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The Effects of High-Dose Toluene on Embryonic Development in the Rat[1]

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ABSTRACT

Developmental disability, intrauterine growth retardation, renal anomalies, and dysmorphic features have been described in offspring of women who abuse toluene during pregnancy. A Sprague-Dawley rat model was developed to study this clinical syndrome. During d 6–19 of gestation, 11 treated dams received daily gavage doses of toluene, 520 mg/kg body weight, diluted in corn oil, and 11 control dams received corn oil. This dose of toluene simulates the blood toluene levels obtained after an inhalation exposure to 3290 ppm toluene, an inhalation level in the lower end of the range experienced by toluene abusers. Maternal weight gain was 24% less in the toluene-exposed group ($p < 0.002$); however, there were no maternal deaths. The fetuses were delivered on d 19 of gestation, and 287 fetuses (148 toluene exposed, 139 control) were examined. Toluene treatment did not affect the number of implantations or stillbirths. There were no toluene-induced major congenital malformations or neuropathologic changes noted. In the toluene-treated group, the weights of the fetuses were reduced by 9.4% ($p < 0.004$) and placental weights were reduced by 10.3% ($p < 0.01$). Toluene exposure also reduced fetal organ weights as follows: brain 4.6%, heart 5.9%, liver 13.2% ($p < 0.02$), and kidney 13% ($p < 0.05$). Organ weight/body weight ratios did not differ significantly, suggesting that prenatal toluene exposure produced a generalized growth retardation. (***Pediatr Res* 36: 811–815, 1994**)

Human exposure to the organic solvent toluene may produce a variety of acute and chronic toxic effects (1–3). A significant percentage of human toluene exposure occurs as a form of substance abuse (inhalant abuse, solvent abuse), and toluene is the preferred solvent of many inhalant abusers (4). Solvent abuse is common among adolescents of lower socioeconomic groups, and adolescent and young adult women represent a significant proportion of abusers (5).

Toluene embryopathy has been described in offspring of toluene-abusing women (3, 6–11). These infants are generally small for gestational age and microcephalic with dysmorphic features including short palpebral fissures, deep-set eyes, small face, low-set ears, micrognathia, spatulate fingertips, and small fingernails (7–11). As these children mature, developmental and neurologic consequences of prenatal toluene exposure become evident. Significant developmental delay, language impairment, hyperactivity, and cerebellar dysfunction have been described (3, 8, 10, 11). Similarities between fetal alcohol syndrome and toluene embryopathy have been noted (8, 10, 11).

Although human teratogenic effects of toluene secondary to solvent abuse are apparent, the risks of occupational toluene exposure to the developing fetus are unclear. In a study of women employed in an electrical insulation factory using varnish containing 70% toluene, Syrovadko (12) reported an increased number of "small babies." McDonald *et al.* (13) examined congenital defects in offspring of women with occupational chemical exposure during at least the first trimester of pregnancy. They found an excess of genitourinary and gastrointestinal defects in the children of toluene-exposed mothers. The genitourinary defects were similar to those noted in some of the offspring of toluene abusers (6, 10). Developmental evaluations of these children have not been reported.

Toluene exposure dosages encountered by solvent abusers and workers can differ by up to two orders of magnitude. It is estimated that abusers may inhale from 4 000 to 12 000 ppm toluene, taking multiple inhalations over several minutes (14, 15). This repetitive dosing may continue for many hours. In the workplace, the threshold limit value for toluene is 100 ppm measured as a time-

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weighted average for an 8-h day. The short-term exposure limit for a 15-min exposure is 150 ppm (16). Measurements taken in a plastic processing factory have ranged from 184 to 332 ppm (17). Industrial accidents have caused toluene levels as high as 30 000 ppm (18).

Although the epidemiologic studies of the effects of occupational toluene exposure on the human fetus are limited, microcephaly and craniofacial dysmorphic features have not been noted. This suggests that high maternal doses of toluene, such as those encountered by solvent abusers, are necessary to induce these defects.

