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Evaluation of the Reproductive Toxicology of 2,4,6-Trichlorophenol in Male and Female Rats

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Evaluation of the Reproductive Toxicology of 2,4,6-Trichlorophenol in Male and Female Rats. BLACKBURN, K., ZENICK, H., HOPE, E., MANSON, J. M., GEORGE, E. L., AND SMITH, M. K. (1986). *Fundam. Appl. Toxicol.* **6**, 233–239. The toxicity of chlorinated organic compounds which may be generated as a by-product of drinking water chlorination has been an issue of increasing concern. Relatively few data are available concerning their reproductive toxicity. The present study was designed to evaluate the reproductive effects of one of these compounds, 2,4,6-trichlorophenol (TCP), in male and female rats. Adult males were treated with either 0, 100, 500, or 1000 mg/kg of TCP (po) for 10 weeks, at which time semen evaluations were conducted on ejaculates recovered from the genital tract of receptive females. Fertility was assessed in the 0- and 1000-mg/kg groups. Females were treated with identical doses for 2 weeks prior to pregnancy then throughout gestation. Dams were allowed to litter and pup development was monitored until Day 42 postpartum. TCP had no effect on any sperm parameter or male fertility. Treatment of females with 1000 mg/kg of TCP produced gross maternal toxicity as reflected in increased lethality and decreased weight gains in the dams. However, no treatment-related differences were seen in litter sizes or pup survival. Male and female birth weights were significantly depressed in the 500- and 1000-mg/kg groups; these differences disappeared by Day 4 postpartum, suggesting that they were a reflection of maternal toxicity. To this extent, the reproductive processes of male and female rats do not appear to be a primary target for the effects of TCP. © 1986 Society of Toxicology.

One of the contaminants detected in drinking water supplies is 2,4,6-trichlorophenol (TCP). TCP may be a by-product of the chlorination process or may be formed by microbial transformation of chlorobenzene (Ballshmitter *et al.*, 1977). In addition, TCP has been identified as a metabolite of the chlorinated pesticide, lindane (Fitzloff *et al.*, 1982). This observation is relevant to the present study since lindane has been shown to produce reproductive toxicity in male and female rodents. Adverse outcomes include spermatotoxicity and infertility in male rats (e.g., Shivanandappa and Krishnakumari, 1983; Lakkad *et al.*, 1982) and prolonged gestation as well as increases in postimplantation loss in pregnant rats (Manetkulieu, 1978). Female offspring of rats treated with lindane during pregnancy had delayed vaginal opening and disruption of the estrous cycle (Petrescu *et al.*, 1974).

The National Cancer Institute has declared TCP to be carcinogenic in rats. Tumors of the hematopoietic system were produced following 106 weeks of treatment with dietary levels of 10,000 ppm TCP (NCI, 1979). TCP has been shown to have spermaticidal properties when tested *in vitro* using human sperm (Brotherton, 1977). Limited data suggest that the kidney and liver are target organs for TCP toxicity (USEPA, 1979). No data concerning

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the reproductive toxicity of TCP with *in vivo* exposure to either sex are available.

The present study was designed to evaluate the potential toxicity of 2,4,6-TCP to the male and female reproductive systems. For the male reproductive segment, animals were treated for 11 weeks by gavage. Copulatory behavior and semen parameters were evaluated in each animal prior to and following 10 weeks of treatment. After the 10-week evaluation, rats from the high-dose and control groups were bred with untreated females. Litters produced from these matings were examined on Day 18 postconception. For the female reproductive segment, rats were treated by gavage 5 days/week for 2 weeks prior to mating and then 7 days/week through Day 21 of gestation. Dams were allowed to deliver and sex ratio, litter size, and litter weights were recorded. Offspring were followed until Day 42 postpartum. In addition to male fertility and semen assessments, TCP organ distribution and the kinetics of TCP absorption and clearance were investigated in male rats. The data from this component are to be reported in a subsequent paper.

