treatment regimen, CC selectively inhibited the follicular development of small follicles, while the dominant follicles were unaffected and developed to the preovulatory stage. The inhibitory effects on ovulation by a high dose of CC continued at least for 120 hours after PMSG; such inhibitory effects could be reversed by treatment with hCG as a preovulatory LH surge substitute at the expected time of the endogenous LH surge. Lower doses of CC did not affect ovulation.

When the length of treatment was reduced from three to two days, treatment with 1.0 mg CC resulted in an enhancement of ovulation in response to PMSG. A single dose of the same level of CC did not affect ovulation, both the proportion of ovulating rats and the mean oocyte number were comparable to that of controls. This indicates that the effects of CC on ovulation change with the length of treatment.

The effects of CC on ovarian growth in terms of ovarian weight were parallel to the effects of CC on ovulation, being both dose- and treatment time-dependent. The ovarian growth induced by PMSG was inhibited by high doses of CC, while lower doses were ineffective. The ovarian weights of rats treated

with lower doses of CC were comparable to those of control animals. The inhibition of ovarian growth was restored by hCG acting as a preovulatory LH surge.

Treatment time with CC appears to be an important factor influencing ovarian growth. Three daily treatments with a high dose (1.0 mg) of CC significantly inhibited ovarian growth in terms of ovarian weight. However, when the number of treatment days was reduced from three to two the opposite results were obtained: clomiphene citrate in this treatment regimen, significantly stimulated ovarian growth.

The effects of CC on ovarian steroidogenesis in response to PMSG were dose-dependent. High doses (1.0 mg) of CC significantly stimulated oestradiol-17β biosynthesis but CC did not show any inhibitory effect on progesterone production as previously reported. Human chorionic gonadotropin stimulated the progesterone production in rats treated with CC. Lower doses (0.05 mg) of CC stimulates progesterone and androgen production but this aspect requires further study.

It is concluded that the effects of CC on ovulation, ovarian growth and ovarian steroidogenesis are dose-dependent and treatment time-dependent and that CC appears to exert its action through the pituitary by altering gonadotropin release.

REFERENCES

Adam, H. K., Douglas, E. G. and Kemp, J. V. (1979) The metabolism of tamoxifen in humans. Biochem. Pharmacol. 27:145.

Adashi, E. Y., Hsueh, A. J. W., Bambino, T. H. and Yen, S. S. C. (1981) Disparate Effect of clomiphene and tamoxifen on pituitary gonadotropin release in vitro. Am J Physiol. (Endocrinology and Metabolism) 240:E125.

Adashi, E. Y. (1986) Clomiphene citrate-initiated ovulation: a clinical update. Thieme Inc., Seminars in Reproductive Endocrinology 4(3):255.

Adashi, E. Y. (1984) Clomiphene citrate: mechanism(s) and site(s) of action-a hypothesis revisited. Fertil. and Steril. 42(3):331.

Adashi, E. Y., Hsueh, A. J. W. and Yen, S. S. C. (1980) Alterations induced by clomiphene in the concentrations of oestrogen receptors in the uterus, pituitary gland and hypothalamus of female rats. J. Endocr. 87:383.

Allen, R. E., Palopoli, F. P. and Schumann, E. L. (1959) U. S. Patent 2:914, 561.

Andrade, A. T. L., Guimaraes, C. S. and Guerra, M. O. (1972) The in vitro effects of clomiphene citrate on 6-day rabbit blastocysts and their subsequent in vivo development. Fertil. Steril. 23:841.

Barraclough, C. A., Collu, R., Massa, R. and Martini, L. (1971). Temporal interrelationships between plasma LH, ovarian secretion rats and peripheral plasma progestin concentrations in the rat: Effect of nembutal and exogenous gonadotropin. Endocrinology 88:1437.

Blankstein, J., Mashiach, S. and Lunenfeld, B. (1986) Ovulation Induction and In Vitro Fertilization. Year Book Medical Publisher, Inc. Chicago. London. p.112.

