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Effects of clomiphene citrate on ovarian function and embryo developmental capacity in the rat

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랫드에 있어서 클로미펜 시트레이트가 난소기능 및 수정란 발육성에 미치는 영향

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초록 : 클로미펜 시트레이트가 배란반응, 난자의 형태, 난소 스테로이드 생성 및 수정란 발육에 미치는 영향을 PMSG 처리한 랫드에서 조사하였다. 먼저 세가지 용량(0.05mg, 0.1mg 및 1.0mg)의 클로미펜 시트레이트 또는 부형제를 미성숙의 Sprague Dawley 암컷에 일령 25일부터 27일까지 3일간 투여하였다. 그후 28일령에 이들 모든 암쥐에게 4IU PMSG를, 30일령에는 1.0mg 클로미펜 시트레이트가 처치된 일부의 암쥐에게 10IU hCG를 추가로 투여하였고, 31일령에 모두 도살하였다. 한편 4IU PMSG와 더불어 0.1mg클로미펜 시트레이트 또는 부형제를 투여한 일부의 암쥐는 숫쥐와 교미시킨 다음 임신 2일부터 5일까지 매일 도살하였다. 클로미펜 시트레이트의 용량을 증가시킴에 따라 배란반응(배란율 및 평균 배란난자의 수)과 난소중량이 대조군에 비하여 현저히 감소하였고 반면 배란난자의 변성율(%)은 그 용량에 비례하여 증가하였다. 클로미펜 시트레이트에 의한 배란반응 및 난소중량의 억제적 반응은 10IU hCG 추가투여에 의하여 완전히 대조군 수준으로 회복되었다. 그리고 클로미펜 시트레이트의 투여용량의 증가는 프로제스테론과 안드로젠의 혈장치 감소와 더불어 에스트라디올의 혈장치를 현저하게 증가시켰다. hCG의 추가투여는 이러한 클로미펜 시트레이트 작용에 의해 증가된 에스트라디올치를 현저하게 감소시키고 감소된 프로제스테론치를 증가시키는데 효과적이었다. 0.1mg의 클로미펜 시트레이트를 투여한 임신 랫드로부터 회수한 수정란은 전기간에 걸쳐 그 변성율(%)의 현저한 증가와 아울러 특히 임신 3일부터 그 수가 유의성있게 감소하였다. 클로미펜 시트레이트 투여에 의한 수정란의 난분할 속도도 임신 3일부터 대조군에 비하여 현저하게 지연되었으며 아울러 회수된 수정란의 난분할율(%)도 전기간에 걸쳐 대조군보다 지속적으로 저하되었다.

위의 결과는 흰쥐에 있어서의 클로미펜 시트레이트 투여에 의한 배란억제반응과 아울러 그 작용기전에 성선자극호르몬의 분비억제 또는 차단작용이 포함됨을 증명하였고 이 약제의 투여에 의한 난자의 형태적 정상성과 수정란발육에 대한 유해효과는 수정이전 시기에 있어서의 난소스테로이드 생성 특히 에스트라디올의 증가에 기인함을 제시한다.

Key words : clomiphene citrate, ovarian function, embryo development, rat.

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Introduction

Since the original study of Greenblatt21 et al in 1961, clomiphene citrate(CC), a synthetic nonsteroidal compound, is the most extensively used drug employed for induction of ovulation in anovulatory and amenorrheic women. The clinical utility of CC has been extensively enhanced through its recent introduction as a superovulatory agent in *in vitro* fertilization programs.[2] Despite its widespread use, the employment of CC in ovulation induction therapy results in a consistent disparity between the rate of ovulation and the incidence of pregnancy in women.[3] This disparity has previously been attributed to the adverse effects of CC on luteinization of unruptured follicles or luteal phase deficiency,[4] and cytotoxic effects on developing zygotes.[5] It appears that CC also affects advancing oocyte maturation and oocyte morphology.[6,7] However, it is unclear whether primary defect(s) following the CC treatment and its associated mechanism(s) occur before or after the time of fertilization.