METHODS

Preparation of TCP solutions. 2,4,6-Trichlorophenol was obtained from Fisher Scientific. This reagent-grade TCP was purified by recrystallization from hot glacial acetic acid. Crystals were precipitated by adding 25% (v/v) distilled water. The precipitate was collected by vacuum filtration and washed with distilled water. Gas chromatography indicated the product was 99% pure 2,4,6-TCP.

Dosing solutions of TCP were dissolved in corn oil by gentle heating, and administered in a volume of 2 ml/kg body wt. Control animals received the same volume of corn oil alone.

Experimental design—male reproductive segment. Eighty male Long–Evans hooded (LEH) rats were obtained from Charles River (Canada) at 70 days of age. Female LEH rats were obtained from the same source as needed for semen and fertility evaluations. Males were singly housed in hanging stainless-steel cages and maintained on Purina Lab Chow (5001) and tap water *ad libitum*. Animals were maintained on a 12:12 reverse light:dark cycle with the light phase commencing at 2100 hr.

At 100 days of age, pretreatment (baseline) copulatory and semen evaluations were conducted on the males. These methods have been described in detail (Zenick *et al.*, 1984a, b). Behavioral evaluations included recording mount and ejaculation latencies, and number of mounts and intromissions. Semen evaluations were conducted on ejaculates recovered from the genital tract of a receptive female (15 min postcopulation) and included assessments of sperm count, motility, and morphology. Animals having baseline ejaculated sperm counts of less than 20 million sperm, or ejaculation latencies longer than 30 min, were eliminated from the study. Remaining animals were assigned to treatment groups (0, 100, 500, 1000 mg/kg TCP) so that baseline, group mean sperm counts and ejaculation latencies were matched across groups. Fifteen animals per group were included in the 0-, 100-, and 500-mg/kg groups with 25 assigned to the 1000-mg/kg group. The additional animals in this group were necessary to ensure adequate sample size for the distribution and kinetic studies conducted at the end of treatment. Animals were dosed by gavage 5 days/week for 11 weeks. Doses were selected on the basis of a 3-week pilot study, wherein males and females receiving 1000 mg/kg exhibited approximately a 5% weight loss. It was concluded that higher doses would produce more pronounced and unacceptable weight losses given the lengthened dosing periods in the present study. Data from NCI bioassay (NCI, 1979) also suggest more pronounced weight loss at higher doses (based upon converting dietary intake into a milligram per kilogram dose).

Following 10 weeks of treatment, copulatory behavior and semen profiles were again evaluated. On the 11th week, males from the high-dose and control groups were mated to unexposed females for fertility evaluations. Females selected had regular estrus cycles as determined by evaluation of vaginal smears for 3 weeks prior to mating. A male was housed with a female until mating was confirmed by a copulatory plug and/or presence of sperm in the vaginal lavage. In the present study, all males mated within a 3- to 5-day period.

Once mating was confirmed females were removed to individual cages. On Day 18 of gestation females were sacrificed by CO_2 asphyxiation. The sex, weight, and viability of all fetuses were assessed. The number of resorption sites was recorded after staining the uteri in 10% ammonium sulfide for 10 min.

One week later, all males were sacrificed by CO_2 asphyxiation. Blood was drawn from the heart and plasma samples were frozen for testosterone analyses. Testosterone analyses were conducted using a Diagnostic Products Kit (Los Angeles, Calif.). In addition, one cauda epididymis from each animal was minced, filtered through a 20-gauge brass screen, diluted to 50 ml, and the sperm count was determined in a hemacytometer.

Experimental design—female reproductive segment. Female LEH rats were obtained from Charles River (Portage, Mich.) at 60 days of age. Males were obtained from the same source for the breeding segment. They were group-caged in hanging wire cages and maintained on

Purina Lab Chow (5001) and tap water *ad libitum*. The females employed were selected from a larger breeding colony population. Females were maintained on a 12:12 light:dark cycle with lights commencing at 0700 hr.

Estrus cycles were monitored for 2 weeks by vaginal lavage, and females not cycling were eliminated. Animals were assigned to treatment groups (0, 100, 500, 1000 mg/kg TCP) so that mean body weights were matched across groups. Forty animals were assigned to the 0- and 1000-mg/kg groups with 30 assigned to the 100- and 500-mg/kg groups.

