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Maternal and Fetal Blood and Organ Toluene Levels in Rats Following Acute and Repeated Binge Inhalation Exposure

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Abstract

Inhalation of organic solvents is a persistent form of drug abuse with particular concern being the abuse of inhalants by women of child-bearing age. While studies have begun assessing postnatal outcomes of offspring exposed prenatally to inhalants, relatively little is known about the distribution of toluene in blood and body tissues of pregnant, inhalant-abusing women, or in the fetuses. The present study assessed the tissue toluene levels attained following brief toluene exposures using a pre-clinical rat model of maternal inhalant abuse. Timed-pregnant Sprague-Dawley rats were exposed to toluene at 8,000 or 12,000 parts per million (ppm) for 15, 30 or 45 min/exposure. Exposures occurred twice each day from gestational day 8 (GD8) through GD20. Immediately following the second exposure on GD8, GD14 and GD20 blood was taken from the saphenous vein of the dams. Following saphenous vein blood collection on GD20, dams were sacrificed and trunk blood was collected along with maternal tissue specimens from cerebellum, heart, lung, kidney and liver. The placenta, amniotic fluid and fetal brain were also collected. Results demonstrated that maternal saphenous blood toluene levels increased as the inhaled concentration of toluene and duration of exposure increased. The maternal cerebellum, heart, kidney and liver appeared to be saturated after 30 min on GD20 such that toluene levels in those organs were equivalent across all ambient concentrations of inhaled toluene. Toluene levels also increased in fetal brain as the inhaled concentration of toluene increased and in placenta and amniotic fluid as the duration of exposure increased. Toluene levels in all tissues at GD20, except maternal lung and amniotic fluid, were higher than in maternal saphenous blood suggesting that toluene concentrated in those organs. Measurement of toluene levels in blood and other tissues following repeated toluene exposure demonstrated that toluene readily reaches a variety of potential sites of action throughout the maternal-placental-fetal unit.

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1. Introduction

Many people inhale vapors from workplace and household products that contain volatile organic solvents or aerosols in search of a quick intoxication. Toluene is found in a wide variety of commonly available products such as gasoline, paint, varnish, glue, shoe and fingernail polish, and rubber cement. Recent national data suggest that greater than half of the commonly abused inhalants may contain varying percentages of toluene and over 30% of inhalant users/abusers under 18 years old inhale toluene [53,55,56]. While earlier surveys showed large increases in the number of new users (> 240% between 1990 and 2002), trends had leveled off and the rates of use had been stable for the last four years, at least until the most recent survey showed a return to increasing use among young people [54]. For people between 18-25 years old, the prime childbearing years, there was a similar increase in the rate of first-time use over the same period. In 2005, 877,000 people 12 years old or older used inhalants for the first time, and while 72.3 percent were under age 18 years at first use (average age was 16.1 years of age), more people continue to abuse inhalants into their 20's. Pearson and colleagues [49] estimated that >50% of chronic solvent abusers were women of reproductive age, a trend that is still holding [54]. The majority of solvent users had been from lower socioeconomic groups [9, 40,47] where there is also a higher risk for adolescent pregnancy [15], but more recent trends show over 30% of new users live at more than four times the federal poverty level [56]. Because such large numbers of inhalant abusers in all groups are young women in their childbearing years [4,40,43], there are rising concerns about the potential negative impact of intentionally inhaled organic solvents on the unborn [10,32].