Clinical preparations of CC possess estrogenic agonist and antagonist properties as a racemic mixture of approximately 38% zuclomiphene and 62% enclomiphene.[8] The effects of this agent on reproductive system are complicate depending upon the species, the target tissue and the length of exposure and its mode of interaction with various extrogen-dependent/-responsive tissues is still controversial.[9] CC has been reported to act as an estrogen antagonist and stimulate the secretion of gonadotropin releasing hormone(GnRH) and the release of gonadotropins(FSH and LH) by negating the negative feedback of endogenous estrogens at the level of hypothalamus and pituitary.[9,10] Thus the induction of ovulation by CC is thought to be due to its effects at hypothalamic pituitary axis. On the other hand, other alternative lines of experiments indicate that CC exerts a direct effect at the ovarian level as an estrogen-agonist by sensitizing granulosa cells to the action of pituitary gonadotropins,[11] since estrogens generally increase the sensitivity of adenylate cyclase to FSH and LH and augment the roles of these gonadotropins in their receptor formation.[12] The effects of CC on the ovary remain poorly understood. CC can exert inhibitory and stimulatory actions on a variety of ovarian function, including alterations of steroidogenesis[6,13] and promotion of oocyte degeneration.[7] It seems most likely that developmental capacity of the embryos following CC treatment depends upon the steroid microenvironment of the ovary and oocyte quality prior to fertilization.

The present study was designed to examine the effects of different doses of CC on ovulatory response, ovarian steroidogenesis and its associated detrimental effects on oocytes and developing embryos in rats.

Materials and Methods

Experimental animals : Immature female Sprague-Dawley rats at the 22 days of age were initially housed under temperature and light-controlled conditions (20-25℃, 12L : 12D) and were provided free access to standard rat chow and water. At 28 days of age, all rats received a single dose of 4IU PMSG s.c., between 0830 and 0900h, a known dose to induce physiological ovulatory response in terms of nuclear maturation and normality of ovulating oocytes and the ovarian steroidogenesis[14,15] and produce normal pregnancy with no significant embryonic or fetal wastage.[16,17]

Experiment 1. Prior to PMSG, twenty one rats received daily subcutaneous injections with 0.05㎎, 0.1㎎ or 1.0㎎ CC in 0.4㎖ sesame oil for 3days starting at the age of 25days and were allotted to three groups(N=7). The remaining seven rats of control group received sesame oil alone, a vehicle of CC, following the same schedule.

Experiment 2. Twenty rats received the 3 consecutive day-injections of 1.0㎎ CC prior to PMSG, and were allotted to two groups. On the 30th day of age, 48 hrs after PMSG, one group(N=7) subsequently received 10IU hCG in 4㎖ of 0.9% NaCl solution and the other group(N=13) received 0.9% NaCl solution vehicle. Controls were eleven rats given the two vehicles alone, following the same schedule.

Experiment 3. Sixty four rats given 0.1㎎ CC and sesame oil alone respectively, as in Experiment 1, were subsequently mated to provide the pregnant rats of two different groups(CC, N=32; Control, N=32). On the 30th day of age 60 hrs after PMSG, the rats had their vaginae gently opened with saline-soaked cotton swabs and were caged with mature fertile Sprague-Dawley males(one male and two females per breeding cage). Females were seperated from males and scored for the occurrence of mating on the following day(Day 1 of pregnancy). A sperm-positive score was noted by the presence of a copulatory plug in the vagina and/or spermatozoa in vaginal smears.

Chemicals and hormones : PMSG and hCG were purchased from Ayerst, McKenna and Harrison Incorporated-

(Vancouver, British Columbia, Canada). CC, hyaluronidase, estradiol-17β, testosterone and progesterone were purchased from Sigma Chemical Company(St Louis, MO, USA). [2, 4, 6, 7, 16, 17-^{3}H]Estradiol-17β(sp. act. 140Ci/mmol), [2, 6, 7-^{3}H]testosterone(sp. act. 80 Ci/mmol) and [1, 2, 6, 7, 16, 17-^{3}H]progesterone(sp. act. 112 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.

Collection of data : Rats in the Experiments 1 and 2 were killed by cervical dislocation on 31st day of age and mated rats in Experiment 3 were killed between 0900 and 1000 h on Days 2, 3, 4 and 5 of pregnancy. In the Experiments 1 and 2, trunk blood was collected and stored at 4℃ prior to separation of serum by centrifugation and subsequent radioimmunoassay(RIA) of estradiol-17β, testosterone and progesterone. Ovaries were cleaned of ovarian bursae and adjacent adipose tissue, blotted and weighed as a pair.

The oviducts were separated from the uterine horns at the uterotubal junction and the oocytes in the Experiments 1 and 2 or the preimplantation embryos in the Experiment 3 were collected in a few drops of Dulbecco's phosphate-buffered saline(DPBS) under a dissecting microscope. In the Experiments 1 and 2, the distended, translucent ampulla region of the oviduct was punctured and the expelled egg mass was exposed to 0.1% hyaluronidase for 5min. In the Experiment 3, the oviduct was flushed with 0.2㎖ DPBS by inserting a blunt-ended 30-gauge needle through the infundibulum and the uterine horn was flushed with 0.5㎖ DPBS by inserting a blunt 21-gauge needle through the wall of its cervical end. The recovered oocytes or embryos were counted under a dissecting microscope(40 × magnification) and examined without staining under a phase-contrast microscope(100 × magnification). The occurrence of fragmentation and other degenerative changes was assessed as described elsewhere.[14,18] Briefly, those eggs showing an irregular cell mass with debris, an armophous opaque mass of vitelline material, empty zona pellucida, a great difference in size of blastomeres and a hazy blastomere outline were classified as abnormal. The developmental stages of preimplantation embryos were also recorded.