Borgstrom, L. (1981) Plasma levels and pharmacokinetics of cyclofenil after oral administration to man. Eur. J. Clin. Pharmacol. 19:213.

Boyar, R. M. (1970) Effects of clomiphene citrate on pituitary FSH, FSH-RF, and release of LH in immature and mature rats. Endocrinology 86:629.

Chang, M. C. (1964) Effects of certain antifertility agents on the development of rabbit ova. Fertil. Steril. 15:97.

Clark, J. H. and Markaverich, B. M. (1982) The agonistic-antagonistic properties of clomiphene: a review. Pharmac. Ther. 15:467.

Clark, J. H. and Peck, E. J., Jr. (1976) Nuclear retention of receptor-oestrogen complex and nuclear acceptor sites. Nature 260(5552): 635.

Clark, J. H., Anderson, J. N. and Peck, E. J., Jr. (1974) Oestrogen receptors and antagonism of steroid hormone action. Nature 251:446.

Costoff, A., Eldridge, J. C. and Mahesh, V. B. (1974). Pituitary ultrastructure and serum gonadotropin levels in the PMS-primed immature rat. Cell Tiss. Res. 151(1):79.

Davidson, O. W., Wada, K. and Segal, S. J. (1965) Effects of clomiphene at different stages of pregnancy in the rat, implications regarding possible action mechanisms. Fertil. Steril. 16:195.

diZerega, G. S., Campeau, J. D., Nakamura, R. N., Ujita, E. L., Lobo, R. and Marrs, R. P. (1983) Activity of human follicular proteins in spontaneous and induced ovarian cycles. J. Clin. Endocr. Metab. 57:838.

Dlugi, A. M., Laufer, N., Botero-Ruiz, W., DeCherney, A. H., M., Haseltine, F. P., Mezer, H.C. and Behrman, H. R. (1985) Altered follicular development in clomiphene citrate versus human menopausal gonadotropin-stimulated cycles for in vitro fertilization. Fertil. Steril. 43:40.

Docke, F. (1969) Ovulation-inducing action of clomiphene citrate in the rat. J Reprod. Fertil. 18:135.

Docke, F. (1971) Studies on the anti-ovulatory action of clomiphene citrate in the rat. J Reprod. Fertil. 24:45.

Eisenfeld, A. J. and Axelrod, J. (1967) Evidence for estradiol binding sites in the hypothalamus-effect of drugs. Biochem. Pharmacol. 16:1781.

Elsden, R. P., Nelson, L. D. and Seidel, G. E., Jr. (1978). Superovulating cows with follicle stimulating hormone and pregnant mare serum gonadotropin. Theriogenology 9:17.

Engels, J. A, Friedlander, R. L. and Eik-Nes, K. B. (1968) An in vitro effect of clomiphene on the rate of conversion of androstenedione-C^{14} to oestrone-C^{14} by the canine ovary. Metabolism 17:189.

Feng, T. B., Yun, W. Y., Ho Yuen, B. and Moon, Y. S. (1989) Inhibitory effects of clomiphene citrate on ovulation in immature rats. Canadian Fertility and Andrology Society Meeting, November 15, 1989. Vancouver, B. C., Canada. (Abstract).

Ferin, M., Tempone, A., Zimmering, P. E. and Vande Wiele, R. L. (1969) Effect of antibodies to oestradiol-17 and progesterone on the oestrous cycle of the rat. Endocrinology 85:1070.

Fromson, J. M., Pearson, S. and Bramah, S. (1973) The metabolism of tamoxifen

in female patients. Xenobiotica 3(11):711.

Goh, H. H., Chew, P. C., Karim, S. M. and Ratnam, S. S. (1980) Control of gonadotropin secretion by steroid hormones in castrated male transsexuals. I. effects of oestradiol infusion on plasma levels of follicle- stimulating hormone and luteinizing hormone. Clinical Endocrinology 12(2):165.

