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Estrogenic and antiestrogenic properties of clomiphene citrate in laboratory mice

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Abstract. The estrogen agonistic and antagonistic properties of clomiphene citrate were investigated in the mice. Clomiphene citrate was tested at various doses of 0·1, 1·0, 10 and 100 µg for three consecutive days in immature and mature bilaterally ovariectomized mice. Clomiphene citrate showed uterotrophic activity in both immature and ovariectomized conditions. The lower doses of 0·1 and 1·0 µg were ineffective to show any uterotrophic stimulation. Clomiphene citrate at 10 µg dose produced 305·56% increase in uterine weight i.e., 27·70 ± 0·24 vs 6·83 ± 0·06 in immature and 182·27% i.e., 42·68 ± 1·12 vs 15·12 ± 0·57 in ovariectomized mice. Clomiphene citrate at 100 µg dose showed significant uterotrophic effect e.g., 435·57% i.e., 36·58 ±0·34 vs 6·83 ± 0·06 in immature and 586% i.e., 103·80 ± 0·60 in ovariectomized mice· When clomiphene citrate was administered in combination with 0·32 µg of estradiol 17-ß it caused significant antagonistic effect (decrease in uterine weight) at 10 and 100 µg respectively. Clomiphene citrate at 10 µg dose produced 32% i·e·, 28·93 ± 0·43 vs 38·04 ± 2·68 in immature and 35% i.e., 59·64±1·44 vs 83·34 ±0·25 in ovariectomized mice respectively. Histological observation clearly showed that clomiphene citrate at 10 and 100 µg doses did not cause any differential hypertrophy of the epithelial layer. Similar doses in combination with estradiol produced significant antagonistic effect on uterine weight and luminal epithelial cell height.

Keywords. Clomiphene citrate; mice; estrogenic; antiestrogenic; uterus.

1. Introduction

Clomiphene citrate (CC) is a nonsteroidal antiestrogen which also possesses weak estrogenic properties. The degree of agonistic and antagonistic activity observed depends on the species, organ, tissues or cell type that is being examined and on the end point assay chosen. CC has been used extensively to induce ovulation in cases of amenorrhea and oligomenorrhea (Greenblatt *et al* 1961, 1962; Charles *et al* 1963; Roy *et al* 1963; Riley and Evans 1964)· It has also been successfully used in the treatment of several clinical complications involving endometrial hyperplasia, persistent lactation and precocious puberty in the human female (Charles 1962; Whitelaw 1963; Kasier 1963). Treatment of adult rats with CC for 21 days results in a transient cessation of estrous cycle (Barnes and Meyer 1962). In immature mice and ovariectomized (OVX) rats CC exhibited the estrogenic activity by increasing uterine weight. CC and its analogue, ethamoxytriphetol (MER -25),

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Abbreviations used: CC, Clomiphene citrate; OVX, ovariectomized; FSH, follicle stimulating hormone; LH, luteinizing hormone; LHRH, luteinizing hormone releasing hormone; E, estradiol.

were initially described as inhibitors of gonadotropin secretion in the rat (Holtkamp *et al* 1960), but when they were tried in women the opposite situation was observed (Greenblatt *et al* 1961). This type of anomaly was attributed to the species differences in treatment protocols (Clark and Markaverich 1982). Inhibition of reproduction was observed with CC in cycling rats (Holtkamp *et al* 1960) which was due to the decreased secretion of gonadotropins. Later it was suggested that high doses of CC inhibit follicle stimulating hormone (FSH) and luteinizing hormone (LH) release and at lower doses stimulate release/facilitate luteinizing hormone releasing hormone (LHRH) action (Schally *et al* 1970). Several other studies showed that low doses of CC not only increase gonadotropins secretion but also cause ovulation in the intact rat (Coppola and Perrine 1965; Koch *et al* 1971; Nagel *et al* 1970). Hsueh *et al* (1978) and Docke (1960) contradicted each other on the effect of CC on ovulation in rats. The effect of CC is very clear and it can induce ovulation and increase blood level of gonadotropins which is also correlated with follicular growth and elevation of estrogen secretion (Rebar *et al* 1976; Ross *et al* 1970; Yen *et al* 1970). Most of the studies on ovulation stimulating effect of CC were done in rats or human. But, unfortunately the effects are just opposite to each other (Holtkamp *et al* 1960; Greenblatt *et al* 1961)· The mechanism of action of CC on ovulation are not yet understood and seems to be more complex than the normal mechanisms by which endogenous hormones function. An antiestrogenic property of this compound may be focussed to the ability of CC to bind to estrogen receptors to stimulate the gonadotropin secretion· In mice it was shown to be mainly estrogenic (Emmens 1965; Pollard and Martin 1968; Terenius 1971).