Determination of steroid hormones : Aliquots of sera were extracted twice with five volumes of diethyl ether, evaporated under nitrogen at 35℃ and reconstituted with 1㎖ redistilled absolute ethanol. Serum levels of estradiol-17β, testosterone and progesterone were determined by specific RIA using the antisera kindly donated by Dr. David T. Armstrong from the University of Western Ontario(London, Ontario). The cross-reactivity of the estradiol-17β antiserum was : estradiol-17β, 100%; estrone, 2.9%; estriol, 0.5%; other major steroids known to be secreted by the follicle, less than 0.2%. The cross-reactivity of the testosterone antiserum was : testosterone, 100%; 5α-dihydrotestosterone, 75%; 5α-androstane-3α, 17β-diol, 13.5%; 5α-androstane-3β, 17β-diol, 10.9%; 19-hydroxytestosterone, 4.7%; other major steroids known to be secreted by the follicle, less than 1%. The cross-reactivity of the progesterone antiserum was : progesterone, 100%; 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-pregnen-3-one, 1.3%; 17-hydroxyprogesterone, 1.2%; other major steroids known to be secreted by the follicle, less than 0.2%. Intra-assay and inter-assay coefficients of variation were less than 10% and 15%, respectively. Since the testosterone antiserum was relatively nonspecific, the steroids measured using the testosterone antiserum are referred to as androgens rather than as testosterone.

Statistical analysis : Experimental data were evaluated by analysis of variance, or where appropriate, by Student's t-test. Differences were considered significant at $p<0.05$.

Results

Dose-dependent effects of CC on ovarian function including ovulation, oocyte normality and ovarian weight in rats are presented in Table 1. All control rats in vehicle group, apart from one rat showing little evidence of ovarian stimulation with 2 oocytes per rat, ovulated a range of 7-11 oocytes per rat. The ovulatory response was unaffected by treatment with 0.05㎎ CC but apparently inhibited by increasing the dose of CC. In 0.1㎎ CC-treated group, the proportion of rats ovulating was slightly reduced to 71.4% with the mean count of 5.7±2.0 oocytes per rat. In 1.0㎎ CC-treated group, the ovulation efficiency was markedly low with 14.3% proportion of rats ovulating and the mean count was significantly($p<0.01$) reduced to 0.9±0.9 oocytes per rat, compared to control rats. The average weight of paired ovaries, indicative of ovarian stimulation, obtained from the rats of 1.0㎎ CC-treated group was also significantly($p<0.01$) reduced by 40% below the mean value of 48.2±3.9㎎ ovarian tissue from the control rats. More than 95% of oocytes recovered from control rats appeared to be morphologically normal; only one or two degenerate oocytes were

Table 1. Dose-dependent effects of CC on ovulation, oocyte normality and ovarian weight

Treatment	Proportion of rats ovulating	No. of oocytes per rat[a]	% degenerate oocytes[a]	mg ovarian tissue[a]
Veh(Control)[b]	7/7(100%)	8.3±2.1	4.7±2.3	48.2±3.9
0.05mg CC[c]	7/7(100%)	8.6±1.6	12.4±7.2	47.6±2.3
0.1mg CC[d]	5/7(71.4%)	5.7±2.0	41.1±7.4[f]	49.0±3.0
1.0mg CC[e]	1/7(14.3%)	0.9±0.9[g]	76.0±11.2[h]	29.9±1.7[i]

a : Results are expressed as the mean±SE for 7 rats in each group.

b~e : Rats were treated with vehicle or three different doses of clomiphene citrate(CC) for three consecutive days prior to 4IU pregnant mare serum gonadotropin(PMSG).

f~i : $p<0.01$ within each parameter, compared to corresponding control group.

f vs h : $p<0.01$.

