

Duration of anti-implantation action of the triphenylethylene anti-oestrogen centchroman in adult female rats

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The duration of the anti-implantation action of a single oral post-coital dose (1.25 mg kg^{-1}) of a triphenylethylene anti-oestrogen, centchroman, was determined in adult rats. The effects of centchroman were compared with those of tamoxifen. In rats undergoing delay, centchroman administered orally on day 7 post-coitum prevented the induction of implantation of delayed blastocysts by an implantation inducing dose ($1 \mu\text{g}$ per rat, s.c.) of oestrone which was administered earlier than 120 h after centchroman treatment. In tamoxifen (0.2 mg kg^{-1} , orally) pretreated rats, oestrone administered at 144 h or later induced implantation. In cyclic rats treated with centchroman at intervals of 168 h and mated with males of proven fertility, implantation was prevented only when the interval between centchroman treatment and nidatory oestrogen secretion was less than 120 h. None of the females conceived when treated regularly at intervals of 120 h during exposure to fertile males. Discontinuation of treatment resulted in the occurrence of normal implantations in rats that mated 48 h or later after the last dose of centchroman, since in these animals the interval between anti-oestrogen treatment and nidatory oestrogen secretion was greater than 120 h. These findings suggest that the duration of the anti-implantation action of a single oral antifertility dose of centchroman in rats is about 120 h. Recovery of normal blastocysts from rats treated continuously with this dose of centchroman at these intervals suggests lack of significant effect on follicular maturation, ovulation, fertilization, preimplantation development or mating behaviour. Tamoxifen appears to be slightly longer acting and the duration of its action was about 144 h.

Introduction

It has been suggested that the long duration of the action of triphenylethylene anti-oestrogens is due to their storage in body fat (Jordan and Gosden, 1983) or to binding to certain anti-oestrogen binding sites (Sudo *et al.*, 1983) and their gradual and constant release into the system from these storage sites. This results in prolonged availability of anti-oestrogens and translocation of anti-oestrogen–oestrogen receptor complexes into the nucleus and a concomitant depletion of the cytoplasmic oestrogen receptor pool rendering the tissue refractory to subsequent oestrogen action (Dix and Jordan, 1980). According to Clark *et al.* (1973) such anti-oestrogens may occupy nuclei of target cells for up to 19 days. However, studies using uterine responsiveness as an index of oestrogen action in anti-oestrogen pretreated animals revealed that the duration of their action was much shorter (12–48 h) (Katzenellenbogen *et al.*, 1977). Triphenylethylene anti-oestrogens inhibit implantation when administered within 24 h of coitus, by inhibiting the action of nidatory oestrogen secreted late on day 4 post-coitum, without significantly affecting development or viability of preimplantation embryos (Singh *et al.*, 1986; Singh and Kamboj, 1992). This finding suggests a comparatively longer duration of action of anti-

oestrogens than that observed in immature rats using large s.c. doses of both anti-oestrogens and oestrogens.

In view of the potential clinical importance of anti-oestrogens, although determination of the duration of their action is a very important factor in maintaining an effective concentration of the ligand at oestradiol receptors, it may be necessary to produce this information under physiological conditions, doses and by the route by which anti-oestrogens have to be used. In this study the duration of action of a potent triphenylethylene anti-oestrogen centchroman was determined at a single oral anti-implantation dose (1.25 mg kg^{-1} ; Singh and Kamboj, 1992) in intact adult rats. Centchroman provides good pregnancy protection in women on post-coital and weekly regimens (Puri *et al.*, 1988) and partial to complete remission of lesions in some breast cancer patients (Misra *et al.*, 1989). It has recently been marketed as a contraceptive in India. Tamoxifen, already in clinical use for treatment of breast cancer (Gorodeski *et al.*, 1992), was used for comparison.