Several studies in both non-pregnant animals and humans have demonstrated that toluene absorbed into the blood is distributed throughout the body with the brain and liver containing the highest levels [1,20,50]. The majority of absorbed toluene is metabolized by cytochrome P-450 (CYP) enzymes in the liver, primarily to benzyl alcohol and is eventually eliminated in the urine as hippuric acid [44]. In humans, exposure to high concentrations of toluene can have numerous deleterious effects including cerebellar damage [18,19,41,42], wide-ranging neuronal atrophy, and white matter abnormalities [35,41,46,48,51,63] as well as neurological syndromes such as ataxia, myopathy, peripheral neuropathy, convulsions, and encephalopathy [17,19,29,36,42]. Children who have been exposed to toluene *in utero* may also suffer from deficits in later development [7,32]. Toluene is lipid soluble, freely crosses the placenta [20, 60], and clinical cases of toluene-related embryopathy and malformations are evident in women who have abused toluene during their pregnancies [3,21,27,28,49,59,60]. Very high concentrations of inhaled maternal solvent exposure typical of abuse can lead to perinatal death [3,61]. These reports, which are mostly case studies, show that affected infants tend to be premature and/or growth retarded, microcephalic, and can display facial dysmorphism including deep-set eyes, small face, low set ears, and micrognathia, as well as spatulate fingertips and hypoplastic fingernails [3,31,59,61]. The term *fetal solvent syndrome* has been proposed to describe this constellation of morphological and behavioral effects, following the model of fetal alcohol spectrum disorders (FASDs) [3,11,49,59], which may be considered special cases of a fetal solvent syndrome, given the comparable phenotypes in toluene embryopathy and FASDs [32]. Up to 3 years of age, children exposed prenatally to toluene resulting from maternal inhalant abuse showed pronounced developmental delays, language impairment, hyperactivity, cerebellar dysfunction, and postnatal growth retardation [3,27,28, 49], a pattern of teratogenicity common to all human studies of excessive *in utero* exposure to toluene [60]. The popularity of these organic solvents, combined with what little is known about their prenatal effects, means that greater and more focused attention should be placed on studying the effects of abused inhaled solvents and pregnancy outcome.

Some studies in animal models have assessed postnatal outcomes of offspring exposed prenatally to inhalants (e.g., [5,8,12,30,33,34,57]), including studies modeling occupational

exposures (e.g., [52]), but little is known about the distribution and accumulation of toluene in blood and body tissues of pregnant organisms, or their fetuses, exposed to abuse patterns of inhaled solvents. It is possible that the brief, repeated, high blood toluene levels (BTLs) associated with toluene abuse, and occurring at certain critical periods of perinatal neural maturation in the rat, may lead to greater teratogenic damage with more severe, life-long biobehavioral consequences than following the relatively constant, lower-concentration exposures that mimic occupational settings [52]. The aim of the present study was to quantify levels of toluene achieved in maternal and fetal blood – as well as several other organ tissues – following repeated, high-concentration, binge patterns of exposure by pregnant rat dams to toluene, modeling exposure characteristic of inhalant abuse [6]. The present study utilized a sensitive gas chromatography method to measure blood and tissue toluene levels. Toluene was used as a prototype organic solvent, recognizing that tissue distributions of this organic solvent with this exposure pattern may be quite different from levels achieved for other commonly abused inhalants.

2. Materials and methods

2.1. Animals

All animal procedures had prior approval by the Wayne State University Institutional Animal Care and Use Committee (IACUC) and were in accordance with the NIH “Guide for the Care and Use of Laboratory Animals” (Institute of Laboratory Animal Resources, National Academy Press 1996; NIH publication No. 85-23, revised 1996). Seventy-nine timed-pregnant Sprague-Dawley rats (Charles River Laboratories, Portage, MI, USA) arrived on Gestation Day 4 (GD4; date sperm-plug positive = GD0), and weighed >200g on arrival. Pregnant rats were housed individually in polypropylene cages (52 × 28 × 22 cm) with hardwood chip bedding and allowed free access to water and Rodent Lab Diet 5001 (PMI Nutrition International, Inc., Brentwood, MO) except for the brief periods in the exposure chambers. The AAALAC-certified vivarium had a 12-hr light/dark cycle (lights on at 0600 hrs) with temperature controlled to 20-22°C and relative humidity levels between 40% and 70%. Four days after arrival at the vivarium, on GD8, dams were assigned to one of three treatment groups counter-balanced for maternal body weight on GD4.

2.2. Prenatal Inhalation Exposure Procedures

Each day from GD8 to GD20, the dams were transported from the vivarium to the lab in their home cages, and placed into chambers where they were exposed via inhalation to toluene vapors (see below). The dams were weighed every other day before placement. Exposures to 8,000 ppm or 12,000 ppm toluene (#T324; Fisher Scientific, Fair Lawn, NJ, USA) were conducted twice a day between 0900 and 1400 hrs with 2 hours between the start of the first and second exposures. These two high toluene concentrations and the binge pattern of exposure were chosen to mimic the range of high-dose “binge” exposures seen in human abusers [9, 60], exposures that have developmental/teratogenic effects in rats [5,8,12] and that contrast with occupational exposures that produce limited developmental effects. For example, pregnant rats exposed to 1,500 ppm toluene vapor for even 6 hrs/day from GD6 to GD20 had only a 7% decrease in fetal weight [52]. Exposure lengths in the current study were 15, 30, or 45 min and each dam was always exposed to toluene for the same duration (i.e., exposure “Length”). Eight to fifteen dams were assigned to each concentration and exposure length (total N=65). Note that for consistency throughout this paper, the independent variable for “dose” – labeled “toluene Concentration” – refers to the amount of ambient toluene in the exposure chamber (i.e., 8,000 or 12,000 ppm), and the dependent variable measuring the amount of toluene in blood (i.e., BTLs) and tissues is called “toluene Levels.” In a preliminary study, three dams were placed in identical chambers at the same time as toluene exposure groups, but