Animals were dosed by gavage for 5 days/week for 2 weeks and then mated. Three females were housed with one male and allowed to cohabit until mating was confirmed by presence of sperm in the vaginal lavage or for a maximum of 10 days (males failing to impregnate a female were replaced after 5 days). Daily intubations continued from the beginning of the mating trials through Day 21 of gestation.

Body weights were recorded daily from the beginning of the treatment period until delivery. Animals were individually housed in plastic shoe-box cages on Day 14 of gestation. Those females which had not delivered by Day 24 of gestation were sacrificed by CO_2 asphyxiation. Their ovaries were examined and uteri stained with 10% ammonium sulfide for 10 min and then evaluated for signs of postimplantation loss.

For animals that delivered, date of delivery, sex ratio of the litter, and body weights of male and female offspring were recorded. Litters were culled to eight pups on Day 4 postpartum with four males and four females retained when possible. At weaning, litters were culled to two males and two females. Body weights by sex were recorded on Days 1, 4, 7, 14, 21, 28, 35, and 42 postpartum. The establishment of vaginal patency was monitored beginning at Day 28. On Day 42 postpartum, all pups were sacrificed.

Statistical analysis. Body weights (adult male, adult female, and pups postpartum by sex) were analyzed first by a repeated measures analysis of variance with one between factor (dose) and one within factor (time) (BMDP, 2V). Subsequently, data were analyzed week by week using a one-way analysis of variance procedure. One-way analysis of variance procedures were also used to evaluate percentage of weight increase data (SAS, GLM). Differences between group means were assessed using Duncan's new multiple-range test.

Male mating behavior and semen parameters were analyzed by one-way analysis of variance procedures. Data on each animal were expressed as percentage of his baseline value (i.e., Week 10 value/baseline). Organ weights, plasma testosterone, and cauda epididymal sperm counts at sacrifice were also evaluated using one-way analysis of variance procedures.

Litter sizes, sex ratios, and resorption data were analyzed using a Kruskal–Wallis test. Fetal and pup weights and mean times of vaginal opening were analyzed using a one-way analysis of variance. Litter means were the units of analysis for these variables.

Female breeding outcomes (e.g., percentage pregnant, percentage sperm positive) were evaluated using χ^2.

RESULTS

Male Reproductive Segment

Death due to treatment occurred in the high-dose group alone during the first 4 weeks of treatment (two, three, one, and two deaths, respectively). Deaths in the other groups (one each in the low- and mid-dose groups) were the result of intubation errors as evidenced by sudden death minutes after gavaging the animal and confirmed at time of necropsy. The only other indication of toxicity was a reduction in rate of weight gain in the 1000-mg/kg group which was significant at Week 3 alone (Fig. 1). The only consistent clinical indication noted was urogenital staining in all TCP-treated groups. No warning signs were noted in the appearance and behavior of the animals which subsequently died. Due to autolysis, necropsy of these animals was limited to an examination of the potential contribution of the intubation procedure to death.

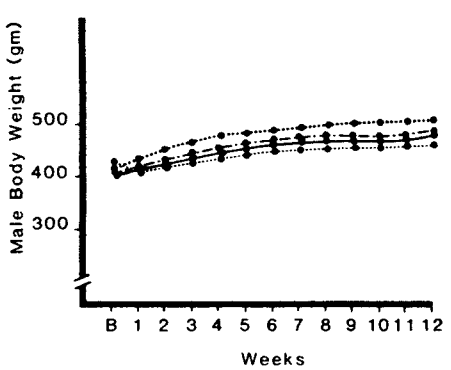

FIG. 1. Group mean body weights for male rats treated with 2,4,6-trichlorophenol for 12 weeks. $^*p \leq 0.05$ at Week 3 only; (●·–·–●), 0 mg/kg; (●····●), 100 mg/kg; (●—●), 500 mg/kg; (●· · · · ·●), 1000 mg/kg.

