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TERATOGENICITY OF A COMMERCIAL XYLENE MIXTURE IN THE MOUSE

Thomas A. Marks, Thomas A. Ledoux

Research Triangle Institute, Research Triangle Park, North Carolina

John A. Moore

National Toxicology Program, Research Triangle Park, North Carolina

Pregnant outbred albino (CD-1) mice received (gavage, three times a day in cottonseed oil) a xylene mixture (60.2% m-xylene, 9.1% o-xylene, 13.6% p-xylene, and 17.0% ethyl benzene) on d 6–15 of gestation (d 1 being the day vaginal plugs were observed). The mice were killed on d 18, the general and reproductive health of the dams evaluated, and the fetuses examined and processed to characterize external, visceral, and skeletal malformations. At 3.6 ml/kg·d, xylene killed 12 of 38 dams and caused a significantly ($p < 0.05$) smaller average weight gain during pregnancy than did the vehicle (cottonseed oil). Fetuses from dams treated with xylene at 2.4 ml/kg·d and higher doses had average fetal weights significantly lower than that of the control fetuses. However, the percent of resorptions for xylene was significantly greater than for the control only at 3.6 ml/kg·d. At 2.4, 3.0, and 3.6 ml/kg·d xylene produced a significantly ($p < 0.01$) greater average percent of malformed fetuses than did the control. Cleft palate was the major malformation at all three doses. When bilateral (multiple) wavy ribs were counted as a malformation, the average percent of malformed fetuses increased from 7.8 to 10.5 at 3.0 ml/kg·d and from 9.1 to 13.4 at 3.6 ml/kg·d. It is concluded that xylene (mixed isomers) is teratogenic to the CD-1 mouse at 2.4 and 3.0 ml/kg·d, doses approaching lethal levels.

INTRODUCTION

Xylene mixtures are used extensively in industry and are present in such household items as degreasing cleaners, insecticides, lacquers, paint removers, and pesticides (Gleason et al., 1969). These solvents are toxic by all routes of exposure, although percutaneous absorption is generally too slow to produce systemic effects. Xylene is reportedly more acutely toxic than toluene or benzene (Gerarde, 1962) and more necrotizing than benzene (Wilson et al., 1948). Xylene poisoning is relatively uncommon, however, probably because xylene is less volatile

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Requests for reprints should be sent to (present address) Thomas A. Marks, Teratology and Reproduction, The Upjohn Company, 301 Henrietta Street, Kalamazoo, Michigan 49001.

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than benzene or toluene. Xylene's chronic effects are less severe than benzene's and human fatalities have not been recorded (Gleason et al., 1969); the mean lethal dose of xylene is therefore unknown. Since xylene and toluene are considered "safe" alternatives to benzene in certain industries (Dean, 1978), industrial xylene use will probably increase. The general public also may be exposed to higher levels of xylene in the future, especially if its concentration in gasoline is significant (Verwilghen et al., 1975).

Kucera (1968) reported that the mothers of five of nine children with agenesis of the sacrum had been exposed to organic solvents (including xylene) during pregnancy. Kucera exposed chick embryos to xylene during the period (HH-3 to HH-14) corresponding to about d 16–24 of the human embryo. In these embryos the incidence of malformations and the mortality rate in the youngest group tested were significantly higher than in the controls. Almost half of the malformed chick embryos were rumpless, and Kučera attributed this condition to the same mechanism that had caused the syndrome of caudal regression in humans.

In 1956, Wolf et al. reported that xylene and ethyl benzene, a major contaminant of mixed xylenes, have low acute toxicities in rats. In 1969, Rumsey et al. included xylene, along with alkylphenoxyethanol (vehicle control), in a reproductive performance study of an organic phosphorous systemic insecticide in rats. The xylene-emulsifier mixture was poured on and rubbed into the backs of the rats 6, 9, or 12 d after breeding, but such treatment did not lead to a significant reduction in the number or size of litters or in pup survival until weaning, or to an increase in the production of malformed offspring. Hudak and Ungvary (1978) continuously exposed CFY rats to xylene (1.0 g/m^3) on d 9–14 of gestation and observed retarded fetal development (low fetal weight, signs of retarded skeletal development) and more skeletal anomalies (extra ribs, fused sternebrae) than normal, but no significant teratogenic effects.