Greenblatt, R. B., Roy, S., Mahesh, V. B., Barfield, W. E. and Jungck, E. C. (1962) Induction of ovulation. Am. J. Obstet. Gynecol. 84:900.

Greenblatt, R. B., Barfield, W. E., Jungck, E. C. and Ray, A. W. (1961) Induction of ovulation with MRL/41 JAMA 178:101.

Hammerstein, J. (1969) Mode of action of clomiphene. I. Inhibitory effect of clomiphene citrate on the formation of progesterone from acetate-1-^{14}C by human corpus luteum slices in vitro. Acta Endocrinol. (copenh) 60:635.

Harman, S. M., Louvet, J. P. and Ross, G. T. (1975) Inhibition of the ovarian augmentation reaction by a chemical antiestrogen. Endocrinology 96:1119.

Hillensjo, T., Barnea, A., Nillson, L., Herlitz, H. and Ahren, K. (1974) Temporal relationship between serum LH levels and oocyte maturation in prepubertal rats injected with pregnant mare's serum gonadotropin. Endocrinology 95:1762.

Ho Yuen, B., Mari, N., Duleba, A. and Moon, Y. S. (1988) Direct effects of clomiphene citrate on the steroidogenic capability of human granulosa cells. Fertil. Steril. 49(4):626.

Holtkamp, D. E., Greslin, J. G., Root, C. A. and Lerner, L. J. (1960) Gonadotropin inhibiting and antifecundity effect of chloramiphene. Proc. Soc. Exp. Biol. Med. 105:197.

Hsueh, A. J. W, Erickson, G. F. and Yen, S. S. C. (1978) Sensitization of pituitary cells to luteinizing hormone releasing hormone by clomiphene citrate in vitro. Nature 273:57.

Huang, E.S. and Miller, W. (1983) Oestrogenic and antiestrogenic effects of enclomiphen and zuclomiphene on gonadotropin secretion by ovine pituitary cells in culture. Endocrinology 112:442.

Igarashi, M., Ibuki, Y., Kubo, H., Kamioka, J., Yokota, N., Ebara, Y. and Matsumoto, S. (1967) Mode and site of action of clomiphene. Am J Obstet. Gynecol. 97:120.

Jordan, V. C., Collins, M. M. and Rowsby, L. (1977) Amonohydroxylated metabolite of tamoxifen with potent antioestrogenic activity. J. Endocrinol. 75:305.

Kahwanago, I., Heinrichs, W. L. and Herrmann, W. L. (1970) Oestradiol "receptors" in hypothalamus and anterior pituitary gland: inhibition of oestradiol binding by SH-group blocking agents and clomiphene citrate. Endocrinology 86:1319.

Kato, J., Kobarashi, T. and Villee, C.A. (1968) Effect of clomiphene on the uptake of oestradiol by the anterior hypothalamus and hypophysis. Endocrinology 82:1049.

Kerin, J. F., Liu, J. H., Phillipou, G. and Yen, S. S. C. (1985) Evidence for a hypothalamic site of action of clomiphene citrate in women. J Clinical. Endocrinol. and Metabolism 61(2):265.

Kinstner, R.W. and Smith, O. W. (1961) Observations on the use of non-steroidal estrogen antagonist MER-25: Effects in endometrial hyperplasia and Stein-Levinthal syndrome. Fertil. Steril. 12:121.

Kistner, R. W., Lewis, J. L. and Steiner, G. J. (1966) Effects of clomiphene citrate on endometrial hyperplasia in the premenopausal female. Cancer 19:115.

Kistner, R. W. (1968) Progress in Infertility. Boston, Little, Brown & Co., 1968, pp.407.

Kostyk, S. K., Dropcho, E. J., Moltz, H. and Swartwout, J. R. (1978) Ovulation in immature rats in relation to the time and dose of injected human chorionic gonadotropin or pregnant mare serum gonadotropin. Biol. Reprod. 19:1102.