There is no report of any mechanism of action of the drug in mice· Most of the works done earlier used CC at very high doses for a prolonged duration, whatever the experimental system may be. But the minimal dose and minimal duration required to elicit an observable and significant basal physiological response is not known. This led us to study the minimal dose and duration required to elicit a physiological response and the impact of this minimal dose on the uterine physiology. Results presented in this paper address the first part of our goal, i.e., to study the minimal dose of CC and the duration required to elicit a physiological response. So we have studied the action of CC in a short regimen schedule to test its estrogenic/antiestrogenic activities to explore in the mouse species.

2. Materials and methods

2.1 *Animals*

Immature and adult female Parkes strain mice weighing 15 and 30 g obtained from the departmental stock and housed under temperature and light controlled (14 : 10 LD) room. They were maintained in a well ventilated animal quarter· They had free access to water and commercially available food (Lipton India)· The adult mice were bilaterally OVX 7 days before the beginning of treatment. The animals were divided at random groups of atleast 5 animals.

2.2 *Treatment conditions*

To determine estrogenic activity, various doses of CC, i.e., 0·1, 1·0, 10 and 100 µg/day/animal was administered intraperitoneally in 0·1 ml saline for 3 consecutive

days and on the 4th day animals were sacrificed. To determine antiestrogenic activity, injections containing a standard dose (0·32 µg daily) of estradiol 17-ß (Sigma, USA) administered subcutaneously in olive oil along with various doses of CC (0·1, 1·0, 10 and 100 µg) for 5 consecutive days· Animals were sacrificed 24 h after the last injection and uteri were removed, weighed and fixed in Bouin's fixative for routine histological studies.

2.5 *Statistics*

Uterine weights were expressed on the basis of 15 g in immature or 30g body weight of adult mature OVX mice presented as mean ± SD. The significance of differences between groups was evaluated by Dunnett test. Percentage changes in the weights of uteri were calculated with the formulae, % inhibition = 100 × [1 – (CE –C)/(E–C)] and % increase = l00×CC–C/C, where symbols indicate mean weights of organs from animals treated with vehicle (C), estradiol (E), CC, and a combination of CC and E (CE).

2.4 *Histology*

Uteri were fixed in Bouin's fixative, dehydrated in alcohol and embedded in paraffin. Three uteri from each of the treatment groups were taken for histological examination by haematoxylin-eosin staining. Five serial sections from the mid-horn of each uterine horn were compared. Luminal epithelial cell heights were determined with an ocular micrometer at × 400, calibrated with a stage micrometer. The results were expressed in µm.

3. Results

3.1 *Immature female mice*

3.1a *Estrogenic effect:* CC administered alone, at a dose of 10 and 100 µg to immature mice caused an increase in uterine weight (table 1). A significant increase in the uterine weight (455%) was observed at a dose of 100 µg when compared to the control. There was no agonistic effect when CC was used at a dose of 1 µg or less.

Uterine histology of animals treated with 0·32 µg estradiol for three consecutive days indicated stimulation of all the three cell types i.e., epithelial, stromal and myometrial. CC treated immature mice showed almost same results like estradiol. As it is indicated in table 5, there is no response in relation to epithelial cell height, to lower doses of CC. No significant difference could be found when the control is compared to the animals treated at doses of 0·1 and 1· 0 µg. However significant difference could be found at higher doses i.e., a two-fold increase was found with 10 µg and four-fold increase with 100 µg when compared to the control.

3.1b *Antiestrogenic effect:* Effect of CC administered in combination with estradiol is indicated in table 1· Antiestrogenic effect of CC on uterine weight at daily doses of 0·1, 1·0, 10 and 100 µg was observed and the results indicate that there is a

Table.1. Estrogenic and antiestrogenic effects of clomiphene citrate administered to immature mice·

	Uterine weight (mg/15 g)	
Vehicle control	06·83 ± 0·06	
Estradiol (E_2) (0·32 µg/animal/day) (E_2 control)	38·04 ± 2·68*	
	$-E_2$	$+E_2$
Clomiphene citrate (µg)		
0·1	06·54 ± 0·12	36·53 ± 0·18 (5)
1·0	07·18 ± 0·78	30·78 ± 1·53 (23)
10·0	27·70 ± 0·24*	28·93 ± 0·43** (32)
100·0	36·58 ± 0·34*	22·36 ± 0·71** (50)

Values are presented as means ± SD.
*P < 0·01 compared with vehicle control.
**P < 0·01 compared with E_2 control (Dunnett test)· Values in parentheses are percentage inhibition calculated with the formula: 100 × [1 - (C E - C) / (E – C)] , where symbols indicate mean uterine weights from animals treated with vehicle (C), E_2 (E) or a combination of clomiphene citrate and E_2(CE). Animals (n = 5) were treated once daily for 3 days with varying doses of clomiphene citrate either alone ($-E_2$) or in combination with + E_2 (+ E_2).