Table 2. Inhibitory effects of CC on ovulation, oocyte normality and ovarian weight and the effects of hCG on overcoming of this inhibition

Treatment	Proportion of rats ovulating	No. of oocytes per rat[a]	% degenerate oocytes[a]	mg ovarian tissue[a]
Veh(Control)[b]	10/11(90.9%)	9.6±1.2	10.2±3.4	40.2±2.0
CC[c]	2/13(15.4%)	0.2±0.2[e]	100.0±0.0[f]	28.0±2.9[g]
CC + hCG[d]	7/7(100%)	7.0±1.4[i]	71.7±28.1[i]	47.6±3.3[j]

a : Results are expressed as the mean±SE for the number of rats : (b) 11 rats, (c) 13 rats and (d) 7 rats in each group.

b~d : Rats were treated with vehicle, 1.0mg clomiphene citrate (CC) for 3 consecutive days prior to 4IU pregnant mare serum gonadotropin(PMSG), or 1.0mg CC(3 doses) puls 10IU hCG(single dose) at an interval of 24hr and 48hr prior and subsequent to 4IU PMSG.

e, f, g, i : $p<0.01$ within each parameter, compared to corresponding control group.

e vs h : $p<0.01$, f vs i : $p>0.05$, g vs j : $p<0.01$

Table 3. Effects of CC on normality and cleavage of preimplantation embryos

Treatment	Day of pregnancy[a]			
	2	3	4	5
Veh(Control)[b]				
No. of ova recovered	8.3±2.7	6.8±1.7	7.3±0.8	8.4±2.3
% degenerate ova	4.5±1.4	3.6±0.2	8.7±2.1	6.6±1.5
% cleaved embryos	87.0±2.4 (2-cell)	90.2±5.4 (2-to 6-cell)	83.2±5.7 (8-cell)	85.7±2.1 (blastocyst)
CC[c]				
No. of ova recovered	6.8±1.1	5.3±0.7[d]	1.4±1.2[e]	---
% degenerate ova	51.1±6.4[f]	46.2±1.8[g]	72.8±5.1[h]	---
% cleaved embryos	43.2±11.6[i] (2-cell)	48.7±6.9[j] (2-to 4-cell)	26.8±11.2[k] (4-to 8-cell)	---

a : Results are expressed as means±SEM for 8 pregnant rats in each group.

b,c : Rats were treated with vehicle or 0.1mg clomiphene citrate (CC) for three consecutive days prior to 4IU PMSG.

d vs e : $p<0.01$, g vs h : $p<0.05$

e~k : $p<0.01$ within each parameter, compared to corresponding control group.

occasionally observed. In contrast, CC treatment considerably elevated the percentage of oocytes exhibiting visible signs of degeneration(Fig 1) in a dose-dependent manner. Although the percentage of degenerate oocytes was intermediate in 0.05mg CC-treated group(12.4±7.2%), it was significantly($p<0.01$) greater with the values of 41.1±7.4% in 0.1mg CC group and 76.0±11.2% in 1.0mg CC group, as compared to that in control group of the rats.

The inhibited ovulatory response in 1.0mg CC-treated rats was completely restored by an additive dose(10 IU) of

hCG(Table 2). In experiment 2, only one or two oocytes were recovered from two of thirteen rats(15.4%) in the 1.0㎎ CC group, indicating a considerable block of ovulation. In contrast, all rats treated with CC plus hCG at an interval of 48 hrs ovulated with the mean count of 7.0±1.4 oocytes per rat, which was significantly($p<0.01$) greater above that in the rats of CC group(0.2±0.2 oocytes per rat) and was comparable to the value of controls(9.6±1.2). The average weight of paired ovarian tissue(47.6±3.3㎎)from the rats of CC plus hCG group was also significantly($p<0.01$) greater above the value of 28.0±2.9㎎ ovarian tissue obtained from the rats of CC group and reached to the comparable value of controls(40.2±2.0㎎) The additive treatment of hCG slightly reduced the percentage of degenerate oocytes recovered from the rats of CC group by 28.3% but was found not to be highly effective.

The changes in serum levels of estradiol-17β, androgens and progesterone after treatment with vehicle or various doses of CC are presented in Figure 2. By increasing doses of CC, the circulating level of estradiol-17β in 1.0㎎ CC-treated group(0.104±0.009n g/mℓ) was significantly($p<0.05$) greater above its level of controls(0.071±0.009n g/mℓ) treated with vehicle alone, but not in the other lower dose regimens(0.05㎎ CC group, 0.160±0.052n g/mℓ; 0.1㎎ CC group, 0.095±0.08n g/mℓ). On the other hand, the mean values for androgens and progesterone were significantly($p<0.05$) greater in 0.05㎎ CC group(0.51±0.08n g/mℓ and 28.2±2.6n g/mℓ, respectively), as compared to control values(0.32±0.04n g/mℓ and 13.6±1.9n g/mℓ, respectively), but not in the other higher dose regimens(0.1㎎ CC group, 0.37±0.03n g/mℓ and 19.4±3.2n g/mℓ; 1.0㎎ CC group, 0.35±0.04n g/mℓ and 19.5±5.3n g/mℓ, respectively).