TABLE 1

ORGAN WEIGHTS AND EPIDIDYMAL SPERM COUNTS FOLLOWING 11 WEEKS OF TCP EXPOSURE

Treatment (mg/kg TCP)	0 (N = 15)	100 (N = 14)	500 (N = 14)	1000 (N = 17)
Liver	18.26 ± 3.01[a]	19.30 ± 3.69	18.89 ± 5.89	19.04 ± 1.89
Kidney	1.97 ± 0.22	2.00 ± 0.33	1.89 ± 0.37	2.04 ± 0.19
Lung	1.13 ± 0.25	1.20 ± 0.20	1.04 ± 0.34	1.11 ± 0.11
Adrenal	0.05 ± 0.01	0.06 ± 0.05	0.05 ± 0.05	0.05 ± 0.02
Spleen	0.75 ± 0.15	0.82 ± 0.22	0.70 ± 0.17	0.68 ± 0.11
Heart	1.41 ± 0.17	1.53 ± 0.18	1.33 ± 0.27	1.39 ± 0.11
Testis	1.69 ± 0.18	1.80 ± 0.18	1.58 ± 0.30	1.62 ± 0.19
Prostate	0.76 ± 0.25	0.66 ± 0.19	0.59 ± 0.14	0.59 ± 0.11
Seminal vesicle	2.73 ± 0.54	2.69 ± 0.30	2.39 ± 0.67	2.48 ± 0.30
Vas deferens	0.23 ± 0.03	0.25 ± 0.07	0.22 ± 0.03	0.22 ± 0.03
Epididymis	0.63 ± 0.08	0.70 ± 0.07	0.64 ± 0.10	0.61 ± 0.09
Sperm counts/g cauda epididymis ($\times 10^6$)	760 ± 150	682 ± 206	795 ± 250	658 ± 119

[a] Mean and standard deviations.

Copulatory Behavior and Semen Evaluations, and Sacrifice Data

No treatment-related differences were seen in any of the copulatory behaviors or semen parameters measured. Seminal plug weight increased over the treatment period for all groups, a maturational increase which has been previously noted (Zenick *et al.*, 1984b). No significant treatment-related differences were seen in male organ weights, plasma testosterone, or caudal sperm counts obtained at sacrifice. Organ weight and cauda epididymal sperm count data are shown in Table 1.

Fertility and Fetal Outcome Evaluations

No male in either the control or 1000-mg/kg group required more than 5 days to mate as noted by the presence of copulatory plugs and positive sperm lavages. Females were sacrificed on Day 18 postconception. Ten of thirteen females bred to control males and all females bred to high-dose males (n = 14) carried litters. Litter size, sex ratio, mean pup weight by sex, number of dead fetuses, and number of resorption and implantation sites were recorded for each litter (Table 2). No treatment-related differences were seen in any of these outcomes.

TABLE 2

MALE REPRODUCTIVE SEGMENT: FETAL OUTCOME PARAMETERS OBTAINED ON DAY 18 OF PREGNANCY (MEAN ± STANDARD DEVIATION)

Treatment (mg/kg/TCP)	Number litters	Litter size[a]	% Postimplantation loss[b]	Sex ratio[c]	Pup body weights (g)	
					Male	Female
0	10	12.9 ± 3.4	9.6 ± 4.8	1.39 ± 0.63	1.64 ± 0.17	1.61 ± 0.14
1000	14	12.9 ± 2.3	6.9 ± 8.9	1.32 ± 0.53	1.53 ± 0.23	1.45 ± 0.22

[a] Number of viable fetuses per litter.
[b] (No. resorptions)/(No. implantation) × 100.
[c] (No. males/litter)/(No. females/litter).

Female Reproductive Segment

The survival of females through the entire treatment period was as follows: 38/39 control animals; 29/29 100-mg/kg animals; 25/30 500-mg/kg animals; and 24/40 1000-mg/kg animals. The cause of death appeared to be primarily intubation errors, with females in the high-dose group exhibiting marked resistance to intubation in the hands of technicians highly experienced in this procedure. Three of the sixteen deaths in the high-dose group appeared to be the result of TCP toxicity since postmortem necropsy eliminated intubation error as a cause. As with the males, females which died as an apparent result of TCP toxicity did not exhibit differences in appearance or behavior which were not apparent in other members of the treatment group (urogenital staining and weight loss). Due to autolysis, necropsy of these animals was confined to an examination of the potential contribution of the intubation procedure to death.

