Disruption of Rat Estrous Cyclicity by the Environmental Estrogen 4-*tert*-Octylphenol

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Abstract. **4-*tert*-Octylphenol (OP) is a prevalent environmental pollutant which binds to estrogen receptors and exerts estrogenic actions *in vitro*. The effects of OP *in vivo* on mammalian female reproduction are not known. We investigated whether (i) exposure of neonatal rats to OP interfered with the onset of vaginal opening or their ability to have regular estrous cycles as adults and (ii) exposure of adult rats to OP interfered with estrous cyclicity and ovulation. Injection of 1 mg OP in corn oil sc on the day after birth did not affect the day of vaginal opening. However, 9 of 11 OP-treated rats were in persistent vaginal estrus when examined at three months after birth compared with 0 of 9 corn oil–injected controls, which cycled regularly. Ten of eleven neonatal rats injected with 1.7 mg of the estrogenic pesticide methoxychlor also were in persistent estrus at 3 months after birth, and all 10 neonatal rats injected with 1 mg of 2,4,5-trichlorophenol, which is apparently nonestrogenic, cycled regularly. Injection of 20 or 40 mg OP in corn oil vehicle sc three times weekly into previously untreated adult cyclic rats caused persistent estrus in 2 of 6 and 16 of 21 rats, respectively. Injections were continued for three more weeks in 5 of the 16 rats rendered persistent estrus by the 40 mg OP treatment. These rats remained in persistent estrus for the additional 3-week period. The other 11 persistent estrous rats in the 40 mg treatment group started to cycle regularly within 5–7 days after the last injection. Unlike pentobarbital, injection of OP into cyclic rats during the afternoon of proestrus did not block ovulation. These results provide strong evidence that OP acts like estrogen *in vivo* in both neonatal and adult female rats to exert effects that block reproductive cyclicity.**

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Large quantities of alkylphenols are manufactured to provide substrate for the production of alkylphenol polyethoxylates (APEOs) (1, 2). The APEOs have widespread use as agricultural and industrial cleaners and as detergents and emulsifiers for a wide variety of products. They have become a major component in wastewater, and they contaminate the environment along with their respective alkylphenols, which are produced during degradation (3, 4). In fact, a number of different APEOs were measured at nanogram-per-liter concentrations in drinking water in New Jersey (5), and 2,4-di-*tert*-pentylphenol was reported to reach 130 μg/g in the sediment of the Detroit River's Trenton Channel (6). In England, nonylphenol concentration approached 10^{-6} *M* in the Aire River, but 4-*tert*-octylphenol (OP) was usually undetectable in rivers, reflecting the low use of OP-APEOs in the United Kingdom (7). However, OP was approximately 5×10^{-8} *M* in the outer Tees estuary in England (7).

There has been considerable concern about the contribution of alkylphenols to the pool of environmental estrogens. Of the alkylphenols that have been tested for their estrogenicity, OP is the most potent. The structure of OP is shown in Figure 1. It has been reported to bind to estrogen receptors and to exert estrogenic actions on piscine, avian, and mammalian cells *in vitro* with a potency of approximately 1000-fold less than that of the potent estrogen, 17β-estradiol (8, 9). However, little is known about the effects of

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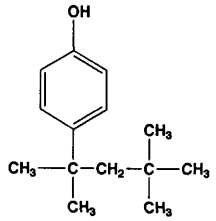

Figure 1. The structure of 4-*tert*-octylphenol (OP).

OP *in vivo*. It has been shown to exert estrogen-like actions when administered to adult male rats (10, 11) and to increase uterine weight when given to immature female rats (12). To our knowledge, the effects of OP on mammalian female reproduction have not been reported.

In the present study, we demonstrate that OP administered to female rats can exert estrogen-like effects that adversely affect the reproductive system. Exposure of neonatal rats to OP had long lasting deleterious effects on estrous cyclicity. Exposure of adult rats to OP also disrupted estrous cyclicity, but the effects were not permanent under the conditions of our study.