Nawrot and Staples (1980) administered (gavage) pure *m*-, *o*-, or *p*-xylene to CD-1 mice (three times a day) from d 6 to 15 of gestation (0.30, 0.75, and 1.0 mg/kg·d). With the *ortho* and *para* isomers this regimen caused an increased incidence of cleft palate among the offspring at the higher two dose levels as well as an increased incidence of resorptions and evidence of maternal toxicity. In one experiment *m*-xylene administration led to a small but statistically significant increase in cleft palate at 1.0 mg/kg·d, but no overt maternal toxicity.

The paucity of teratogenicity, reproduction, or even mutagenicity studies on xylene (Dean, 1978) prompted the mixed xylene study reported here. A commercially used mixture of xylene (*m*-, *o*-, and *p*-isomers and ethyl benzene) was administered to mice on d 6–15 of gestation, and its effects on the dams and their offspring were evaluated.

METHODS

Male and nulliparous female albino mice (CD-1, 60–90 d old) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.). Females were grouped 10 per cage in 19 X 10.5 X 6 in polypropylene cages. Males were housed individually in another room in 11.5 X 7.5 X 5 in polypropylene cages. Animals were fed Purina Mouse Chow and allowed tap water *ad libitum*. Animal rooms were maintained on a 12-h light cycle at 72–75°F and 45–50% relative humidity. After 1 wk, the mating period was begun by placing two females into each male's cage. The following morning mice with vaginal plugs (d 1 of gestation) were caged together. By d 6 of gestation, the dams had been divided into experimental and control groups (≤10 mice per cage) in such a way that body weight differences between the groups were minimized.

Xylene (mixed isomers) was obtained from Shell Development Co. (Houston, Tex.) through the National Toxicology Program (NTP). This batch of xylene (lot F-309, batch 01) was analyzed for the NTP (Midwest Research Institute, Kansas City, Mo.) and found to contain 17.0% ethyl benzene, 13.6% *p*-xylene, 60.2% *m*-xylene, 9.1% *o*-xylene, and <0.3% other volatile impurities. The xylene was dissolved in cottonseed oil at concentrations (v/v) of 0 (vehicle control), 2, 4, 8, 10, 12, and 16% and administered by gavage in individual doses three times a day (9:00 a.m., 12:00 noon, and 3:00 p.m.) on d 6–15 of gestation. The volume of each cottonseed oil solution, given to each dam in a single dose, was equivalent to 1% of body weight (i.e., 10 ml/kg) on the day of administration. Thus the actual doses of xylene administered were 0.6, 1.2, 2.4, 3.0, 3.6, and 4.8 ml/kg·d, or 0.52, 1.03, 2.06, 2.58, 3.10, and 4.13 mg/kg·d (density, 0.86 g/ml).

The study was carried out in a total of five replicates, spaced 7 or 14 d apart. The 0.6, 1.2, and 2.4 ml/kg·d groups were included in the first three replicates, the 3.0 and 3.6 ml/kg·d groups in the fourth and fifth replicates, and the 4.8 mg/kg·d group in the third replicate only. A vehicle control group was included in each replicate.

On d 18 of gestation, the mice were coded numerically so that laboratory personnel evaluating teratogenic effects knew neither the test agent nor the test group. Each dam was killed by cervical dislocation and weighed. A cesarean section exposed the gravid uterus, which was weighed. The reproductive status of each dam was determined. Implantation sites in each uterine horn were counted, and the general condition of each conceptus was recorded. Implantation sites in the uteri of apparently nonpregnant females were examined after staining with ammonium sulfide (Kopf et al., 1964). Live fetuses were weighed individually and checked

for external anomalies (variations and malformations).[1] Live fetuses weighing ≤0.5 g, or less than two-thirds of the mean weight of their larger littermates, were designated "stunted." If cleft palate or open eye was observed in stunted fetuses, it was not scored as a malformation.