In Figure 3, the elevated level of estradiol-17β(0.127±0.011n g/mℓ) by 1.0㎎ CC treatment was significantly($p<0.01$) reduced to the level(0.075±0.003n g/mℓ) comparable to that of controls(0.076±0.008n g/mℓ) by an additive treatment of 10IU hCG. The other significant effect by the hCG treatment was observed for the progesterone with its level of 66.2±5.7n g/mℓ, which was considerably($p<0.01$) higher than that in CC group(40.1±4.0n g/mℓ), but not for the androgens.

Data for the capacity of developing preimplantation embryos after 0.1㎎ CC treatment are presented in Table 3. The number of ova recovered from the oviducts and/or uteri of CC-treated group of rats diminished rapidly after Day 3 and reached 0 on Day 5 of pregnancy : Day 2, 6.8±1.1 ova per rat; Day 3, 5.3±0.7 ova per rat; Day 4, 1.4±1.2 ova per rat; Day 5, 0 ova per rat. In contrast, the recovery from control rats treated with vehicle was consistent through all the periods of preimplantation stage(Days 2 to 5 of pregnancy) with a mean range of 6.8−8.4 ova per rat. Gross morphology of the ova obtained from the control rats was slightly deteriorated with a mean range of the percentage of degenerate ova from 4.5% on Day 2 to 6.6% on Day 5 of pregnancy. However, as compared to control regimen, CC treatment significantly($p<0.01$) increased the percentage of degenerate ova consistently from Day 2 to Day 4 with the mean values : Day 2, 51.1±6.4%; Day 3, 46.2±1.8%; Day 4, 72.8±5.1%. The percentage of degeneration in CC group was significantly($p<0.05$) elevated after Day 3. CC treatment also significantly($p<0.01$) decreased the percentage of cleaved embryos below that in the control group of the rats with a consistency through all the periods examined. Control rats showed a mean range of percentage of cleaved embryos from 87.0% on Day 2 to 85.7% on Day 5 of pregnancy, but CC-treated rats displayed it from 43.2% on Day 2 to 26.8% on Day 4 of pregnancy. On the other hand, the stage of preimplantation embryos recovered from each Day of pregnancy was unaffected by CC treatment.

Discussion

The results of the present study show both a dose-dependent inhibitory effect of CC and an overcoming effect of hCG on ovarian function in response to a "physiologic" dose of PMSG in immature rats. In addition, a marked alteration of ovarian steroidogenesis prior to fertilization in conjunction with a promotion of degenerate oocytes in CC-treated rats has been associated with the subsequent observation of progressive perturbation of embryo development in preimplantation stage.

Administration of various doses of CC resulted in a dose-dependent inhibition of the ovulatory response with an asscciated decrease in ovarian weight; a dose of 1.0㎎ CC completely blocked the PMSG-induced ovulation. It is generally accepted that CC stimulates the secretion of endogenous gonadotropins via the hypothalamic-pituitary axis[9,10] and consequent induction of ovulation in the human female.[2] However, in the present study, CC did not stimulate but rather substantially inhibited the ovulatory response in rats. This finding reflects the discrepancy of CC effects on ovulation between the species. Furthermore, an intriguing observation of the overcoming effect of hCG on the inhibited ovulation in

CC-treated rats strongly suggests a different action mechanism of CC in the rat model. It seems most likely that CC could inhibit a positive cooperativeness triggered by binding of endogenous estrogens to their receptors at the hypothalamic-pituitary level and may consequently attenuate or block the secretion of endogenous gonadotropins. This concept may also be accounted for by a significant elevation of serum estradiol following the CC treatment.

It appears that an early phase of follicular development ensuring the gonadotropin-induced ovulation is estrogen-dependent and that the administration of CC has detrimental effects on this process. Estrogens have been shown to stimulate ovarian function directly and to promote follicular growth[19,20] and to prevent follicular atresia.[21] The antiovulatory and ovarian weight-limiting actions of CC observed in the present study may, therefore, be viewed as estrogen-antagonistic. Indeed, while CC has been recorded as a mixed estrogen agonist/antagonist on a variety of ovarian function,[7,9] most of its documented actions are of an estrogen-antagonistic nature.[11] It is believed that CC binds to estrogen receptors and translocates these receptors to the nucleus. CC-estrogen receptor complexes appear to remain in the nucleus for a prolonged period of time with a resultant long-term depletion of the cytoplasmic estrogen receptor pool.[22,23] Consequently, it is postulated that estrogen-agonistic effects of CC are observed within the first day of its administration, while estrogen-antagonistic effects are apparent only later. The results of the present study, in which CC was administered over 3 days and its effects were observed even later, seem representative for the proposed delayed antiestrogenic action.