Materials and Methods

Animals. Pregnant and 8-week-old female CD (Sprague Dawley) rats were purchased from Charles River Laboratories, Inc. (Wilmington, MA). These rats were used for studying the effects of OP on vaginal opening and estrous cyclicity. For study of the acute effects of OP on ovulation, 8-week-old female Sprague-Dawley rats were purchased from Simonsen Laboratories (Gilroy, CA). We have used this strain of rat successfully in previous studies conducted on the timing of the preovulatory surge of luteinizing hormone (LH) in blood and subsequent ovulation (13–15). The 8-week-old rats and rat pups weaned at 21 days after birth were housed individually in rooms with controlled temperature (20°–22°C) and given Teklad Rodent Diet 8604 and tap water *ad libitum*. The CD rats were kept in a room with the lights on 0700–1900 hr daily. The Simonsen rats were kept in another room with the lights on 0500–1900 hr daily to be consistent with our previous studies on timing of the LH surge, which employed a 14:10-hr light:dark schedule. Rats were maintained and utilized in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and with the approval of the University of South Carolina's Animal Care and Use Committee.

Chemicals. Stripped corn oil was purchased from Acros Organics (Geel, Belgium; lot number 80682/2). The OP was purchased from Aldrich Chemical Co., (Milwaukee, WI; lot number BN15223AN, 98.1% pure). Methoxychlor, which has been used as a pesticide and reported to be a weak estrogen in the rat *in vivo* (16), was purchased from Sigma (St. Louis, MO; lot number 124F0575, >95% pure). The chlorophenol, 2,4,5-trichlorophenol (TCP), was purchased from Sigma (lot number 101H0740, >99% pure). It is yet another environmental contaminant that to our knowledge is not estrogenic. These chemicals were dissolved in 100% ethanol and then a measured amount of corn oil was added. Ethanol alone was added to corn oil to prepare a vial for vehicle injection. The mixtures were placed in a hood and stirred with magnetic stirring bars until the ethanol evaporated. The vials were then sealed. Fresh solutions were made weekly when needed. Gloves, apron, and mask were used as protective clothing while handling these compounds. For some of the ovulation studies, OP was dissolved in 100% ethanol and then diluted with 0.9% saline to yield OP in 30% ethanol.

Estrous Cyclicity. Estrous cyclicity was monitored by examination of vaginal smears prepared daily. An eyedropper containing a drop of 0.9% saline was inserted a few millimeters into the vagina. The saline was flushed gently into the vagina, collected, and placed onto a glass slide. Leukocytes, epithelial cells, and cornified cells were identified through a light microscope. The stage of the estrous cycle was determined as described previously (17). According to Everett (17), a state of persistent estrus occurs when cornified cells persist beyond 2 days. He also pointed out that "once persistent estrus has become established, the vaginal smears do not remain fully cornified day after day. Commonly there is a pseudocyclicity such as that occurring in ovariectomized rats receiving a steady supply of extrinsic estrogens. The population of cornified cells is accompanied from time to time by considerable numbers of nucleated epithelial cells and leukocytes" (17).

Ovulation. Rats were anesthetized with sodium pentobarbital (35 mg/kg body wt) between 0800 and 1000 hr on estrus. The fallopian tubes were excised and placed between two glass slides. The ampullae were examined through a light microscope for swelling and the presence of ova.

Effects of Exposure of Neonates to OP, Methoxychlor, or TCP on Vaginal Opening and Estrous Cyclicity. Twelve timed pregnant rats delivered their pups within a period of 12 hr. On the day after birth (Day 1), all pups from the 12 litters were mixed together. Ten pups consisting of four to seven females and three to six males were chosen randomly and placed with each of the 12 mothers. Thus, the pups may or may not have been placed with their genetic mother. Three litters were assigned to each treatment group. All pups within a single litter were injected sc with 0.1 ml corn oil alone (50 μl in each of two sites) or containing 1.0 mg OP, 1.7 mg methoxychlor, or 1.0 mg TCP. This resulted in near equal molar amounts of each chemical being injected and approximately 140 times the molarity of 10 μg of testosterone proprionate. Administration of this dose of testosterone proprionate to female rats sc on Day 1 after birth is highly effective in inducing sterility as observed at 45 and 90–120 days after birth (18). The male rats were used to collect tissues for future examination. After weaning, female rats were checked daily for vaginal opening. At 40 days of age, we selected 9–11 rats at random in each group to be kept for monitoring of vaginal estrous cyclicity starting at 3 months after birth. At that time, vaginal smears were prepared and examined daily for 3 weeks.