The sex of each fetus was determined by internal (surgical incision below navel) examination. At least one-third of the fetuses in each litter, as well as all stunted fetuses and those with external malformations, were examined for visceral alterations (Staples, 1974). The bodies of all fetuses were then processed for skeletal examination (Staples and Schnell, 1964). The heads of fetuses subjected to visceral examination (with the exception of any fetuses that had external head malformations) were cut off at the base and prepared for freehand sectioning (Wilson, 1965).

The litter was considered the experimental unit for analysis of data on embryotoxicity and teratogenicity. The statistical significance of differences between groups was determined by the Mann-Whitney *U*-test or Fisher's exact test (Siegel, 1956); the statistical significance of dose-response relation was measured by Jonckheere's test (Jonckheere, 1954). Two-tailed tests were performed and $p < 0.05$ was selected as the level of statistical significance.

RESULTS

At 4.8 ml/kg·d-xylene killed all 15 dams; at 3.6 ml/kg·d it killed 12 of 38 dams (Table 1). At 3.6 ml/kg·d xylene also caused a significantly ($p < 0.05$) smaller average weight gain (maternal weight minus gravid uterine weight on d 18) during pregnancy than did the vehicle alone. Lower xylene doses did not significantly affect maternal weight gain. However, dams treated with xylene at 2.4 and 3.0 ml/kg·d had significantly higher liver weights than those in the control group.

Fetuses from dams treated with xylene at 2.4 ml/kg·d and higher doses had average fetal weights significantly lower than that of control fetuses. The numbers of stunted fetuses from the dams treated at these doses, however, were not significantly greater than the numbers from the control dams. The numbers of implants were not significantly affected at any of the doses tested, and the percent of resorptions for xylene was significantly higher than for the control only at 3.6 ml/kg·d.

Wavy ribs, in most instances bilateral or at least involving several ribs, were found at 3.0 and 3.6 ml/kg·d. Since a single wavy rib is usually

[1] This laboratory routinely classifies anomalies frequently seen in the offspring of untreated dams (i.e., the historical control) as variations; we do not consider them a threat to the survival or well-being of the pups. When found in significantly increased numbers, these effects are considered indicators of embryotoxicity but not usually of teratogenicity. Although malformations are also indicators of embryotoxicity, these teratogenic events are classified separately, as they are generally irreversible and frequently reduce the chances that the offspring can cope with its environment. These classifications are not mutually exclusive, and it would be a mistake to dismiss variations altogether.

TABLE 1. Effect of Xylene on Reproduction in Mice[a]

Observation	Dose (ml/kg·d)[b]						
	0	0.6	1.2	2.4	3.0	3.6	4.8
Number of dams receiving test agent	66	24	23	26	28	38	15
Number of dams alive on d 18	66	24	23	26	28	26	0
Number of dams pregnant	62[c]	22[d]	23[d]	23[c]	26[e]	20[f]	
Average weight gain (g) during pregnancy (d 1–18)[g]	4.9 ± 0.26	5.0 ± 0.43	5.2 ± 0.49	5.1 ± 0.72	4.4 ± 0.47	2.4 ± 0.76[h]	
Average gravid uterine weight[g]	15.6 ± 0.60	14.1 ± 1.06	15.1 ± 0.94	13.2 ± 1.16[h]	11.6 ± 1.06[h]	5.3 ± 1.51[h]	
Average dam liver weight[g]	2.2 ± 0.03	2.2 ± 0.57	2.3 ± 0.05	2.4 ± 0.08[h]	2.6 ± 0.07[h]	2.3 ± 0.11	
Average number of implants[i]	12.7	11.8	13.2	12.7	11.6	13.0	
Number of resorptions	88	38	49	56	61	162	
Percent resorptions of total number of implants[g]	11.2	14.7	16.2	19.2	20.3	62.3[h]	
Number of fetal deaths	40	13	22	8	16	7	
Percent fetal deaths of total number of implants	5.1	5.0	7.3	2.8	5.3	2.7[h]	
Male/female live fetuses	324/334	108/100	111/121	117/109[j]	113/111	33/46	
Number of stunted fetuses	4	0	3	4	1	1	
Average number of live fetuses per dam[j]	11.2	9.9	10.5	11.4	10.2	11.3	
Average fetal weight (g)[g,k]	0.982 ± 0.010	0.982 ± 0.014	0.975 ± 0.014	0.861 ± 0.021[h]	0.785 ± 0.017[h]	0.708 ± 0.024[h]	