Kurl, R. N. and Morris, I. D. (1978) Differential depletion of cytoplasmic high affinity oestrogen receptors after the in vivo administration of the antioestrogens, clomiphene, MER-25 and tamoxifen. Br. J. Pharmacol. 62:487.

Labhsetwar, A. P. (1970). Role of oestrogens in ovulation: a study using the oestrogen-antagonist ICI46, 474. Endocrinology, 87:542.

Laufer, N., Reich, R., Braw, R., Shenker, J. G. and Tsafriri, A. (1982) Effects of clomiphene citrate on preovulatory rat follicles in culture. Bio. Reprod. 27:463.

Laufer, N., Pratt, B. M., DeCherney, A. H., Naftolin, F., Merino, M. and Markert, C. L. (1983) The in vivo and in vitro effects of clomiphene citrate on ovulation, fertilization, and development of cultured mouse oocyte. Am J Obstet. Gynecol. 147:633.

Linkie, D. M. and Niswender, G. D. (1972). Serum levels of prolactin, luteinizing hormone, and follicle stimulating hormone during pregnancy in the rat. Endocrinology 90:632.

Littma, B. A. and Jodgen, G. D. (1985) A comprehensive dose-response study of clomiphene citrate for enhancement of the primate ovarian/menstrual cycle. Fertil. Steril. 43:463.

MacGillivary, I. and Klopper, A. (1968) Clomiphene. Current Therapeutics 200:727.

Marut, E. L. and Hodgen, G. D. (1982) Antiestrogenic action of high-dose clomiphene in primates: pituitary augmentation but with ovarian attenuation. Fertil. Steril. 38:100.

Masala, A., Delitala, G., Alagna, S., Devilla, L. and Lotti, G. (1978) Effect of clomiphene citrate on plasma levels of immunoreactive luteinizing hormone-releasing hormone, gonadotropin, and testosterone in normal subjects and in patients with idiopathic oligospermia. Fertil. Steril. 29:424.

Maurer, R. and Woolley, D. (1971) Distribution of ^{3}H-estradiol in clomiphene-treated and neonatally androgenized rats. Endocrinology 88:1281.

Maxson, W. S., Pittaway, D. E., Herbert, C. M., Garner, C. H. and Wentz, A. C. (1984) Antiestrogenic effect of clomiphene citrate: correlation with serum estradiol concentration. Fertil. Steril. 42:356.

Mayer, H. G. K. (1973) Experimental investigation of the effect of clomiphene on the gonadotropin secretion of the rat pituitary. Endocrinology Exp. (Britisl) 7:177.

Meyer, R. K., Karavolas, H. J., Klausing, M. and Norgard, D. W. (1971) Blood progesterone and pregnenolone levels during phenobarbital block of PMS-induced ovulation in immature rats. Endocrinology 88:983.

Mikkelson, T., Kroboth, P. D., Cameron, W. J., Dittert, L. W., Chungi, V. and Manberg, P. J. (1986) Single-dose pharmacokinetics of Clomiphene citrate in normal volunteers. Fertil. Steril. 46(3):392.

Miller, B. G., and Armstrong, D. T. (1982) Infertility in superovulatory doses of pregnant mare serum gonadotropin cause delayed implantation and infertility in immature rats. Biol. Reprod. 25:253.

Miyake, A., Tasaka, K., Sakumoto, T., Kawamura, Y., Nagahara. Y. and Aono, T. (1983) Clomiphene citrate induces luteinizing hormone release through hypothalamic luteinizing hormone-releasing hormone in vitro. Acta Endocrinol. (Copenh) 103:289.

Miyake, A., Aono, T., Minagawa, J., Kawamura, Y., and Kurachi, K. (1980) Changes in plasma luteinizing hormone-releasing hormone during clomiphene-induced ovulatory cycles. Fertil. Steril. 34:172.