Body weights for females during the 2 weeks of pretreatment and gestation are shown in Fig. 2. Repeated measures analysis of variance (BMDP, 2V) showed a significant effect of dose on female body weight ($p \leq 0.05$) at the end of both the first and second week of dosing as well as Days 1, 7, and 14 of pregnancy. Comparison of group means using Duncan's new multiple-range test (SAS, GLM) showed that only the 1000-mg/kg group differed significantly from the control group. No significant effects were seen on Day 21 of pregnancy. As a consequence of the "catch up" in weight between Days 14 and 21 of pregnancy in the 1000-mg/kg group, no group differences were seen in percentage of weight gain during gestation. Mean percent weight gains during gestation were 30, 29, 29, and 28% for the 0-, 100-, 500-, and 1000-mg/kg groups, respectively.

Breeding success was somewhat low across all groups. The percentage of females that were pregnant was 63, 72, 60, and 50% for the 0-, 100-, 500-, and 1000-mg/kg groups respectively. These differences were not significantly different ($\chi^2 = 2.87$, $p \geq 0.40$). Historically, control breeding data range from 70 to 85% in this laboratory. There did not appear to be any differences in housing, season, diet, or protocol that would account for the lower rates seen in the present study. Moreover, we had selected only cycling females for inclusion in the study. That the general condition of the animal colony was adequate is also supported by the fact that breeding in the concurrent male reproductive segment was quite good (76–100%).

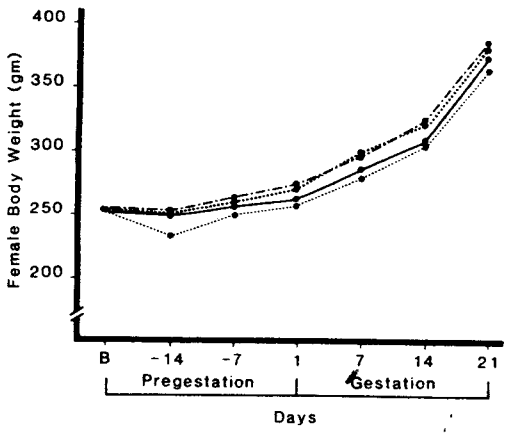

FIG. 2. Group mean body weights for female rats treated with 2,4,6-trichlorophenol for 2 weeks prior to gestation (−14, −7 days) and then during gestation. B = baseline weights prior to initiating treatment: $^*p \leq 0.05$; (●·–·–●), 0 mg/kg; (●····●), 100 mg/kg; (●—●), 500 mg/kg; (●· · · · ·●), 1000 mg/kg.

Evaluation of Offspring

Mean litter sizes at birth and survival from birth to Day 4 postpartum are shown in Table 3. No treatment-related differences were apparent.

Mean litter body weights by sex from birth until weaning (Day 21 postpartum) are shown in Table 4. Both the 500- and 1000-mg/kg groups showed significantly depressed body weights for both sexes on Day 1 postpartum. By Day 4 no differences were detectable. No treatment-related differences were seen in survival or pup body weights from weaning

TABLE 3

FEMALE REPRODUCTIVE SEGMENT: LITTER SIZE AND SURVIVAL FROM BIRTH TO DAY 4 POSTPARTUM

Dam dose (mg/kg TCP)	N	Litter size at birth (±SD)	Percentage survival on day 4 (±SD)
0	22	10.2 (3.7)	95.2 (10.6)
100	20	10.6 (3.6)	98.9 (2.8)
500	15	12.3 (2.5)	96.1 (7.5)
1000	11	11.5 (2.4)	99.3 (2.3)

through postnatal Day 42. No treatment-related differences were seen in mean day of vaginal patency of female pups. Means and standard deviations were 34.0 ± 2.0, 32.9 ± 1.8, 33.3 ± 1.7, and 34.5 ± 2.8 days postnatal for the 0-, 100-, 500-, and 1000-mg/kg TCP groups, respectively.