Current observation of the increased serum estradiol and the decreased progesterone in 1.0㎎ CC-treated rats is thought to be due to its disparate actions on the ovarian cells. Previous studies with the cultured rat granulosa cells have shown that increasing concentrations of antiestrogens including CC augmented the stimulatory effect of FSH on estrogen production in a dose related manner and inhibited the stimulatory effect of FSH on progesterone biosynthesis.[24] Although the precise mode of actions of CC is unknown, it has been indicated that CC enhances FSH-stimulated aromatase activity and disparately suppresses the biosynthesis of pregnenolone, a precursor metabolite of progestins. A decrease in serum androgens observed by increasing the doses of CC in the present study is probably a secondary effect resulted from the insufficient production of progesterone and may, in part, comprise the non-aromatizable androgens, since serum levels of estradiol remained high. On the other hand, the elevated serum progesterone by a single dose of hCG in CC-treated rats is thought to result from the action of this exogenous gonadotropin on the ovarian theca cells and to be a secondary effect of the overcome ovulation and thus increased number/mass of corpus luteum tissue.

At the gamete level, high doses of CC have been reported to exert a direct toxic effect on the developing embryos when administered in the preimplantation stage of the pregnant rats[25] and rabbits.[5] This embryo toxicity was not recovered by estradiol treatment. It has been, therefore, suggested that the direct cytotoxicity following CC treatment may be responsible for the differences between ovulation and conception rates and for the increased spontaneous abortion rates reported in some women treated with CC. However, an exposure of rabbit blastocysts to low doses of CC *in vitro*[5] and the administration of CC to the rats on Day 1 of pregnancy[26] had no adverse effects after subsequent transplantation of blastocysts into the pseudopregnant animals. Although the present study did not examine a direct effect of CC on the developing embryos, the increasing abnormalities in embryonic morphology and development as well as early embryonic loss observed in the CC-treated rats may result most likely from the disturbed ovarian steroid milieu rather than the direct cytotoxicity of this compound. It has been shown that a sustained elevation of circulating preovulatory estradiol is detrimental to embryo development presumably through a direct action on the preovulatory oocytes; the effect could be partially restored by an antisera to estradiol.[27] Our recent study also indicates that administration of tamoxifen citrate, related to CC in its chemical structure, in PMSG-primed rats promotes the degeneration of ovulating oocytes presumably by an elevating the circulating estradiol.[28] In the present study, it is evidenced that a promotion of degenerate oocytes after the CC treatment has been followed by a significant increase in serum estradiol. Similarly, an exposure of CC to the cultured preovulatory follicles exhibited dose-dependent atretic-like changes in the rats.[29] On the other hand, the oviducts under the influence of high levels of ovarian and circulating estradiol have been shown to secrete a low molecular weight substance to inhibit embryo development in mice[30] and rabbits.[31] Thus, it may be possible that an elevated or sustained preovulatory estradiol following the CC treatment contributes to the abnormalities of preimplantation embryo development by producing a hostile oviductal environment. Such a secondary impact on the oviduct, in association with increased cir-

culating estradiol, has recently been suggested by a study using another antiestrogen, tamoxifen citrate in the rats.[28]

Summary

The effects of CC the ovulatory response, oocyte normality, ovarian steroidogenesis and subsequent embryo developmental potential were examined in PMSG-treated rats. On Days of 25～27 of age, immature female Sprague Dawley rats were treated with three different doses(0.05, 0.1 or 1.0㎎/day) of clomiphene citrate or vehicle. The females subsequently received 4IU PMSG on Day 28 and/or 10IU hCG on Day 30, and were killed on Day 31. Some females given 0.1㎎ CC or vehicle with 4IU PMSG were then mated and killed on Days 2, 3, 4 and 5 of pregnancy. Compared to vehicle(control) group, by increasing the doses of CC, there were a significant decrease in the ovulatory response as judged by both the proportion of rats ovulating and the mean number of oocytes per rat and a marked reduction of ovarian weight. The increasing doses of CC substantially promoted the degeneration(%) of oocytes ovulating in a dose-dependent manner. The CC-mediated inhibitions of the ovulatory response and ovarian weight were completely overcome by a subsequent treatment of hCG. Increasing doses of CC resulted in a siginificant elevation of serum estradiol with the decreased levels of progesterone and androgens. The additive treatment with hCG was effective to reduce the elevation of estradiol and to increase the reduction of progesterone produced by high dose(1.0㎎) of CC. The preimplantation embryos recovered from 0.1㎎ CC-treated pregnant rats demonstrated a progressive early loss from Day 3 of pregnancy with a significant increase in the percentage of degeneration during all periods examined, compared to controls. The rate of progressive embryo cleavage in the CC-treated rats were slower than that in controls from Day 3 of pregnancy. Additionally, the percentage of the cleaved embryos recovered from the CC-treated rats remained significantly lower consistently from Day 2 of pregnancy, compared to control regimen.