dose dependent inhibition of uterine weight when compared to estradiol treated uterine horns. Comparison between the animals treated with different doses indicates a general increase in inhibitory effect from 0·1 to 100 µg and the difference of inhibitory effect is notable between 0·1 and 1·0 and between 10 and 100 µg. Table 3 shows the effect of CC on luminal epithelial cell height. As is seen in the case of uterine weight, here also CC showed inhibitory effect. No antiestrogenic activity was seen at doses 0·1 and 1·0 µg, but there was a considerable decrease in luminal epithelial cell height at doses 10 and 100 µg when compared to estradiol treated mice. The epithelial cell height of vehicle control and estradiol treated mice was 12·32 ± 0·51 and 35·28 ± 1·60 respectively.

3.2 *OVX adult mice*

3.2a *Estrogenic effect:* The estrogenic effect of CC in OVX mice is given in table 2 and it indicates that the stimulatory effect of CC at doses 0·1 and 1·0 µg is insignificant. Whereas, at doses of 10 µg or more it stimulated significant increase in uterine growth and it increases 586% of the control at a dose of 100 µg.

Table 4 shows the effect of CC on uterine luminal epithelial cell height. CC stimulates all uterine cell layers like estradiol is also observed in immature mice. Though a slight decrease in the luminal epithelial cell height is seen in the animal treated with 1·0 µg when compared to the one treated with a dose of 0·1 µg, there

Table 2. Estrogenic and antiestrogenic effects of clomiphene citrate administered to ovariectomized mice.

	Uterine weight (mg/30 g)	
Vehicle control	15·12 ± 0·57	
Estradiol (E_2) (0·32 µg/animal/day) (E_2 control)	83·34 ± 0·25*	
	$-E_2$	$+E_2$
Clomiphene citrate (µg)		
0·1	13·92 ± 0·52	—
1·0	14·89 ± 0·71	—
10·0	42·68 ± 1·12*	59·64 ± 1·44** (35)
100·0	103·80 ± 0·60*	46·71 ± 1·93** (54)

Same notations as in table 1.

Table 3. Effect of clomiphene citrate on uterine luminal epithelium cell heights (µg) in immature mice after 3 days of treatment.

Treatment	Luminal epithelial cell height	
Vehicle control	12·32 ± 0·51	
Estradiol 0·32/µg/animal/day (E_2)	35·28 ± 1·60	
	$-E_2$	$+E_2$
Clomiphene citrate (µg/animal/day)		
0·1	12·94 ± 1·70	36·77 ± 2·70
1·0	13·54 ± 1·26	39·32 ± 1·32
10·0	24·22 ± 0·51	24·22 ± 0·47
100·0	37·52 ± 2·42	19·74 ± 1·24

Values represented mean ± SEM for 20 determinations. Animals were treated once daily for 3 days with varying doses of clomiphene citrate either alone ($-E_2$) or in combination with ($+E_2$).

is a general increase in the luminal epithelial cello height as there is increase in the doses applied when compared to the vehicle control.

3.2b *Antiestrogenic effect*: Table 2 indicates the antiestrogenic effect of CC in combination with estradiol on uterine weight At lower doses of 0·1 and l·0 µg, CC had no antiestrogenic activity. However, the effect was significant at doses 10 and 100 µg when compared to estradiol treated mice· Animals treated with 10 and 100 µg showed 35% and 54% decrease in uterine weight respectively.

Table 4. Efect of clomiphene citrate on uterine luminal epithelium cell heights (μg) in ovariectomized mice after 3 days of treatment.

Treatment	Luminal epithelial cell height	
Vehicle control	12·74 ± 0·52	
Estradiol 0·32 μg/animal/day (E_2)	34·58 ± 2·90	
	$-E_2$	$+E_2$
Clomiphene citrate (μg/animal/day)		
0·1	22·26 ± 1·34	36·18 ± 0·96
1·0	20·58 ± 1·69	37·16 ± 1·29
10·0	23·80 ± 1·14	23·00 ± 0·96
100·0	36·12 ± 2·55	21·56 ± 0·96

Same notations as in table 3.

CC, at lower doses of 0·1 and 1·0 μg, did not show any antiestrogenic effect on luminal epithelial cell height (table 4). However, the antiestrogenic action was prominent at higher doses of 10 and 10 μg when compared to estradiol control. The luminal epithelial cell height of vehicle control and estradiol control was 12·74 ± 0·52 and 34·58 ± 2·90 respectively.