DISCUSSION

Data from the present study indicate that the male reproductive system is not a critical target system for TCP toxicity. No treatment-related differences were found in any of the reproductive parameters that were evaluated. Treatment-related mortality at the high dose (1000 mg/kg) was 28%, indicating that evaluation of the compound at higher treatment levels would not be productive. Other than mortality and a significant depression in body weight at Week 3 of treatment (1000-mg/kg group only), no differences between groups were observed in terms of copulatory behaviors, semen parameters, organ weights, fertility, and a number of measures of fertility and fetal outcome. The depression in body weight at Week 3 in the high-dose males did not appear to be biased by the animals which subsequently died. Six of the eight deaths had already occurred by this time point and the two animals which subsequently died did not show body weight depression that was more severe than that seen in other members of the group.

The effects of TCP on the female reproductive system appeared primarily at the highest dose. However, at this dose (1000 mg/kg) 2,4,6-TCP caused gross signs of toxicity in

TABLE 4

FEMALE REPRODUCTIVE EXPOSURE SEGMENT: MEAN LITTER WEIGHTS FOR MALE AND FEMALE PUPS FROM BIRTH UNTIL WEANING (MEAN ± SD)

Dam dose group (mg/kg TCP)	N	Postpartum day 1	4	7	14	21
Males						
0	22	6.94 (0.77)	9.02 (0.94)	14.10 (2.05)	30.93 (3.47)	50.08 (5.74)
100	20	6.57 (0.72)	9.38 (1.03)	14.64 (1.46)	30.71 (2.44)	47.70 (4.09)
500	15	6.24 (0.35)*	9.35 (1.71)	14.35 (1.33)	30.50 (1.98)	48.76 (3.00)
1000	11	6.21 (0.62)*	8.70 (1.26)	13.85 (1.11)	30.69 (1.25)	46.43 (6.73)
Females						
0	22	6.57 (0.94)	8.61 (0.96)	13.38 (2.06)	29.87 (3.64)	47.07 (5.09)
100	20	6.22 (0.61)	8.97 (0.84)	14.11 (1.44)	29.43 (2.39)	44.65 (5.91)
500	15	5.88 (0.31)*	8.54 (0.95)	13.63 (1.21)	29.32 (1.71)	46.42 (2.95)
1000	11	5.91 (0.59)*	8.05 (1.48)	13.40 (1.09)	29.69 (1.77)	47.00 (2.81)

* Significantly different from control $P \leq 0.05$.

treated dams including alopecia, reduction in weight gain prior to and during gestation, lethargy, irregular breathing, and, in a few instances, death (3/40). In addition, marked resistance to intubation was exhibited, resulting in an unexpectedly high number of fatalities. These observations suggest that the highest dose was a maximum tolerated dose in the present study.

Although breeding success appeared somewhat lower in the 1000-mg/kg group (50%), fertility rates were actually suppressed in all groups (controls = 63%) relative to historical norms in this laboratory. However, in terms of fetal outcomes, no treatment-related differences were seen in live litter sizes or pup survival. In the 500- and 1000-mg/kg litters, birth weights were significantly depressed for both sexes. However, all pups had caught up by Day 4 postpartum. Subsequent weight gain and survival were not affected. These data suggest that the initial depression of pup body weights may have been a secondary manifestation of maternal toxicity. Moreover, in litter-bearing animals, fetal weight appears to be directly related to litter size. The slight increase in litter size (Table 3) in the 500- and 1000-mg/kg groups may have contributed to the small but significant reduction in body weight at birth. In fact, when analysis of covariance was applied to this data (correcting for litter size) the significant weight differences between groups disappeared. Correlations between litter size and birth weight were −0.53, −0.55, −0.66, and −0.64 for the 0-, 100-, 500-, and 1000-mg/kg groups, respectively ($p \leq 0.001$).

In conclusion, when tested at levels which appear to be maximally tolerated for this strain and route of adminstration, neither female reproductive function and subsequent postnatal development nor male reproductive function and associated fetal outcomes appear to be selectively impaired by TCP treatment. These findings also suggest that TCP is not the metabolite responsible for the reproductive toxicity associated with lindane treatment.

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