Materials and Methods

Animals and treatments

Adult female Sprague-Dawley rats (180–220 g) maintained at $22 \pm 1^\circ\text{C}$ with a 12 h light:12 h dark photoperiod

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Table 1. Duration of anti-implantation action of centchroman and tamoxifen in adult female rats under experimental delay

Treatment on day 7 post-coitum	Day post-coitum of oestrone administration	Time after anti-oestrogen treatment (h)	Rats pregnant/treated	Implantations
Vehicle	9	48	10/11	65
Centchroman			0/8 ^b	—
Tamoxifen			0/8 ^b	—
Vehicle	10	72	5/6	31
Centchroman			0/6 ^b	—
Tamoxifen			0/6 ^b	—
Vehicle	11	96	5/5	29
Centchroman			0/6 ^b	—
Tamoxifen			0/6 ^b	—
Vehicle	12	120	5/7	14 ^d
Centchroman			4/7 ^{c,d}	7
Tamoxifen			0/6 ^b	—
Vehicle	13	144	4/5	13 ^d
Centchroman			4/17 ^a	5 ^a
Tamoxifen			3/16 ^a	4 ^a
Vehicle	16	216	2/6 ^d	3 ^{e,f}
Tamoxifen			1/6	3

Centchroman: 1.25 mg kg⁻¹, orally; tamoxifen: 0.2 mg kg⁻¹, orally; oestrone: 1 µg per rat, s.c.; —: none. ^a*P* < 0.05,

^b*P* < 0.01 versus corresponding vehicle pretreated group. ^c*P* < 0.05 versus corresponding tamoxifen-pretreated group.

^d*P* < 0.05, ^e*P* < 0.01 versus corresponding days 9, 10 or 11 groups. ^f*P* < 0.05 versus corresponding day 13 group. All other relevant comparisons were statistically insignificant.

and showing at least two regular 5 day cycles were used. Females were mated with males (4:1) of proven fertility and the day on which a sperm positive vaginal smear was obtained was designated day 1 of pregnancy. Animals received pellet diet (Lipton India Ltd, Bangalore) and tap water *ad libitum*.

Centchroman (synthesized at this Institute) and tamoxifen (Sigma Chemical Co., St Louis, MO) suspended in distilled water using gum acacia were administered orally. Progesterone (Schering AG, Berlin) and oestrone (Sigma) were administered s.c. in olive oil. Control animals received vehicle(s) alone.

Experimental design

The time of luteal phase nidatory oestrogen secretion was determined by ovariectomizing mated rats at timed intervals beginning at 10:00 h on day 4 post-coitum, followed by administration of progesterone (4 mg day⁻¹). Animals without implantation(s) on day 10 post-coitum were supplemented with oestrone (1 µg day⁻¹) on days 10–12 post-coitum. The number and status of implantations were recorded on day 13.

The duration of action of centchroman was determined in rats under experimental delay as well as in cyclic rats. Delayed implantation was induced by ovariectomy on day 3 post-coitum followed by administration of progesterone (4 mg day⁻¹). Since preimplantation rat embryos do not enter the uterus until late on day 4 post-coitum (Singh *et al.*, 1986), care was taken not to disturb oviducts during ovariectomy. Each rat received a single oral anti-implantation dose of centchroman (1.25 mg kg⁻¹ body weight; Singh and Kamboj, 1992), tamoxifen (0.2 mg kg⁻¹ body weight; Harper and Walpole, 1967) or vehicle on day 7 post-coitum and an implantation-inducing dose of oestrone (1 µg per rat; Sreenivasulu *et al.*, 1992) between day 9 and day 16 post-coitum (Table 1). The number and status of implantations were recorded 48 h later. Tamoxifen was used for comparison in this study only.

Adult cyclic rats were treated orally with centchroman (1.25 mg kg⁻¹ body weight) or vehicle at intervals of 168 h. After at least two such treatments, females were caged with males. Females mated on each day after vehicle or centchroman treatment were divided into two groups. Mated animals of only one group received respective treatment on the next scheduled day (Table 2; Fig. 1a). Animals of the second group did not receive any treatment after coitus (Table 3).