Limited animal research addressing this clinical problem has concentrated on the teratogenic effects of exposure to low levels of toluene (19–24). By exposing pregnant rats to gavage doses of toluene that simulate blood toluene levels similar to those achieved after high-level toluene inhalation exposures, we have initiated the development of an animal model of the teratogenic effects of this solvent that are seen in the offspring of toluene-abusing women.

METHODS

Young female Sprague-Dawley rats (160–180 g) were purchased from Simonsen Laboratories, Inc. (Gilroy, CA) and housed in wire mesh cages. They were provided with water and Rat Chow (Purina, St. Louis) *ad libitum* and maintained in a temperature-controlled room at 22°C with a 12-h light/dark cycle. For breeding, proven male rats obtained from the same vendor were placed with the females overnight. The presence of copulation plugs the following morning was taken as evidence of successful mating, and that day was designated as pregnancy d 0. For this study, there were 11 control and 11 toluene-exposed dams. Throughout gestation, daily maternal weight was monitored in all dams. Gestational food consumption was monitored during the treatment period in the final 10 pregnancies produced (five control, five toluene exposed). From d 6 through 19 of gestation, toluene-treated dams were given a daily dose of toluene by transoral gavage. Toluene (Fisher, San Francisco, CA) was diluted in corn oil to give a final concentration of 520 mg/mL. Animals in the treatment group received 1 mL/kg of this toluene/corn oil solution, and animals in the control group received an identical daily gavage dose of corn oil. Previous studies have demonstrated that this gavage dose of toluene will produce blood toluene levels that are equivalent to those obtained after a 3-h inhalation exposure to 3290 ppm toluene (25).

On d 19, 2 h after receiving toluene or corn oil, the dams were killed by CO_2 vapor inhalation. A heparinized blood specimen was obtained by cardiac puncture and was frozen for later determination of toluene concentration by gas chromatography/mass spectroscopy (26). The gravid uterus and maternal liver were then removed and weighed. The uterus was then dissected and examined for live, stillborn, and resorbed fetuses.

Each fetus was then identified by its intrauterine location, removed along with its placenta, and sexed. The placenta and associated membranes were weighed separately from the fetus. Two male and two female fetuses from each litter were placed in formalin for later evaluation, and the remainder were examined for major craniofacial and external malformations and were then dissected. The heart, liver, and kidneys were removed and weighed. The brain was removed and divided into forebrain and hindbrain at the mesencephalic-pontine junction, and each portion was weighed.

The fetuses that had been placed in formalin were examined externally and internally for malformations using the method of Barrow and Taylor (27). The heads of these fetuses were embedded in paraffin using standard techniques. These specimens were then sectioned in the coronal plane at 6 μm from the level of the anterior horns of the lateral ventricles to the level of the fourth ventricle. Every 25th section was affixed to a glass microscope slide and stained with hematoxylin and eosin. These sections were examined for malformations and neuropathologic changes. The coronal section at the level of the anterior commissure, corresponding to level E19–16 of the atlas of Paxinos *et al.* (28), was photographed. With the use of a digitizing tablet and Sigma Scan software (Jandel Scientific, San Rafael, CA), the cross-sectional areas of the brain, ventricular system, cortex, germinal matrix, caudate nucleus, and septal nucleus were measured.

One-way analysis of variance was used to compare the control and toluene-exposed groups. The litter was chosen as the statistical unit for these analyses. A p value < 0.05 was selected for determining statistical significance.

RESULTS

Toluene exposure did not result in any maternal deaths, although gestational weight gain between d 6 and 19 was reduced by 24% ($p < 0.002$). There was no significant difference in the weights of the gravid uteruses, suggesting that the reduced maternal gestational weight gain of the toluene-exposed dams was due to a decrease in overall body weight gain. During the treatment period, toluene-exposed dams consumed 12% less food; however, this difference was not statistically significant. There was no significant difference in either maternal liver weight or liver/body weight ratio (Table 1). All toluene-exposed dams had detectable blood toluene levels at the time of death (7–61 μg/mL).