Effects of Exposure of Adults to OP on Estrous Cyclicity. Starting at 10 weeks of age (2 weeks after arrival) vaginal smears were prepared and examined daily to monitor the estrous cycle. Rats showing at least two regular 4-day estrous cycles were used. They were injected sc with 0.2 ml corn oil vehicle or 20 or 40 mg of OP in vehicle. In preliminary studies, adult rats that had been ovariectomized for 1 week were injected sc with different concentrations of OP in oil. A single dose of 80 mg OP consistently induced estrus within 3 days as evidenced by the appearance of numerous cornified cells in vaginal smears. A dose of 20 mg OP had little or no effect on inducing estrus. Based on these results, we selected doses of 20 and 40 mg OP for the present studies which involved multiple injections.

The first injection was given without regard to the day of the estrous cycle. Injections were given three times weekly on Mondays, Wednesdays, and Fridays for 2 weeks. Sixteen of the rats in the 40 mg OP group entered persistent estrus during the 2 weeks of treatment. Five of these sixteen rats continued to receive the 40 mg OP treatment three times weekly for an additional 3 weeks. The remaining 11 rats rendered persistent estrus in the 40 mg OP group were not given additional injections during this 3-week period. Vaginal smears were prepared and examined daily throughout the experiment.

Effects of Injections of OP on Proestrus on Ovulation during Estrus. Starting at 10 weeks of age (two weeks after arrival) vaginal smears were prepared and examined daily. Rats showing at least two regular 4-day estrous cycles were used on proestrus. One group of control rats was injected ip with sodium pentobarbital (35 mg/kg body wt) at 1400 hr. Ovulation was blocked in 5 of 5 rats, indicating that sufficient LH was not released prior to 1400 hr to induce ovulation. Other rats were injected sc with 0.2 ml corn oil vehicle or 40 mg OP in corn oil vehicle at 1300, 1430, and 1600 hr. The remaining rats were injected sc with 100 mg OP in 30% ethanol (0.1 ml) at 1300, 1430, and 1600 hr. Multiple injections of OP were given, and OP was administered in two different vehicles in attempts to uncover any acute effect that OP might have on ovulation. The occurrence of ovulation was determined between 0800 and 1000 hr the next morning.

Statistics. Comparisons between groups with regard to the day of vaginal opening or the number of ova shed per ovulating rat were performed by one-way analysis of variance. The remaining data were compared with chi-square tests.

Results

Effects of Exposure of Neonates to OP, Methoxychlor, or TCP on Vaginal Opening and Estrous Cyclicity. None of the treatments had a statistically significant effect on altering the day of vaginal opening compared with that seen in corn oil–treated controls (Table I).

Of 9 corn oil– and 10 TCP–treated neonatal rats examined starting at 3 months after birth, all had regular 4- or 5-day estrous cycles during the 3 weeks in which vaginal smears were prepared (Table I). A different situation was seen for rats that had been injected neonatally with OP or methoxychlor. Nine of 11 OP-treated rats and 10 of 11 methoxychlor-treated rats were in persistent estrus (17) as exhibited by the presence of epithelial and/or cornified cells daily during the 3-week examination period. The other three rats in these two groups appeared to have irregular estrous cycles with leukocytes being the predominant cell type in the smears for periods of 1–3 days interspersed between 2 and 3 days when epithelial or cornified cells predominated.

Effects of Exposure of Adults to OP on Estrous Cyclicity. Injection of corn oil into six 4-day cyclic adult rats for 2 weeks did not interrupt the regular pattern of estrous cyclicity (Table II). By contrast, the 20 mg and 40 mg OP treatments caused the appearance of cornified cells within 3 days in 2 of 6 and 16 of 21 rats, respectively. These cell types persisted and were occasionally accompanied by epithelial cells in these rats throughout the remainder of the 2-week injection period. The remaining 4 of 6 rats in the 20 mg OP treatment group and 5 of 21 rats in the 40 mg OP treatment group had 4- or 5-day estrous cycles during the 2-week injection period.