Table 2. Effect of centchroman (1.25 mg kg^{-1} , orally) administered at intervals of 168 h on fertility performance in cyclic rats: centchroman also administered on next scheduled day in mated animals

Day of mating ^a	Number of mated rats	Number of pregnant rats ^b	Total corpora lutea ^b	Total implantations ^b	Unimplanted embryos ^c					Fetal weight (g)
					Total embryos	Stage of development	Zona pellucida (+/-)	Number of rats in litter	Duration of gestation (days)	
2	8	0	88	0	6 (7) ^d	Expanded blastocysts	+ 3	—	—	—
3	10	6	108	51 (47) ^d	1 (1)	Expanded blastocysts	- 3	6	22.4 ± 0.6 ^e	4.9 ± 0.6 ^e
4	7	5	78	53 (68)	0	Expanded blastocysts	- 1	5	22.0 ± 0.0	5.1 ± 0.6
5	6	0	74	0	13 (18)	Expanded blastocysts	- 13	—	—	—
6	5	0	53	0	15 (28)	Expanded blastocysts	- 15	—	—	—
7	7	0	62	0	7 (11)	Expanded blastocysts	- 7	—	—	—
8	5	0	47	0	3 (6)	Expanded blastocysts	- 3	—	—	—

In vehicle control group, 42 (91%) of 46 rats mated at different times became pregnant and had an average of 10.4 ± 2.2 corpora lutea and 10.0 ± 2.1 implantations, 86% of which developed to term fetuses.

Mean gestation duration was 22.0 ± 0.0 days and fetal weight was 5.0 ± 0.2 g.

^aDay after centchroman treatment. ^bDay 10 post-coitum. ^cOnly uteri of rats without implantations were flushed. ^dPercentage of total corpora lutea. ^eMean ± SD. ^fPercentage of normal implantations in rats that produced litters.

Zona pellucida: +, present; -, absent.

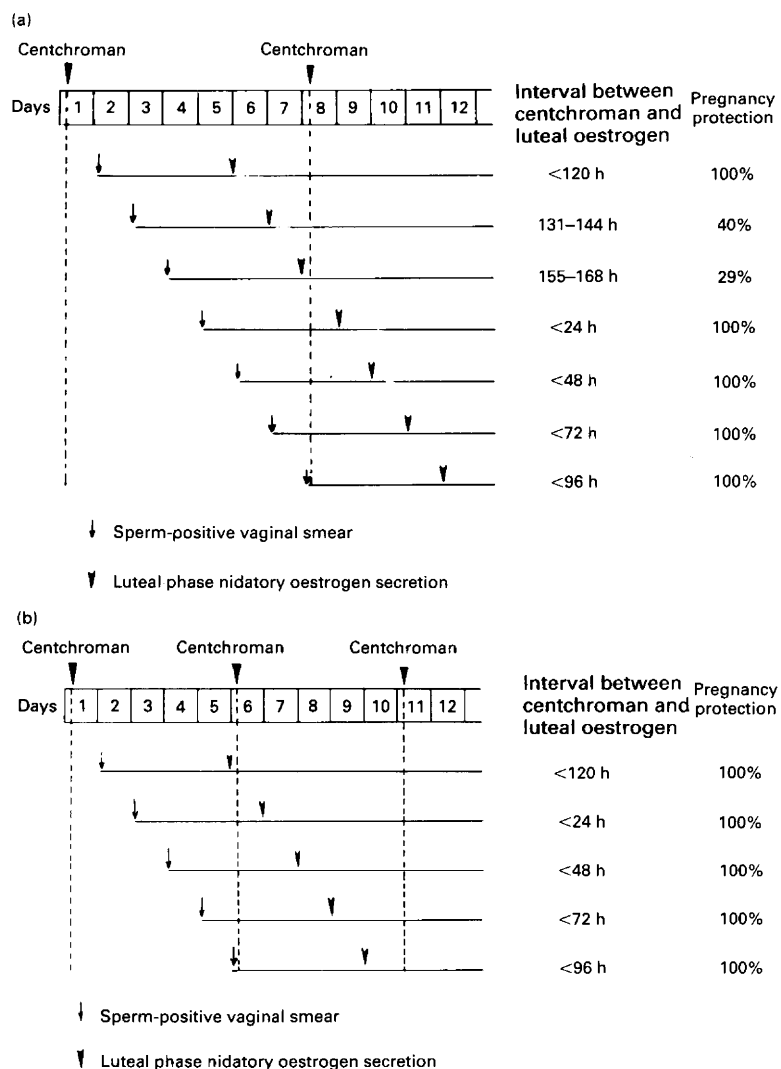


Fig. 1. Pregnancy protection in rats treated continuously with centchroman (1.25 mg kg^{-1} , p.o.) at (a) 168 h or (b) 120 h intervals. Centchroman was administered also on next scheduled day in mated rats. Note 100% pregnancy protection was obtained only when the interval between anti-oestrogen administration and nidatory oestrogen secretion was < 120 h.