[a]Killed on d 18 of gestation after receiving xylene (mixture of *o*-, *m*-, and *p*-isomers on d 6–15).
[b]Dams received one-third daily dose at 9:00 a.m., 12:00 noon, and 3:00 p.m.
[c]Three dams had all resorptions.
[d]One dam had all resorptions.
[e]Four dams had all resorptions.
[f]Thirteen dams had all resorptions.
[g]Significant trend ($p < 0.05$) as dose increased (Jonckheere's test).
[h]Two-sided $p < 0.05$ versus vehicle control (Mann-Whitney *U*-test).
[i]Per pregnant dam.
[j]Excludes dams with all resorptions.
[k]Stunted and dead fetuses were excluded. Average of mean fetus weights per litter ± standard error of the mean.
[l]The sex of one fetus was not determined.

TABLE 2. Effect of Xylene on Incidence of Malformed Mouse Fetuses[a]

Observation	Dose (ml/kg·d)					
	0	0.6	1.2	2.4	3.0	3.6
Number of fetuses examined externally	658	208	232	227	224	79
Number of fetuses with external malformations	1[b]	0	1[c]	7[d]	11[c]	7[c]
Number of litters containing fetuses with external malformations	1	0	1	5	5	4
Number of fetuses examined viscerally	242	81	98	97	97	42
Number of fetuses with visceral malformations	0	0	0	0	0[e]	0[f]
Number of litters containing fetuses with visceral malformations	0	0	0	0	0	0
Number of fetuses examined skeletally	658	208	232	227	224	79
Number of fetuses with skeletal malformations	1[g]	2[h]	2[i]	0	1[j]	0[k]
Number of litters containing fetuses with skeletal malformations	1	2	2	0	1	0
Total number of malformed fetuses	2	2	3	7	12	7
Total number of litters with malformed fetuses	2	2	3	5	6	4
Average percent malformed fetuses[l,m]	0.3	1.0	1.0	3.4[n]	7.8[n] (10.5)[o]	9.1[n] (13.4)[o]

[a]Dead fetuses were excluded from all malformation computations.
[b]Meningoencephalocele; two stunted fetuses with open eye were not included.
[c]Cleft palate; one stunted fetus with cleft palate and a partially open eye was not included.
[d]Cleft palate (6); bilateral open eye; exencephaly. One fetus with kinked tail but no bone change and two stunted fetuses with bilateral open eye were not included.
[e]One fetus had cream-colored liver lobes; another had a white spot in the left ventricle.
[f]One fetus had a white spot in the left ventricle.
[g]Fused arches and ribs.
[h]Fused sternebrae.
[i]Fused arches (2); fused ribs; missing arches and ribs.
[j]Fused ribs; six fetuses with bilateral wavy ribs were not included.
[k]Three fetuses had bilateral wavy ribs.
[l]100 × Σ (no. of malformed fetuses in litter/total no. of litters)/total no. of fetuses in litter.
[m]Significant trend ($p < 0.001$) as dose increased (Jonckheere's test).
[n]Two-sided $p < 0.01$ versus vehicle control (Mann-Whitney U-test).
[o]Average percent of malformed fetuses if bilateral wavy ribs were scored as a malformation.