These results demonstrate a possible mechanism of CC-mediated inhibition of ovulatory response in the rats which may include the attenuation or blockade of the endogenous secretion of gonadotropins and also suggest that its detrimental effects observed on oocyte normality and embryonic development may be caused by abnormal follicular steroidogenesis(especially elevated estradiol) preceding fertilization.

Legends for figures

Fig 1. Morphology of normal and abnormal oocytes recovered from oviducts after treatment with vehicle (a) or clomiphene citrate (b-f), × 400. a) Normal appearing oocyte with the first polar body ; b-d) Abnormal oocytes with different sizes of ooplasmic fragments ; e) Abnormal oocyte with contracted ooplasm. A part of ooplasm is pulled away from the fractured zona pellucida ; f) Abnormal oocyte with "empty zona" structure.

Fig 2. Serum levels of estradiol-17β, androgens and progesterone after treatment with Veh or different doses(0.05㎎, 0.1㎎, 1.0㎎) of CC in 4IU PMSG-treated immature rats. Values are given as the mean±SE(n=7). $^{*}p<0.05$ compared to control.

Fig 3. Serum levels of estradiol-17β, androgens and progesterone after treatment with Veh, CC or CC+hCG in PMSG-treated immature rats. Values are given as the mean±SE(n=7-13). $^{**}p<0.01$ compared to control(Veh).