The number and status of corpora lutea and implantations were recorded on day 10 post-coitum. Rats with at least one normal implantation were kept and the duration of their gestation and number and weight of fetuses were recorded. Animals without implantation(s) were autopsied on day 10 and their uteri flushed (Singh *et al.*, 1986) and the developmental stage and status of embryos were recorded. Ovaries of each animal were fixed in Bouin's fixative and sections ($5 \mu\text{m}$) were stained with haematoxylin and eosin.

Details of this experiment were the same as in the preceding experiment, except that here centchroman (1.25 mg kg^{-1}) or vehicle were administered at intervals of 120 h (Table 4; Fig. 1b). In addition to embryo collection and ovarian histology, plasma oestradiol and progesterone concentration on day 10 post-coitum were measured by radioimmunoassay using kits supplied by WHO (Geneva) and an LKB 1217 RACKBETA Liquid Scintillation Counter.

Statistical analysis

Results were analysed by Mann–Whitney *U* test.

Results

Time of luteal phase nidatory oestrogen secretion

Ovariectomy performed up to 21:00 h on day 4 post-coitum induced delay of implantation, since in most rats implantation of delayed blastocysts could be induced by oestrone administration on days 10–12 post-coitum. No delay was evident when the time of ovariectomy was postponed until 10:00 h on day 5 and apparently normal implantations were observed on day 10 in rats supplemented with progesterone *per se*.

Table 3. Effect of centchroman (1.25 mg kg^{-1} , orally) administered at intervals of 168 h on fertility performance in cyclic rats: no centchroman treatment post-coitally

Day of mating ^a	Number of rats that mated	Number of pregnant rats ^b	Total number of corpora lutea ^b	Unimplanted embryos ^c					Duration of gestation (days)	Number of fetuses	Fetal weight (g)
				Total implantations ^b	Number of rats with embryos	Total number of embryos	Stage of development	Zona pellucida (+/-)			
2	6	0	73	0	4	18 (25) ^d	Expanded blastocysts	+6 -12	—	—	—
3	6	4	50	26 (52) ^d	1	6 (12)	Expanded blastocysts	-6	4	19 (73) ^f	5.0 ± 0.0 ^e
4	5	4	41	27 (66)	1	1 (2)	Expanded blastocysts	-1	4	22 (82)	5.0 ± 0.0
5	6	6 ^g	54	52 (96)					5	25 (61)	4.9 ± 0.6
6	7	7 ^h	64	59 (92)					6	41 (77)	5.1 ± 0.6
7	8	8	73	73 (100)					8	55 (75)	5.0 ± 0.2
8	6	4	42	38 (91)	0				4	30 (79)	4.9 ± 0.4

^aDay after centchroman treatment. ^bDay 10 post-coitum. ^cOnly uteri of rats without implantations were flushed. ^dPercentage of total corpora lutea. ^eMean ± SD. ^fPercentage of normal implantations in rats that produced litters. ^gOne rat with 11 normal implantations died before delivery date. ^hOne rat with six normal implantations died before delivery date.

Zona pellucida: +, present; -, absent.

Table 4. Effect of centchroman (1.25 mg kg^{-1} , orally) administered continuously at intervals of 120 h on fertility performance in cyclic rats: centchroman also administered on next scheduled day in mated animals

Day of mating ^a	Number of rats that mated	Number of pregnant rats	Total number of corpora lutea ^b	Number of implantations ^b	Unimplanted embryos ^b			
					Number of rats with embryos	Total number of embryos	Stage of development	Zona pellucida (+/-)
2	8	0	97	0	0 ^c			
3	3	0	29	0	1	4	Expanded blastocysts	- 4
4	6	0	65	0	3	8	Expanded blastocysts	- 8
5	5	0	49	0	0			
6	6	0	54	0	0			

^aDay after centchroman treatment. ^bDay 10 post-coitum. ^cUteri of only four rats were flushed.