scored as a variation in this laboratory, whereas multiple wavy ribs in a fetus are scored as a single malformation, the average percent of malformed fetuses was tabulated both ways in Table 2. Whether or not multiple wavy ribs were scored as a malformation, xylene administration caused a statistically significant ($p < 0.01$) increase in the average percent of malformed fetuses at 2.4 ml/kg·d and above. The dose-response effect on the average percent of malformed fetuses also followed a significant ($p < 0.001$) trend as the dose increased (Jonckheere's

test). Cleft palate was the major malformation at 2.4, 3.0, and 3.6 ml/kg•d. When multiple wavy ribs were counted as malformations, the average percent of malformed fetuses increased from 7.8 to 10.5 at 3.0 ml/kg•d and from 9.1 to 13.4 at 3.6 ml/kg•d.

The presence of a white lesion in the left ventricle of two fetal hearts was also observed. This variation is found infrequently, although it was observed in large numbers in another study (Marks et al., 1981). Some of the variations observed are noted in Table 2. However, most of the variations were not out of the ordinary and have been frequently observed in past control groups. Therefore, these variations were not detailed.

DISCUSSION

Inhalation is the most likely route of human exposure to mixed xylene. However, since our laboratory does not have inhalation facilities, we frequently administer compounds orally in our teratology studies, as we did in the study reported here. We feel that the high lipophilicity of mixed xylene will most likely cause significant blood levels regardless of the route of administration. Since the top two dosages tested produced lethal effects, the concentrations used were apparently high enough. The effects of mixed xylene on embryo development indicate transplacental passage, which is consistent with what Dowty et al. (1976) reported for humans, as they found xylene in umbilical cord blood and maternal blood samples collected at birth.

Although at 2.4 and 3.0 ml/kg•d xylene produced a significantly greater average percent of malformed fetuses than did the vehicle alone, these concentrations were not lethal to any of the dams, nor was there an adverse effect on maternal weight gain. Both of these doses caused significantly higher mean maternal liver weights than did the control. However, such an effect has not been shown to cause teratogenicity. In an attempt to identify the toxic effects on the pregnant dam that lead to teratogenicity, we routinely weighed the maternal livers in several studies. We did this in addition to observing the dams for signs of toxicity, performing general necropsies at cesarean section, and determining weight gain during pregnancy after subtracting grand uterine weight from the final weight of the dam. However, we were unable to correlate statistically significant differences in mean liver wieght between groups receiving various compounds and their respective vehicle controls with teratogenic events (unpublished observations).

Some of the dams receiving mixed xylene at doses of 3.0 ml/kg•d and below experienced minor toxicity. Two dams at 2.4 ml/kg•d and two at 3.0 ml/kg•d had bloody vaginal discharges on one occasion, but this problem was not apparent on subsequent days. Other minor problems, such as diarrhea (also seen in the controls, and thus most likely caused by the relatively high intake of cottonseed oil) and rough coat (two dams

on d 9 in the 3.0 ml/kg·d test group) also occurred at doses below 3.6 ml/kg·d. Toxic effects in the 3.6 ml/kg·d group were of a similar nature but more general and more pronounced. In addition, in this test group blood was found on the end of the oral intubation needle on one occasion after dosing two of the dams, and one dam was hyperactive over 2 d during the dose period.

Cleft palate or open eye or both occurred in stunted and nonstunted fetuses in the litters of both control and treated dams. We consider these anomalies in a stunted fetus indicative of delayed development and we did not score them as malformations in these fetuses (Table 2, footnotes *b*, *c*, and *d*). In the nonstunted fetuses with cleft palate or open eye, scored as malformations, we felt that the significant decrease in average fetal weight in groups receiving xylene at 2.4 mg/kg·d and above could not be held accountable for these anomalies. We concluded, therefore, that mixed xylene at 2.4 and 3.0 ml/kg·d is teratogenic as well as embryotoxic in the CD-1 mouse.

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