A total of 287 fetuses were examined (139 control and 148 toluene treated). There were no differences in the number of implantations or resorbed fetuses. Gestational exposure to toluene resulted in significant reductions in fetal and placental weights. Fetal weights in the toluene-exposed group were 9.4% less than those in controls ($p < 0.005$), whereas placentas from the toluene-treated pregnancies weighed 10.3% less than those from controls ($p < 0.01$). Fetal organ weights were less in the toluene-

Table 1. *Maternal effects of toluene exposure during d 6–19 of gestation (mean ± SD)*

Effect	Control	Toluene exposed	ANOVA*
Food consumption during treatment period (g)	255.8 ± 31.2	224.8 ± 7.05	NS, $F_{1,8} = 4.70$
Weight gain during treatment period (g)	92.88 ± 16.13	70.37 ± 13.35	$p = 0.002$, $F_{1,20} = 12.71$
Uterus weight (g)	53.29 ± 12.82	51.99 ± 7.58	NS, $F_{1,20} = 0.08$
Liver weight (g)	14.92 ± 1.79	13.94 ± 1.03	NS, $F_{1,20} = 2.48$

* ANOVA, analysis of variance.

exposed group, with significant reductions in the weights of fetal liver and kidney (Table 2).

Ratios of fetal organ weight/body weight were not affected by toluene exposure, except for the brain weight/body weight ratio. This ratio was increased by 6% ($p < 0.05$) in the toluene-exposed group, whereas the encephalization index, calculated for the neonatal rat as brain weight/body weight$^{0.88}$ (29), was not significantly different (Table 3).

Toluene exposure did not produce any major fetal malformations. Examination of serial sections of the brain did not reveal any significant neuropathologic findings. Specifically there was no evidence of hydrocephalus, heterotopia, necrosis, inflammation, or hemorrhage. Morphometric analysis of brain sections indicated a trend toward smaller brains and reduced subcortical nuclei; however, these changes did not reach statistical significance (data not shown).

DISCUSSION

This study has demonstrated several effects of daily maternal oral exposure to toluene given during d 6–19 of gestation. Toluene treatment caused a significant decrease in fetal and placental weights, together with reductions in fetal organ weights, most significantly in liver and kidney. These reductions in fetal organ weights were not associated with a change in the organ weight/body weight ratio. This suggests that toluene exposure caused generalized growth retardation, rather than organ-specific developmental toxicity. The fetal brain weight/body weight ratio was actually increased; however, this was likely due to the much larger reduction in body weight seen in the toluene-exposed fetuses. In support of this conclusion, we noted that there was no significant change in the encephalization index. This is a more specific measurement of the effect of a teratogen or treatment on brain growth (29).

Maternal gestational weight gain was also reduced by toluene treatment. Despite these effects, toluene exposure did not cause maternal deaths, stillbirths, or fetal resorptions.

Previous animal research on the teratogenic effects of toluene in rats, rabbits, and mice has focused primarily on fetal growth and gross abnormalities (19–24). The effects of toluene exposure on fetal organ weights were not reported. In general, an increase in major malformations, including those of the CNS, has not been observed at inhalation toluene exposures ranging from 133 to 1596 ppm (19–22). Some effects of prenatal toluene exposure were specific to the animal selected for study.

In rats, a decrease in fetal weight and retarded skeletal development were seen after dams were exposed to 399 ppm toluene for 24 h/d on d 1–8 of gestation (19). An increase in maternal deaths was also observed. These maternal and fetal effects were not present when the dams were exposed to 399 ppm toluene during d 9–14; an increase in extra ribs was noted. When dams were exposed to 266 ppm toluene for 8 h/d on d 1–21 of gestation, only a retardation in fetal skeletal ossification was found (19). In a separate study, dams were exposed to 1596 ppm toluene for 24 h/d during either d 1–8, d 9–14, or d 9–21 of gestation (20). A decrease in fetal and placental weights and retarded skeletal development were found to result from toluene exposure during d 9–21.