4. Discussion

Our results indicate that CC manifests both estrogenic and antiestrogenic properties in immature and OVX animals. In contrast to earlier reports we observed responses with very low doses i.e., 10 and 100 μg instead of 40 mg/kg/day for 20 days (Roy *et al* 1964). The minimal concentration used by Roy *et al* (1964) was 0·5 mg/kg/day in rats. The statistical calculation indicates that the minimum concentration used by others is the maximum in our experimental design.

Earlier reports indicated that estrogenic action of CC, as measured by uterotrophic effect, was very weak in comparison to natural estrogen in rats. The present study clearly shows that responses of CC is similar to that of 0·32 μg of estradiol on promoting uterine stimulation. In case of luminal epithelial cells, there was a general and significant increase of height. When the immature mice were treated with 0·1 and 1·0 μg of CC there was no significant effect on luminal epithelial cell height in comparison to the vehicle control. But, when they were treated with 10 and 100 μg of CC, the cell height increased. If the data so obtained is compared to that of OVX mature mice, it becomes clear that the animals responded to all the doses administered by increasing the luminal epithelial cell height progressively. The results obtained indicate that the immature mice have not developed a stronger competence for CC (estrogenic action) and in the mature mice, the luminal epithelial cells developed a stronger competence as evidenced by the response shown by OVX mature mice to all the doses treated, starting from. 0·1 to 100 μg and the response shown by immature mice only to higher doses i.e., 10 and above. In general, it indicated that even if the hormonal status of an immature mice is

changed it may not respond to the expected reproductive status, or else, it may require very high dose of CC (for agonistic action) to elicit a proper response.

The most important observation we find is that CC, like estradiol, stimulates all the three layers of the uterus i.e., epithelium, stroma and myometrium. Lower doses (0·1 and 1·0 µg) were ineffective but 10 µg showed first evidence of such trophic effect. Dose level of 100 µg showed a significant uterotrophic effect in comparison to 0·32 µg of estradiol 17-β. However, it is clear from our results that though CC shows hypertrophy of uterine layers comparable to estradiol its action on epithelium cell hypertrophy is same as that of the action of estradiol (tables 3 and 4) in both immature and OVX mice. This finding is contrary to the observation of the rat (Clark and Guthrie 1981) where uterine growth obtained with estrogen far exceeds than that of CC. In rat CC can induce differential hypertrophy of the luminal epithelial cell layer but in mouse at least at the dose level of 100 µg no differential stimulation of CC is observed. Though the property of differential stimulation of uterine epithelial cells appear to be common for all triphenylethylene compounds (Emmens and Carr 1973; Kang *et al* 1975; Terenius and Ljungkvist 1972; Karkun and Mehrotra 1973; Jordan *et al* 1979; Clark and Peck 1979), it is not observed in mice as indicated in our results.

Another inference that can be derived from the results is that CC is estrogenic and antiestrogenic over the same dose ranges in the uterus (tables 1 and 2). The suggestion given earlier by many investigators that CC is antagonist at lower doses to stimulate gonadotropins secretion and ovulation is not true in mice· In rat Clark and Guthrie (1981) also observed same trend in CC response· In mice estrogen antagonism is found only when both are given conjointly for atleast three days. Antagonistic effect is not seen by single injection of CC (unpublished data). However, the antiestrogenic effect of CC is more prominent in the diminution of the epithelial cell height than in reduction of uterine weight.

Earlier it has been proposed that CC is antagonistic at lower doses and agonistic at higher doses (Boyar 1970; Koch *et al* 1971; Nagel *et al* 1970; Schally *et al* 1970; Schulz *et al* 1972) in many species. CC binds to estrogen receptors at low doses and block negative feedback of endogenous estrogens resulting gonadotropins secretion. At high doses it binds the estrogen receptors and act as estrogen and blocks gonadotropin secretion.

From our results it reveals that the action of CC is like a true mixed agonist and antagonist because it showed both estrogenic and antiestrogenic action over the same range of doses. From the literature it is evident that in mice CC is antiestrogenic at a comparably very low doses· Thus we can expect a progonadotrophic activity at this dose range because CC is believed to stimulate gonadotropins at low concentration. CC is agonist when applied alone but in combination with estradiol it is antagonist at the doses which were agonist when given alone. This observation is in sharp contrast to that of Martin *et al* (1973) and Martin (1980) who claimed no antagonistic activity of CC in mice. We again emphasize that effect of CC depends on the species, tissue type and experimental design to interpret its action. We have started this work on CC presuming it as progonadotropin to explore its embryotoxic and antiimplantation effect/side effect in 'mouse model'. This is the first evidence to establish CC as antiestrogenic i.e., antagonist in mice. We are now studying whether this antagonistic action of CC as can be extrapolated as a progonadotropic action of it. Its progonadotrophic activity is common in many

species including rat· From our present findings it appears that the mechanism of CC action in mice may be different from that of rat· It needs more clarification.

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