Zona pellucida: +, present; -, absent.

Duration of action of centchroman and tamoxifen in rats under experimental delay

Oestrone failed to induce implantation of delayed blastocysts in rats pretreated with a single anti-implantation dose of centchroman or tamoxifen when administered 48, 72 or 96 h after treatment with anti-oestrogen. In centchroman pretreated rats, normal implantations were observed after oestrone administration at 120 or 144 h, and with tamoxifen implantations could be induced only in rats receiving oestrone at 144 h or later. Generally, rats pretreated with anti-oestrogen exhibited lower pregnancy and implantation rates (Table 1). Some reduction in pregnancy and implantation rates was also observed in control rats with advancing delay. Implantations in all groups were normal and showed no apparent sign of resorption.

Duration of action of centchroman administered at 168 h intervals in cyclic female rats

In cyclic rats treated orally with a single anti-implantation dose of centchroman at 168 h intervals, pregnancy was prevented in 100% of rats that mated within 24 h of anti-oestrogen treatment (Tables 2 and 3). In these rats, the interval between the secretion of nidatory oestradiol and the preceding dose of centchroman was less than 120 h (Fig. 1a). Normal expanded blastocysts were flushed from their uteri on day 10 post-coitum, but the number of rats with blastocysts (38% versus 66%) and the mean number of blastocysts (2.0 versus 4.5) recovered were significantly lower in animals that received another dose of centchroman on day 7 post-coitum (i.e. at 168 h after the preceding dose of centchroman).

Most (87%) of the rats that had mated between days 3 and 8 after centchroman treatment, which did not receive any centchroman after coitus, conceived and had apparently normal implantations (Table 3). In comparison, in rats that received centchroman on the next scheduled day after coitus, only rats that mated on days 3 and 4 after centchroman treatment became pregnant (Table 2). In animals of all such groups, the interval between the preceding dose of centchroman and secretion of nidatory oestradiol was more than 120 h. The percentage of mated rats that became pregnant and the average

number of corpora lutea and implantations in these animals were similar to those of the control group (Tables 2 and 3). Centchroman provided complete protection from pregnancy in animals that mated between days 5 and 8 and received a scheduled dose of centchroman after coitus, since in these animals the interval between the nidatory oestradiol secretion and the preceding dose of anti-oestrogen (which here refers to centchroman administered post-coitally, Fig. 1a) was between 24 and 96 h only. Normal expanded blastocysts were flushed from uteri of most rats without implantations that received centchroman in either schedule and mated between days 3 and 8.

Anti-fertility action of centchroman administered at intervals of 120 h in cyclic female rats

Continuous administration of centchroman to rats at intervals of 120 h provided complete protection from pregnancy, since none of the animals that mated ($n = 28$, Table 4, Fig. 1b) during this period had implantations on day 10 post-coitum. Normal blastocysts were flushed from uteri of some of these rats (Table 4).

Continuous administration of centchroman to cyclic rats at intervals of 120 or 168 h did not affect their mating behaviour, as evidenced by almost similar numbers of females that mated in various periods in vehicle and anti-oestrogen pretreated rats, ovulation, fertilization, blastocyst formation, percentage of implantations developing to term, duration of gestation or weight of fetuses (Tables 2–4). No change was observed in histoarchitecture of ovaries or plasma oestradiol and progesterone on day 10 post-coitum.

Discussion

The results of the study reported here indicate that the duration of action of the triphenylethylene anti-oestrogen centchroman when administered orally at a single anti-implantation dose to adult female rats under experimental delay or to cyclic rats exposed daily to male rats of proven fertility was about 120 h. Tamoxifen was slightly longer acting with a duration of action of about 144 h. The results also

confirm that such anti-oestrogens prevent implantation by inhibiting endometrial sensitivity to the blastocyst stimulus for decidualization by inhibiting the action of nidatory oestrogen secreted late on day 4 post-coitum, because of their potent anti-oestrogenic property, but without affecting preimplantation development or viability of embryos or ovarian function (Singh *et al.*, 1982, 1986; Singh and Kamboj, 1992). The study reported here, in addition, provides evidence of a lack of any effect of centchroman administered at intervals of 120 h or 168 h on mating behaviour, ovulation or fertilization. No significant difference in fetal development rate, duration of gestation or fetal weight was evident in animals that conceived after an interval between centchroman treatment and nidatory secretion of more than 120 h or when centchroman was not administered on the scheduled day after mating.