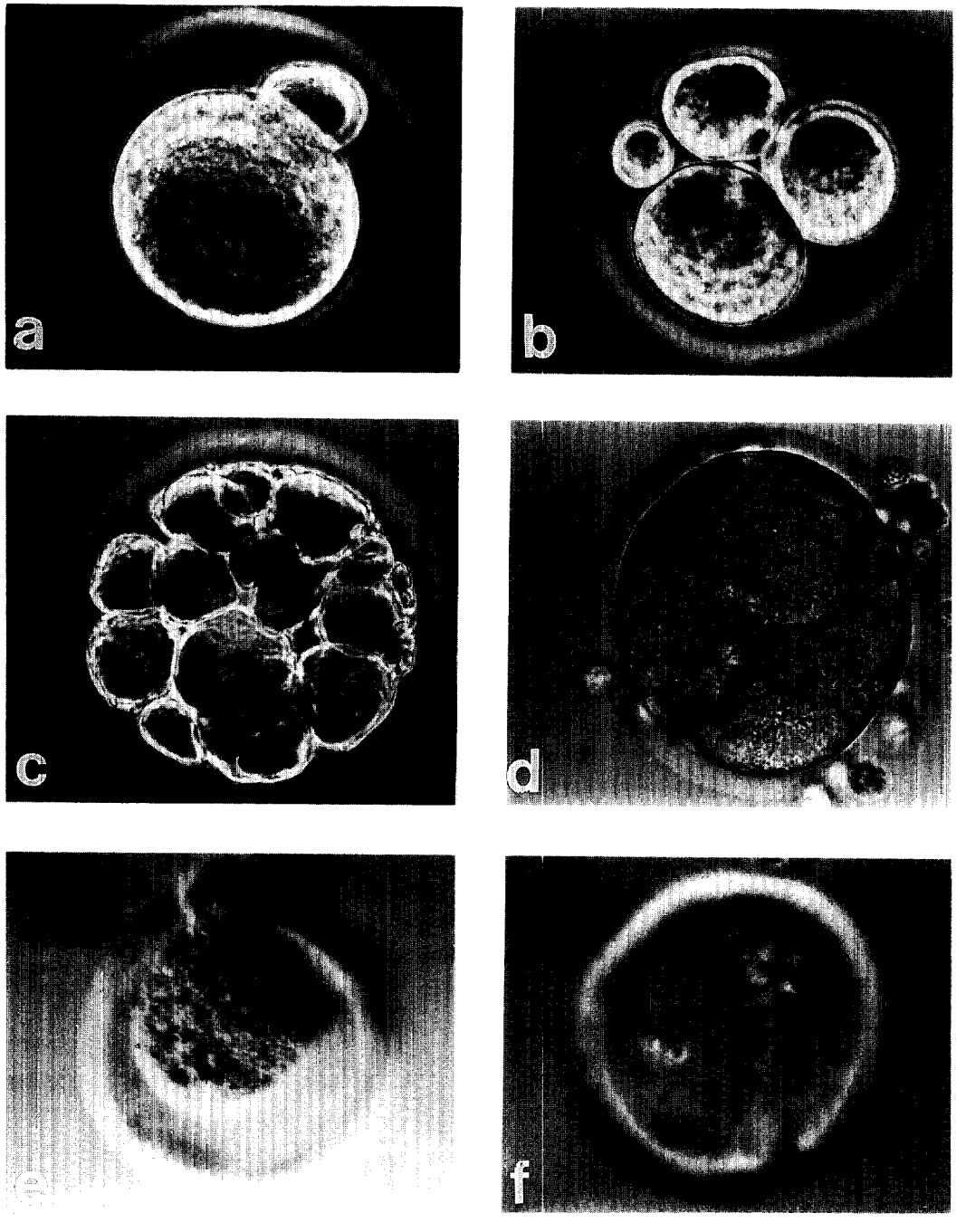

Fig. 1.

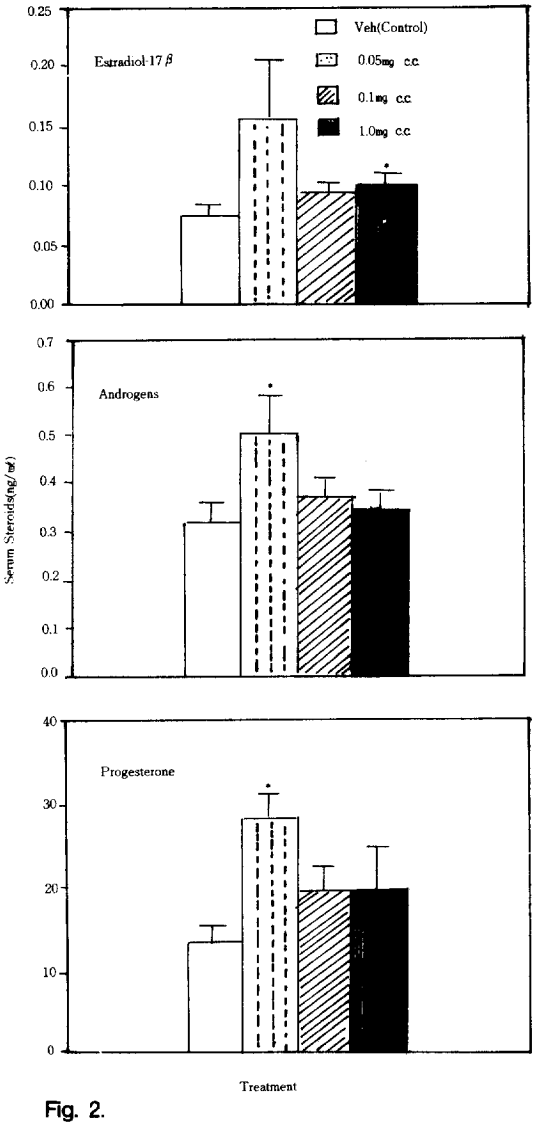

Fig. 2.

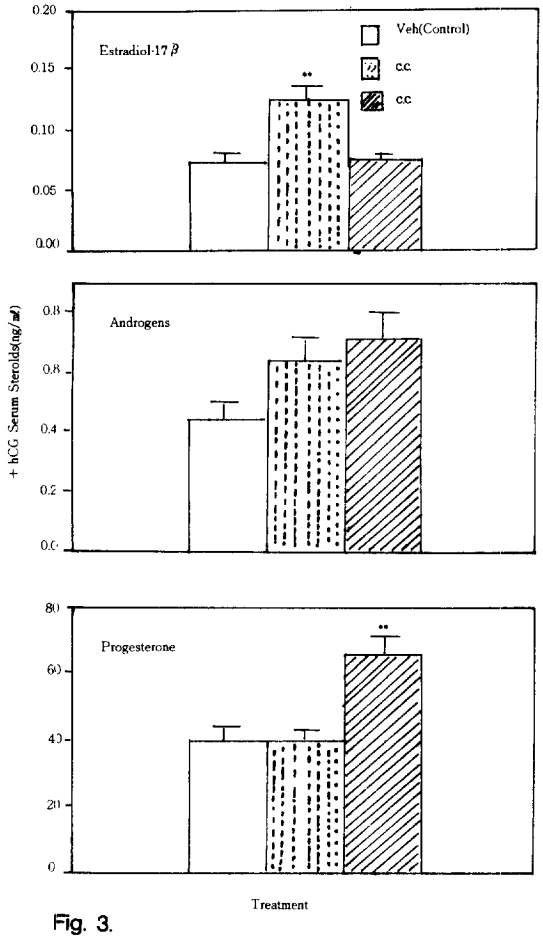

Fig. 3.

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