Moon, Y. S., Duleba, A. J., Yun, Y. W. and Ho Yuen, B. (1989) Effects of

clomiphene citrate on ovarian function in hypophysectomized rats. J Reprod. Fertil. 86:753.

Morris, I. D. (1969) Changes in brain, pituitary and uterine cytoplasmic oestrogen receptors induced by oestradiol-17 in the ovariectomized estrogen-progesterone blocked rats. Endocrinology 84:946.

Morris, I. D. (1976) Changes in brain, pituitary and uterine cytoplasmic oestrogen receptors induced by oestradiol-17 in the ovariectomized rat. J Endocrinol 71:343.

Murray, M. and Osmond-Clarke, F. (1971) Pregnancy results following treatment with clomiphene citrate. J. Obstet Gynecol. Br. Commonw. 78:1108.

Nagel, S., Baier, H. and Taubert, H-D. (1970) Contrasting actions of cis- and trans-clomiphene on the release of FSH and FSH-RF in the female rat. Horm. Metab. Res. 2:344.

Nakano, R., Nakayama, T. and Iwao, M. (1982) Inhibition of ovarian follicle growth by a chemical antiestrogen. Horm. Res. 16:230.

Nuti, K. M., Sridharan, B. N. and Meyer, R. K. (1975). Reproductive biology of PMSG-primed immature female rats. Biol. Reprod, 13:38.

O'Keefe, R. K. and Marrone, B. L. (1986) Inhibition of androgen and oestrogen production by clomiphene citrate in avian theca cells. J. Reprod. Fertil. 78:541.

Palopoli, F.P., Feil, V.J., Allen, R.E. Holtkamp, D.E. and Richardson, A., Jr. (1967) Substituted aminoalkoxytriaryhaloethylenes. J. Med. Chem. 10:84.

Papkoff, H., Bewley, T. A. and Ramachandran, J. (1978). Physicochemical and biological characterization of pregnant mare serum gonadotropin and its subunits. Biochem. Biophys. Acta. 532(1):373.

Papkoff, H. (1981) Variation in the properties of equine gonadotropin. Theriogenology 15:1.

Papkoff, H. (1974) Chemical and biological properties of the sub-units of pregnant mare serum gonadotropin. Biochem. Biophys. Res. Comm. 58:397.

Penchartz, R. L. (1940) Effect of oestrogens and androgens alone and in combination with gonadotropin on the ovary of the hypophysectomized rat. Science, N.Y. 91:554.

Persson, B. H. (1965) Clinical effect of bis-(acetoxyphenyl)-cyclohexlidenemethane (compound F 6066) on menstrual disorders. Acta Sco. Med. Ups. 70:71.

Pfaff, D. W., Gerlack, J. L., McEwen, B. S., Ferin, M., Carmel, P. and Zimmerman, E.A. (1976) Autoradiographic localization of hormone-concentrating cells in the brain of the female Rhesus monkey. J Comp. Neurol. 170:279.

Prasad, M. R. N., Kalra, S. P. and Segal, S. J. (1965) Effect of clomiphene on blastocysts during delayed implantation in the rat. Fertil. Steril. 16:101.

Richards, J. S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S., Durica, J.M. and Goldring, N.B (1987) Ovarian follicular development: from physiology to molecular biology. Rec. Prog. Horm. Res. 43:231.

Richards, J. S., Ireland, J. J., Rao, M. C., Bernath, G. A., Midgley, A. R., Jr. and Reichert, L. E. Jr. (1976) Ovarian follicular development in the rat: hormone receptor regulation by oestradiol, follicle stimulating hormone and luteinizing hormone. Endocrinology 99:1562.

Richards, J. S. (1980) Maturation of ovarian follicles: Actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. Physiological Reviews 60:51.

Robson, J. M. and Schonberg, A. (1937) Oestrous reaction, including mating, produced by triphenylethylene. Nature 140:196.