The long duration of action of triphenylethylene anti-oestrogens is attributed to their storage in body fat (Jordan and Gosden, 1983) or binding to certain anti-oestrogen binding sites (Sudo *et al.*, 1983). Continuous availability of anti-oestrogens is known to maintain cytoplasmic oestrogen receptor concentrations that are depleted in target tissue over prolonged periods, rendering it refractory to subsequent oestradiol action (Katzenellenbogen *et al.*, 1977). Clark *et al.* (1973), using a single large (100 µg, s.c.) dose of nafoxidine (which is at least ten times greater than the dose needed to prevent implantation in adult rats by oral route; Duncan *et al.*, 1963), demonstrated retention of anti-oestradiol-oestrogen receptor complexes in immature rat uterine nuclei for 19 days, with a concomitant depletion of the cytoplasmic oestradiol receptor pool. But later studies, using uterine responsiveness to oestradiol action in terms of a block in oestrogen induced increase in uterine weight, synthesis of induced protein or [³H]oestradiol uptake by uterine nuclei in immature animals pretreated with an anti-oestrogen, revealed a much shorter duration of anti-oestrogen action, varying between 12 and 36 h for nafoxidine, 24 h for CI 628 (Katzenellenbogen *et al.*, 1977) and 48 h for tamoxifen (Jordan *et al.*, 1978). Abolition of oestrogen action was usually associated with depressed cytoplasmic oestrogen receptors during the duration of their action. It is not known whether such variations in the duration of action of anti-oestrogens are related to use of large (non-physiological) doses of both anti-oestrogens and oestradiol or to their ratio (Jordan and Gosden, 1983; Agarwal and Bindal, 1991). Jordan *et al.* (1978) also observed a relationship between dose of anti-oestrogen and depletion of available cytoplasmic oestrogen receptors in the rat uterus. In view of these observations, it was necessary to obtain data under physiological conditions, doses and the route by which anti-oestrogens would be used to determine a dose-schedule relationship.

The occurrence of a normal number of corpora lutea in all mated rats and implantations in rats in whom the interval between centchroman treatment and nidatory oestrogen secretion was greater than 120 h, together with recovery of normal expanded blastocysts from rats without implantations, provide evidence of a lack of significant effect on follicular maturation, ovulation, fertilization or blastocyst formation in rats treated with this anti-oestrogen in these schedules. It has been suggested (Vaidya *et al.*, 1977) that there is an alteration in the rate of sperm transport based on altered cervical mucus

score and karyopycnotic index pattern in women receiving 60 and 120 mg of centchroman per week (i.e. twice and four times, respectively, the minimum effective dose; Puri *et al.*, 1988). Although it is not possible to compare data on rats with those of humans, in this study centchroman did not appear to affect gamete transport in the female genital tract as fertilization was not affected. Significantly reduced recovery of unimplanted embryos on day 10 post-coitum from the uteri of nonpregnant mated rats is in agreement with our earlier observations (Singh *et al.*, 1986) and may be related to decreased uterine progesterone receptors owing to anti-oestrogenic activity (Castellano-Diaz *et al.*, 1989) of centchroman resulting in reduced tissue availability of progesterone, which is well known to cause myometrial quiescence during pregnancy.

Anti-oestrogens also induce delayed implantation in rats (see Singh *et al.*, 1986). A delay in loss of zonae pellucidae by delayed blastocysts was reported by Rumery and Blandau (1971). We showed that there was a delay in the loss of zonae pellucidae in rats treated post-coitally with centchroman. Recovery of some zona encapsulated blastocysts from uteri of centchroman treated rats even on day 10 post-coitum may be significant in terms of the contraceptive action of such anti-oestrogens.

Clearly, in view of their longer duration of action, suggesting prolonged availability at oestradiol receptors, anti-oestrogens have an advantage in the development as potential contraceptives (Puri *et al.*, 1988) or in treatment of clinical disorders including breast cancer (Misra *et al.*, 1989).

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