Toluene has significant fetotoxicity in rabbits. After dams were exposed to 399 ppm toluene for 24 h/d during d 7–20 of gestation, maternal weight gain was reduced and all fetuses were aborted (21). No adverse effects to the dam or fetuses were found after exposure to 133 ppm toluene.

A variety of maternal and fetal effects of toluene exposure in mice have been reported. No adverse effects on the fetuses were seen after dams were exposed to 133 ppm toluene for 4 h, three times per day, on pregnancy d

Table 2. *Fetal effects of toluene exposure during d 6–19 of gestation (mean ± SD)*

Effect	Control	Toluene exposed	ANOVA*
Fetus weight (g)	2.239 ± 0.105	2.029 ± 0.188	$p = 0.004$, $F_{1,20} = 10.46$
Placental weight (g)	0.464 ± 0.035	0.416 ± 0.044	$p = 0.01$, $F_{1,20} = 8.02$
Forebrain weight (g)	0.0903 ± 0.0051	0.0859 ± 0.0087	NS
Hindbrain weight (g)	0.0397 ± 0.0026	0.0382 ± 0.0021	NS
Whole brain weight (g)	0.1301 ± 0.0058	0.1241 ± 0.0098	NS
Heart weight (g)	0.0118 ± 0.0014	0.0111 ± 0.0018	NS
Liver weight (g)	0.1768 ± 0.015	0.1535 ± 0.024	$p = 0.013$, $F_{1,20} = 7.46$
Kidney weight (g)	0.0138 ± 0.002	0.0120 ± 0.0019	$p = 0.043$, $F_{1,20} = 4.68$

* ANOVA, analysis of variance.

Table 3. *Effects of toluene exposure during d 6–19 of gestation on fetal organ weight/fetal body weight ratios and on encephalization index (mean ± SD)**

Ratio	Control	Toluene exposed	ANOVA
Heart (g)/body (g)	0.00528 ± 0.0005	0.00554 ± 0.0007	NS
Liver (g)/body (g)	0.0790 ± 0.0061	0.0761 ± 0.0069	NS
Kidney (g)/body (g)	0.00619 ± 0.0007	0.00598 ± 0.0002	NS
Brain (g)/body (g)	0.0584 ± 0.0032	0.0620 ± 0.0042	$p = 0.035$, $F_{1,20} = 5.11$
Encephalization index	0.0642 ± 0.0033	0.06730 ± 0.0043	NS

* Encephalization index is calculated as brain weight (g)/body weight$^{0.88}$ (g) (29). ANOVA, analysis of variance.

6–15 (21). However, a 266-ppm exposure caused fetal weight reduction and retarded skeletal development, and a 399-ppm exposure resulted in abortions. In a separate study, when toluene was given for 24 h/d on d 6–13 of pregnancy, a 133-ppm exposure resulted only in reduced fetal weight (19). Maternal death occurred when the toluene exposure level was increased to 399 ppm. In a third study, dilated renal pelves were seen after exposure to 200 ppm toluene for 7 h/d on d 6–16 of gestation (22). This effect was not seen after a 400-ppm exposure. However, increased rib count was present at the higher dose. The maternal liver weight/body weight ratio was reduced at both exposure levels. The renal effect seen in this study may relate to the human genitourinary anomalies noted in some of the offspring of toluene-exposed women (6, 8, 11, 13).

Oral administration of toluene has been used in one previous study. Increased embryonic lethality, but no maternal toxicity, was seen after mice were given either 258, 433, or 866 mg/kg body weight toluene per dose, three times a day, on pregnancy d 6–15 (23). However, the two higher doses caused a decrease in fetal weight, and the highest dose resulted in a statistically significant increase in cleft palate. Although the pharmacokinetics of orally administered toluene have not been studied in the mouse, it is likely that a thrice daily dosing schedule would result in accumulation, leading to high sustained maternal toluene levels. If we assume that the pharmacokinetics of oral toluene administration in the mouse are similar to those found in the rat (25), the toluene doses used in this mouse study (23) likely resulted in higher maternal toluene levels than those attained in rats reported in the current study.