Roy, S., Mahesh, V. B., anf Greenblatt, R. B. (1964) Effects of clomiphene on the physiology of reproduction in the rat: III. Inhibition of uptake of radioactive oestradiol by the uterus and the pituitary gland of immature rat. Acta Endocrinology 47:669.

Sahu, A. (1987) Effect of clomiphene citrate on the ovary of a wild rat, bandicota bengalensis. Acta anat. 129:248.

Sasson, S., and Notides, C. A. (1982) The inhibition of the estrogen receptors positive cooperative[^{3}H] oestradiol binding by the antagonistic clomiphene. J. Biol. Chem. 25(19):11540.

Schally, A. V., Carter, W. H., Parlow, A. F., Saito, M., Arimura, A., Bowers, C. Y. and Holtkamp, D. E. (1970) Alteration of LH and FSH release in rats treated with clomiphene or its isomers. Am J Obstet. Gynecol. 107:1156.

Schams, D., Menzer, C., Schallenberger, E., Hoffman, B. and Hahn, R. (1978) Some studies on pregnant mare serum gonadotropin and on endocrine responses after application for superovulation in cattle. Control of Reproduction in the Cow. J. M. Screenan ed., The Hague, Martinus Nijhoff, pp. 122-143.

Schreiber, E., Johnson, J. E., Plotz, E. J. and Wiener M. (1966) Studies with ^{14}C-labelled clomiphene citrate. Clin. Research 14:287.

Schulz-Amling, W., Abraham, R. and Taubert, H-D. (1973) Studies of the mechanism of action of clomiphene: attenuation of oestradiol uptake in the hypothalamus and pituitary of the rat. Arch Gynaekol. 214:125.

Schultz, K. D., August, S., Wustenberg, B. and Holzel, F. (1973) Oestrogen-like and antiestrogenic potencies of clomiphene citrate: biochimical investigation. Infertility and Sterility. Hasegawa, T., Harashi, M., Ebling, F.J. and Hendesson, I. W. eds., Excerpta Medica, Amsterdam, pp. 642-645.

Seegar-Jones, G., Maffezoli, R. Q. and Rose, G. T. (1970) Pathophysiology of reproductive failure after clomiphene-induced ovulation. Am. J. Obstet. Gynecol. 108:847.

Seki, M., Seki, K., Takayanagi, H., Yoshihara, T. and Maeda, H.R. (1972) Effects of clomid and sexovid on FSH and LH secretion in mature female rats. Endocrinology 91:845.

Sgarlata, C. S., Todd, H., Hertelendy, F. and Mikhail, G. (1984) Clomiphene and tamoxifen inhibit progesterone synthesis in granulosa cells: comparison with oestradiol. Presented at the Thirty-First Annual Meeting of the Society for Gynecologic Investigation, March 21-24, 1984, San Francisco, California. Abstract 161P.

Shelton, R. S., Vancampen, M. G., Jr. and Meisner, D. F. (1953) Synthetic oestrogen: Halotriphenylethylene derivatives. J. Am. Chem. Soc. 75:5491.

Shirley, B., Wolinsky, T. and Schwartz, N. B. (1968) Effects of single injection of an estrogen antagosist on the oestrous cycle of the rat. Endocrinology 82:959.

Smith, O. W., Smith, G. V. and Kistner, R.W. (1963) Action of Mer-25 and of clomiphene on the human ovary. JAMA 184:122.

Smith, O. W. (1962) Chemical induction of ovulation. JAMA 179:197.

Sorentino, S., Jr., Reiter, R. J., Lee., L. A. and Schalch, D. S. (1972) Timing and neural pathways involved in release of luteinizing hormone after pregnant mare serum administration. Neuroendocrinology 9:341.

Speroff, L., Glass, R. H. and Kase, N. G. (1983) Clinical Gynaecologic Endocrinology and Infertility. Williams and Wilkins, Baltimore. pp. 523-544.