Five of the 16 rats rendered persistent estrus with the 40 mg OP treatment remained persistent estrus during an additional 3 weeks of injection. The remaining 11 rats in this group which were not given further injections had leukocytes as the predominant cell type in vaginal smears within 5–7 days after the last injection. These rats then displayed

Table I. Effects of sc Injection of 1 mg OP or TCP or 1.7 mg Methoxychlor on Day 1 after Birth on the Day of Vaginal Opening and Estrous Cyclicity at 3 Months after Birth

Treatment	No. of rats	Day of vaginal opening	No. of rats examined for estrous cyclicity	No. of rats with estrous cyclicity	No. of rats in persistent estrus
Corn oil	17	32 ± 1	9	9	0
OP	13	33 ± 1	11	2	9[a]
Methoxychlor	15	34 ± 2	11	1	10[a]
TCP	16	33 ± 1	10	10	0

Note. OP, 4-*tert*-octylphenol; TCP, 2,4,5-trichlorophenol. Only 9–11 rats in each group were kept after vaginal opening for monitoring of estrous cyclicity starting at 3 months after birth.

[a] Treatment with OP or methoxychlor had a significant effect ($P < 0.05$) on inducing persistent estrus.

Table II. Effects of sc Injection of OP Three Times Weekly into Adult Cyclic Rats on Estrous Cyclicity

Treatment	No. of rats with estrous cyclicity	No. of rats in persistent estrus
Corn oil (2 weeks)	6	0
20 mg OP (2 weeks)	4	2
40 mg OP (2 weeks)	5	16[a]

Note. OP, 4-*tert*-octylphenol. Five of the 16 rats in the 40 mg OP treatment group that were in persistent estrus were continued with the 40 mg OP treatment for an additional 3 weeks. They remained in persistent estrus. The other 11 rats rendered persistent estrus with the 40 mg OP treatment were not given further injections. They showed 4- or 5-day estrous cycles starting 5–7 days after the last injection of OP.
[a] The 40 mg OP treatment had a significant effect ($P < 0.05$) on inducing persistent estrus within 3 days.

regular 4- or 5-day estrous cycles during the subsequent 2 weeks.

Effects of Injections of OP on Proestrus on Ovulation during Estrus. All rats injected with corn oil or with OP, regardless of whether it was delivered in corn oil or ethanol, ovulated (Table III). Moreover, there were no differences between groups with respect to the number of ova shed. By contrast, administration of sodium pentobarbital blocked ovulation in all rats.

Discussion

Our results clearly demonstrate that OP can disrupt reproductive processes in the female rat. Exposure of neonatal animals to OP caused changes that became evident when they were examined as adults and found to be in persistent estrus. Exposure of adult animals to OP also disrupted estrous cyclicity by inducing persistent estrus. These effects mimicked those induced by administration of estrogen to the neonatal or adult rat in previous studies.

Exposure of the neonatal female rat to OP had long-lasting effects, as observed by the continued presence of epithelial and/or cornified cells in the vagina of these rats at three months of age. This effect also has been observed when neonatal female rats are androgenized by injection of testosterone or estrogen (19). Androgenized female rats have been observed either to be in anovulatory persistent vaginal estrus after vaginal opening or to cycle and ovulate a few times and then enter anovulatory persistent estrus. The latter situation is referred to as the delayed anovulatory syndrome (19). In the present study, we did not prepare vaginal smears until nearly 2 months after vaginal opening. Thus, we do not know whether the dose of OP employed caused persistent estrus by the time of vaginal opening or a delayed anovulatory syndrome. We also observed the same situation in neonatal rats administered the estrogenic pesticide methoxychlor, but not after administration of TCP, which is apparently nonestrogenic.

Table III. Effects of ip Injection of Sodium Pentobarbital (PB) at 1400 hr or sc Injection of OP at 1300, 1430, and 1600 hr on Ovulation

Treatment	No. of rats ovulating	Mean (±SE) ova per rat
Corn oil	5/5	9 ± 2
PB (35 mg/kg body wt)	0/5[a]	—
40 mg OP in oil	5/5	10 ± 3
100 mg OP in 30% ethanol	5/5	9 ± 2

Note. OP, 4-*tert*-octylphenol. Each OP-treated rat received three injections totaling 120 or 300 mg of OP.
[a] Pentobarbital had a significant effect ($P < 0.05$) on blocking ovulation.

Estrogen formed by aromatization of testosterone in brain is believed to mediate androgenization during the neonatal period in the rat. One obvious morphological change that occurs with androgenization is an increase in size of the sexually dimorphic nucleus (SDN) in the preoptic area of the male (19, 20). In a previous study in which estrous cyclicity was not monitored, injection of neonatal female rats with OP did not affect the size of the SDN (12). This could be due to insufficient OP reaching the SDN. However, it should be pointed out that alteration of the SDN in the neonatal female has not been shown to function in causing the persistent estrous state in adults.