were exposed to air only (i.e., 0 ppm) and used to verify the absence of “endogenous toluene” in blood and other tissues.

Vapor exposures were given in sealed 36-liter cylindrical glass jars with acrylic lids described in detail in Bowen et al. [5]. Briefly, the lids were equipped with an injection port, a fan, and a stainless steel mesh box holding filter paper. During toluene exposures, two dams were placed onto a steel grid floor 20 cm from the bottom and 30 cm from the filter paper in the lid of the chamber. The lid was sealed and a calculated amount of solvent was injected through the port onto filter paper from which the fan volatilized the solvent throughout the chamber. Toluene vapor concentrations were confirmed by single wavelength-monitoring infrared spectrometry (Miran 1A, Foxboro Analytical). For all concentrations of toluene, mean ambient concentrations were within 3% of nominal 2.5 min after the toluene was added and remained within 2% of the nominal concentrations throughout the sessions. Chamber concentrations were verified periodically. Levels of CO₂ and water vapor were monitored during pilot studies and changes during sessions were negligible. Each dam was exposed to the same single solvent concentration throughout gestation. At the conclusion of each exposure period, dams were removed immediately and returned to their home cages to await the next exposure, the same procedure repeated 2 hrs later. After both daily exposures, animals were returned to the vivarium in their home cages.

2.3. Blood Collection

Prior to the first exposure on GD8, the hind legs of the dams were shaved to allow for easier access to the saphenous vein. Blood was collected from the saphenous vein of the dams immediately following removal from the exposure chamber after the second exposure on GD8 (first day of exposure), GD14 (middle of exposures), and GD20 (last day of exposure). These days were chosen to assess changes in BTL and tissue toluene levels over the course of repeated exposures across gestation. The shaved rear leg was extended and the saphenous vein was briefly occluded. Clear petrolatum (Vaseline[®]) was applied to the dam's leg to facilitate local pooling of the blood. A 26.5 gauge needle was used to pierce the saphenous vein and 2-3 drops of blood were collected directly into pre-weighed glass vials and immediately sealed using crimp-top vial caps with PTFE-coated butyl rubber septa, re-weighed, and frozen at -20°C until analyzed. Gauze was pressed firmly against the puncture site on the dam's leg until hemostasis. Sample weights were used to correct the calculation of final toluene levels. All blood samples were collected and run in duplicate.

2.4. Tissue Dissections

Following the saphenous blood draw on GD20, dams were decapitated and trunk blood was collected immediately. Selected maternal and fetal tissues were rapidly dissected for determination of toluene levels. From the dam, one lobe of the liver, the cortex of the left kidney, ventricles of the heart, left lung, and cerebellum were rapidly removed, within 2-3 mins, and placed immediately on dry ice. Small tissue samples (i.e., ~50 mg) were removed from each organ and placed into pre-weighed glass vials, crimp sealed, re-weighed to assess exact sample weight, and then kept frozen at -20°C until analyses. All maternal organ samples were run in duplicate.

Both uterine horns were exteriorized and the most distal fetuses from each of the horns, as well as the two fetuses closest to the fundus, were removed from the uterus. The four fetuses from these positions allowed determination of a toluene concentration gradient along the length of the uterine horns. The remaining pups were euthanized by decapitation. For fetuses used in analyses, the amniotic sac was ruptured and the fluid was collected into glass vials. The placentas were trimmed of membranes and the umbilicus and placed into glass vials. The whole fetal brain was removed by making an incision along the midline of the skull and applying

pressure to the sides of the fore-skull. All fetal samples were sealed, re-weighed, and frozen at -20°C until they were processed and analyzed for tissue toluene levels. All fetal tissue and fluid samples were run in duplicate except for fetal brain which was too small (~50mg) to run in duplicate.