Stewart, F., Allen, W. R. and Moor, R. M. (1976) Pregnant mare serum gonadotropin: ratio of follicle-stimulating hormone and luteinizing hormone activities measured by radioreceptor assay. J. Endocrinol. 71(3):471.

Stumpf, W. (1968) Oestradiol-concentrating neurons: topography in hypothalamus by dry-mount autoradiography. Science 162:1001.

Sugawara, S. and Takeuchi, S. (1970) Ovulatory response to a single dose of human chorionic gonadotropin in the immature rat. Endocrinology 86:965.

Sugawara, S., Umezu, M. and Takeuchi, S. (1969) Effect of a single dose of human chorionic gonadotropin on the ovulatory response of the immature rat. J. Reprod. Fertil. 20:333.

Swyer; G. I. M. (1965) Clomiphene. Biological Council Symposium on Agents Affecting Sterility. Austin, C. R., and Perry, J. S. (eds.) Churchill Edinburgh. pp.180-190.

Taubert, H-D, Kessler, R., Busch, G. and Werner, H-J. (1970) The effect of clomiphene and cyclofenil upon pituitary LH and hypothalamic LH-releasing-factor content in the female rat. Experientia 26:98.

The Wm. S. Merrell Company (1967) Official literature on new drug clomiphene citrate (Clomid). Clinical Pharmacology and Therapeutics 8(6):891.

Trounson, A. O., Leeton, J. F. and Wood, C. (1981) Pregnancies in human by fertilization in vitro and embryo transfer in the controlled ovulatory cycle. Science 212:681.

Trounson, A. O. and Conti, A. (1982) Research in human in vitro fertilization and embryo transfer. British Med. J. Clinical Res. 285:244.

Tyler, E. T., Winer, J., Goltlib, M., Olson, H. J. and Nakabayashi, N. (1962) Effects of MRL-41 in human male and female fertility studies. Clin. Res. 10:119.

Vandenberg, G. and Yen, S. S. C. (1973) Effect of anti-estrogenic action of clomiphene during the menstrual cycle: evidence for a change in the feedback sensitivity. J. clin. Endocrinol. Metab. 37:356.

Welsh, T. H., Jia, X-C., Jones, P. B. C., Zhuang, L.Z. and Hsueh, A. J. W. (1984) Disparate effects of triphenylethlene antiestrogens on estrogen and progestin biosynthesis by cultured rat granulosa cells. Endocrinology 115:1275.

Westfahl, P. K. and Resko, J. A. (1983) Effects of clomiphene on luteal function in the nonpregnant cynomolgus macaque. Biol. Reprod. 29:963.

Willams, P. C. (1940) Effect of stilboestrol on the ovaries of hypophysectomized rats. Nature Lond. 145:388.

Wilson, C. A., Horth, C. E., Endersby, C. A. and Mcdonald, P. G. (1974) Changes in plasma levels of estradiol, progesterone and luteinizing hormone in immature rats treated with pregnant mare serum gonadotropin. J. Endocrinol. 60:293.

Wu, C. H. (1977) Plasma hormones in clomiphene citrate therapy. Obstet. Gynecol. 49:443.

Yoshimura, Y., Kitai, H., Santulli, R., Wright, K. and Wallack, E. E. (1985) Direct ovarian effect of clomiphene citrate in the rabbit. Fertil. Steril. 43:471.

Yun, Y. W., Ho Yuen, B. and Moon, Y. S. (1987) Effects of superovulatory doses of pregnant mare serum gonadotropin on oocyte quality and ovulatory and steroid hormone responses in rats. Gamete Res. 16:109.

Zhuang, L-Z., Adashi, E. Y. and Hsueh, A. J. W. (1982) Direct enhancement of gonadotropin-stimulated ovarian estrogen biosynthesis by estrogen and clomiphene citrate. Endocrinology 110:2219.