Our study, together with previous research, suggests that prenatal toluene exposure causes a decrease in fetal growth and skeletal development. Mice and rabbits are more susceptible to the toxic effects of this solvent, with abortions and maternal death resulting from certain exposure methods (19, 21). The only major malformation reported was cleft palate in mice, resulting from oral exposures to toluene (23). Teratogenic interactions of concurrent administration of toluene and benzene (30) and toluene and acetylsalicylic acid (31) have also been reported.

The mechanisms by which toluene produces teratogenic effects have not been examined. In toluene abusers, clinical renal tubular acidosis, resulting in hyperchloremic metabolic acidosis, has been seen frequently (3). Because similar biochemical abnormalities have been detected in two affected newborns, Goodwin (6) suggests that this alteration in serum electrolytes and acid-base balance may, in part, affect the developing fetus. Direct toxic effects of toluene on the developing fetus cannot be ruled out. Due to high lipid solubility, toluene is transferred across the placenta. Transplacental transfer of toluene was demonstrated in one newborn infant (6). Accumulation of toluene in the placenta of pregnant mice has been demonstrated, as has toluene deposition in developing fetal tissues (32).

The possible interaction of maternal nutrition and prenatal toluene exposure has been investigated. In a study by da Silva *et al.* (33), daily s.c. administration of toluene to pregnant rats during the third week of gestation produced growth retardation similar to that seen in our animals. These dams had a 4% decrease (statistically insignificant) in food consumption. Toluene was also administered to dams whose food consumption was restricted by 50%. In these malnourished dams, the effects of toluene were augmented. In our study, prenatal exposure to toluene caused a 12% decrease in maternal food consumption; however, this change did not reach statistical significance. In a study previously reported in abstract form (34), we noted that a somewhat higher gavage dose of toluene, 620 mg/kg body weight, reduced maternal food intake by 14%. In that study, a pair-fed group of dams was also evaluated. Prenatal toluene exposure resulted in fetal growth retardation and smaller placentas; however, the pair-fed group did not differ from the control group. For example, the control and pair-fed fetal weights were 2.077 ± 0.128 g and 2.066 ± 0.259 g, respectively, whereas the toluene-exposed fetuses weighed 1.619 ± 0.122 g (mean ± SD) ($p < 0.001$). Similarly, control and pair-fed placentas weighed 0.429 ± 0.033 g and 0.418 ± 0.041 g, respectively, whereas placentas from the toluene-exposed group weighed 0.335 ± 0.033 g ($p < 0.001$). This suggests that the fetal growth retardation reported in the current study is due to the prenatal toluene exposure rather than the decrease in maternal food consumption.

It has been suggested that toluene abusers are exposed to inhalation toluene levels of 4 000 to 12 000 ppm or higher (14, 15). In this study, we exposed pregnant dams to oral doses of toluene that are similar, in a pharmacokinetic manner, to 3-h toluene inhalations at the low end

of this range. This study clearly shows a systemic growth retardation effect of prenatal toluene exposure without preferential changes to the developing CNS. Because the clinical embryopathy seen in the offspring of toluene-abusing women is primarily a neurodevelopmental teratogenic syndrome, doses of toluene that are in the upper part of the proposed clinical exposure range may be necessary to produce specific teratogenic effects on the CNS.

Because animal studies using low to moderate doses of toluene do not produce specific neurodevelopmental teratogenic effects, and because the well-described clinical syndrome has only been observed in the offspring of toluene-abusing women, the term toluene embryopathy is somewhat misleading. This term implies that any type of maternal exposure to toluene (from occupational contact to inhalant abuse) may have teratogenic consequences. Therefore, we suggest that the term toluene-abuse embryopathy be used in future reports describing the clinical and experimental teratogenic effects of this organic solvent.

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