2.5. Gas Chromatography Materials and Methods

Analyses of toluene levels in the blood and tissues were performed by static headspace gas chromatographic analyses. First, a 7-point internal calibration curve was established using toluene concentrations ranging from 0-250 µg/ml (250 ppm) in distilled water or whole blood from a non-exposed animal. Toluene standards and quality control samples were prepared in both distilled water and in toluene-free blood. Fifty-µl samples of blood were used in each vial for establishing the calibration curve and were analyzed in duplicate. Biological specimens (50 µl of fluid or ~50 mg of tissue) were placed in 22-ml vials containing 1.95 ml of distilled water. Calculation of toluene levels in all test specimens was corrected for exact sample weight. The sealed vials were placed in the headspace rack and gas chromatography (GC) analysis was carried out using a Perkin Elmer Clarus 500 Gas Chromatograph (Wellesley, MA, USA) with Flame Ionization Detection (FID) and a Turbo Matrix 40 headspace sampler (Perkin Elmer Instruments, Wellesley, MA, USA). The GC was fitted with a 30-m Supelco column (model #Equity-1 fused Silica Capillary Column, (I.D. = 0.32mm) using He as the carrier gas. The sequence used for this study began by heating the GC oven to 50°C for 2.5 min when the temperature was increased by 25°C/min to a final temperature of 175°C where it was held for 2.5 min. Vials were heated in the headspace sampler to 85°C for 30 min which allowed for complete inter-phase equilibration. The needle (90°C) was then injected through the septum into the space above the sample and the vial was pressurized for 1.5 min. After pressurization, the vials were vented to the GC at a temperature of 95°C for 30 sec to allow headspace gas to enter the GC column. The FID detector temperature was set at 275°C. The cycle time between samples was 20 min.

2.6. Statistical Analyses

Maternal saphenous blood toluene levels (BTLs) were analyzed using a $3 \times 3 \times 2$ repeated-measures analysis of variance (ANOVA), with Gestational Age (GD8, GD14 & GD20) as the within-subjects factor, and exposure Length (15, 30 & 45 min) and toluene Concentration (8,000 & 12,000 ppm) as between-subjects factors. Tukey's *post hoc* test was used as appropriate. For all fluids and tissues that were collected only on GD20, a MANOVA was used to examine the differences among toluene Concentrations, exposure Lengths, and among the fetuses as a function of intrauterine position (cf., [62]). For the fetal measures, the data for the two medial fetuses were combined and a mean was calculated for them. The same was done for the two most lateral fetuses. For the maternal tissues, a mean was calculated from the duplicate samples, and the mean was used in statistical analysis. If during examination of the duplicate samples an outlier was detected, the sample that exceeded two SEM's from the mean of the group with that value included was discarded. Tukey's *post hoc* test was used as appropriate to elucidate significant findings on the ANOVAs. A $p \leq 0.05$ was the threshold for statistical significance.

3. Results

3.1. Controls / Standards

Under the chromatographic conditions used, there was no interference with any endogenous materials in the blood or other tissues examined and there were no blank effects. The standard curve for toluene was linear across the full concentration range used with correlation coefficients above $r = 0.99$ for standards prepared in either water ($r = 0.9990$) or blood ($r = 0.9986$; Figure 1). The slopes and the intercepts of the water- and blood-prepared standards

were essentially identical. Under the conditions described in the Methods, toluene was well separated from water and blood and no potential interferants in the toluene analysis were detected (Figure 2).

3.2.1. Maternal

Dams exposed to toluene were initially very active, usually circling the perimeter of the exposure jar for the first few minutes of exposure. As toluene exposure continued, general sedation was observed in all animals with mild body tremors and occasional jerking of the legs. Animals exposed to the 12,000 concentration of toluene became sedated more quickly and salivated profusely during the exposures. Upon removal from the exposure chamber, all toluene-exposed animals continued to have mild body tremors, occasional jerking of the legs, nose twitching, and salivation. Mean latency to regain normal postures were ~15 min and ~25 min for animals exposed to 8,000 and 12,000 ppm, respectively. ANOVAs for maternal body weight showed that, as expected, there was significant maternal weight gain in both toluene groups across pregnancy ($F(6,348)=414.73$, $p<0.001$). There was no significant main effect of toluene Concentration ($p>0.27$) or exposure Length ($p>0.22$). There was a significant gestation Day $\times$ exposure Length interaction ($F(12,348)=4.70$, $p<0.001$) and *post hoc* analyses revealed that increases in maternal weight were not as great for animals exposed for 45 min in either toluene group as for animals exposed for shorter lengths.