The mechanism involved in OP induction of persistent vaginal estrus when OP is first administered to rats as adults is likely due to OP exerting estrogenic action directly on the vagina. Serum 17β-estradiol, which rises in concentration during the rat estrous cycle, is known to change the vaginal epithelia to epithelial cells at early proestrus and then cornified cells at late proestrus and estrus, and administration of exogenous estrogen during diestrus can advance the changes in vaginal epithelia (17). Also, administration of exogenous estrogen can induce persistent vaginal estrus in ovariectomized rats (21). Such changes in vaginal epithelia would be expected to occur due to the estrogenicity of OP regardless of whether chronic administration of OP suppressed serum gonadotrophin concentrations as it did in adult male rats (10). The predominance of leukocytes in vaginal smears within 5–7 days after cessation of treatment with OP strongly suggests that OP cleared substantially from the circulation during that period and that it was no longer exerting appreciable estrogenic effects. However, the observation that the OP-induced effect on estrous cyclicity was reversible should not be interpreted to mean that more prolonged exposure to OP than that which occurred in the present study could not have permanent effects on disrupting estrous cyclicity.

The failure of OP to act acutely to block ovulation indicates that OP had no major effect on the neural mechanisms responsible for luteinizing hormone–releasing hormone (LHRH) release, the anterior pituitary gland response to LHRH during the afternoon and evening of proestrus, or the ovarian response to gonadotropins. We would not expect OP to have any such effects if its actions were solely estrogenic in nature. Serum 17β-estradiol concentrations

rise during the rat estrous cycle and peak on proestrus. They then decline during the afternoon and evening of proestrus to reach low levels during estrus (22). Prevention of this decline in serum 17β-estradiol levels by administration of estrogen during the early afternoon of proestrus did not alter the preovulatory surges of LH or FSH in blood (14). If OP exerted additional actions *in vivo* that were not estrogenic in nature, such actions were not disruptive to the ovulatory process under the conditions of this study which addressed the acute effects of OP on ovulation.

The present study was not designed to simulate the levels of OP in the circulation that humans or wildlife might attain due to exposure to OP in the environment. Our purpose was to determine whether OP could exert estrogen-like actions in the neonatal or adult female rat that were disruptive to reproduction. The present results on female rats and those of previous studies on male rats (10, 11) clearly show that OP can disrupt reproduction dramatically in both sexes. These results are of concern because several observations point to the likelihood of exposure to OP in the environment with possible effects on reproductive processes. Significant concentrations of APEOs and alkylphenols exist in environmental water. Individual APEOs have been measured in nanogram-per-liter concentrations in drinking water (5), and individual alkylphenols have been measured in microgram-per-liter concentrations in river water (7) and microgram-per-gram concentrations in river sediment (6). The significance of these levels may be suggested by recent observations that as little as 10^{-9} *M* OP in drinking water of adult male rats for 4 months caused a small but significant increase in the incidence of sperm with gross malformations (23). The additional observation that alkylphenols may bioaccumulate in fat (6) and the suggestion that xenoestrogens may have potentiating effects (24) point to greater possible harm caused by OP in the environment. Indeed, xenoestrogens have been shown to exert additive effects (25). Although there is a report that xenoestrogens have synergistic effects in *in vitro* systems (26), several groups of investigators have been unable to confirm such synergistic phenomena employing several *in vitro* and *in vivo* systems (25).

A particular concern is the level of exposure to OP during the perinatal period and the long-term effects of this exposure which are observed in adulthood. Placement of OP-APEO or OP in female rat drinking water for 2 weeks prior to mating and throughout pregnancy and lactation at a concentration of approximately 5×10^{-6} *M* caused small but significant decreases in testis size of the male offspring as adults (27). The effect of the OP-APEO was attributed to its metabolism to OP *in vivo* (27). These decreases in testes size were accompanied by small but significant decreases in sperm production, at least in the rats exposed to OP. As evidenced in the present study, a single exposure during the neonatal period can have long-lasting effects on female reproduction as well. Thus, maternal exposure to OP and potential transfer of OP to fetuses through the placenta and to offspring through the milk may be of greater concern than exposure of adults to the xenoestrogen.

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