3.2.2. Maternal Saphenous Blood

A significant main effect of toluene Concentration was found with maternal BTLs from the 12,000 ppm toluene groups being higher than the 8,000-ppm groups ($F(1,39)=7.20$, $p<0.05$). There was also a significant main effect of exposure Length ($F(2,39)=38.79$, $p<0.001$) resulting from the 30- and 45-min/exposure animals having significantly higher BTLs than animals exposed for 15-min/exposure (Figure 3). A toluene Concentration $\times$ exposure Length interaction was not significant ($p>0.05$). There was also a significant main effect for gestational Day ($F(2,78)=6.72$, $p<0.01$) resulting from an overall decrease in BTLs across gestation between GD8 and GD20. A significant gestation Day $\times$ exposure Length interaction ($F(4,78)=5.12$, $p<0.01$) and *post hoc* analyses also revealed that decreases in BTLs were pronounced in animals exposed for 30 min and 45 min and were not observed in groups exposed for 15 min, which did not change significantly across gestation.

3.3. Maternal Trunk Blood

As seen in Figure 4, toluene levels in maternal trunk blood for the 8,000 ppm toluene groups were significantly higher than the 12,000-ppm groups ($F(1,29)=8.16$, $p<0.01$). There was also a significant main effect of exposure Length ($F(2,29)=16.77$, $p<0.001$) in that animals exposed for 30 and 45 min had higher BTLs than the animals exposed for 15 min. A toluene Concentration $\times$ exposure Length interaction was also significant ($F(2,29)=12.26$, $p<0.001$), reflecting a higher BTL at 15 min after 12,000 ppm toluene compared to the 8,000-ppm group at 15 min ($p<0.05$). While there was a small significant overall difference in BTLs between maternal saphenous and trunk blood at GD20 ($F(1,29)=4.14$, $p=0.05$), the patterns of differences varied non-systematically with toluene Concentration and exposure Length.

3.4. Maternal Lung

Examination of the lung demonstrated no significant main effect for toluene Concentration, ($p>0.58$), or for exposure Length, ($p=0.09$). There were no significant interactions, nor were there differences in toluene levels between maternal lung and saphenous or trunk blood levels at GD20 after any exposure duration ($p>0.05$).

3.5. Maternal Liver

In general, the changes in all the other maternal organs – liver, kidney, heart and cerebellum – were similar, with increases as a function of Length of exposure. For liver, toluene levels in the dams exposed to 8,000 ppm were not significantly different from dams exposed to 12,000 ppm ($p>0.32$; Figure 4). Hepatic toluene levels increased as exposure Length increased ($F(2,29)=14.78, p<0.001$) in that animals exposed for 45 min exhibited higher toluene levels than the animals exposed for 15 min. The toluene Concentration $\times$ exposure Length interaction was not significant ($p>0.35$). Overall, toluene levels in liver were significantly greater than those in either maternal saphenous ($F(1,37)= 61.38, p<0.001$) or trunk blood ($F(1,36)= 32.00, p<0.001$) at GD20 following exposures longer than 15 min.

3.6. Maternal Kidney

In kidney, there was no difference between the two toluene exposure groups ($p>0.27$). There was a significant main effect of exposure Length ($F(2,29)=28.46, p<0.001$) with dams exposed for 30 or 45 min exhibiting significantly higher renal toluene levels than the dams exposed for 15 min (see Figure 4). The 30- and 45-min exposure groups did not differ significantly from each other ($p>0.4$) and there was no significant toluene Concentration $\times$ exposure Length interaction ($p=0.07$). Overall, toluene levels in kidney were greater than those in either maternal saphenous ($F(1,38)= 62.76, p<0.001$) or trunk blood ($F(1,36)= 64.82, p<0.001$) at GD20 following exposures longer than 15 min.

3.7. Maternal Heart

Similar to other tissues, exposure to 12,000 ppm of toluene did not increase cardiac toluene levels as compared to 8,000 ppm ($p=0.13$). A significant main effect of exposure Length ($F(2,29)=18.16, p<0.001$) was also observed and *post hoc* analyses revealed that 30- and 45-min exposures resulted in significantly higher heart toluene levels than did the 15-min exposure (see Figure 4). The toluene Concentration $\times$ exposure Length interaction was not significant ($p>0.46$). Overall, toluene levels in heart were greater than those in either maternal saphenous ($F(1,36)= 55.76, p<0.001$) or trunk blood ($F(1,35)= 30.241, p<0.001$) at GD20 following exposures longer than 15 min.

3.8. Maternal Cerebellum

As seen in Figure 4, examination of cerebellar tissue demonstrated no difference for toluene Concentration between the 8,000- and 12,000-ppm groups ($p>0.86$). There was, however, a significant main effect of exposure Length ($F(2,29)=11.34, p<0.001$) and *post hoc* analyses revealed that dams exposed to 30 min and 45 min of toluene had significantly higher cerebellar toluene levels than dams exposed for 15 min ($p<0.05$). The toluene Concentration $\times$ exposure Length interaction was not significant ($p>0.97$). Again, toluene levels in cerebellum were greater than those in either maternal saphenous ($F(1,31)= 82.09, p<0.001$) or trunk blood, ($F(1,31)= 23.98, p<0.001$) at GD20 following exposures longer than 15 min.

3.9. Amniotic Fluid

As seen in Figure 5, there were measurable levels of toluene detected in amniotic fluid. There were no differences in toluene levels in amniotic fluid between fetuses located laterally on the uterine horns compared to those located at the midline, so the data from all fetuses from a given litter were averaged prior to subsequent analyses. Toluene exposure resulted in no difference observed between the 8,000- and 12,000-ppm groups ($p>0.16$). No main effect was observed for exposure Length for amniotic fluid ($p>0.15$) nor was there a significant toluene Concentration $\times$ exposure Length interaction ($p>0.76$). Overall, toluene levels in amniotic fluid were much lower – at most, 10% of maternal levels – than either maternal saphenous ($F(1,45)$

=90.91, $p<0.001$) or trunk blood ($F(1,36)=123.46$, $p<0.001$) at GD20 following exposures of any length.

3.10. Placenta

Maternal toluene exposure resulted in no differences between the 8,000- or 12,000-ppm groups ($p>0.22$). There was a significant main effect of exposure Length for placenta ($F(2,29)=16.58$, $p<0.001$) reflecting the higher levels with longer duration exposures (Figure 5). There was no significant toluene Concentration $\times$ exposure Length interaction ($p>0.54$). Overall, toluene levels in placenta were greater than those in maternal saphenous blood ($F(1,38)=13.45$, $p<0.01$) but not trunk blood ($p>0.27$) at GD20 following exposures longer than 15 min.

3.11. Fetal Brain

Maternal exposures to inhaled toluene produced elevated levels of toluene in fetal brain with a significant main effect observed for toluene Concentration ($F(2,29)=4.02$, $p=0.05$) (Figure 5). There was no main effect for exposure Length ($p>0.27$). While the toluene Concentration $\times$ exposure Length interaction did not reach significance ($p=0.26$), Figure 5 shows that fetal brain toluene levels at 12,000 ppm were relatively similar over time while levels at 8,000 ppm gradually increased with exposure length. Overall, at GD20 toluene levels in fetal brain approximated maternal saphenous BTLs ($p>0.54$) but were lower than maternal trunk BTLs ($F(1,34)=12.55$, $p<0.01$) for both concentrations after the 30- and 45-min exposures ($p<0.05$).

4. Discussion

The primary objective of this study was to use a valid preclinical model of organic solvent inhalant abuse to examine the distribution of toluene into the blood and body tissues of pregnant rats and their fetuses after repeated exposure to high ambient concentrations of toluene. Under the current exposure conditions and chromatographic methods, exposure to abuse patterns of inhaled toluene resulted in elevated levels of toluene in saphenous blood that were dependent on both the inhaled concentration of toluene and the duration of exposure. These data show that circulating levels of toluene reach an apparent asymptotic level by about 30 minutes, and reach that asymptote quicker with higher concentration exposures. While the levels of toluene in maternal blood increased as the duration of exposure was extended from 15 to 30 minutes, generally levels did not increase any further with a 45-min exposure, especially for the 12,000-ppm group. One possible reason for this “maxing out” of blood toluene levels (BTLs) is that very high concentrations of toluene are known to be irritating to membranes and could interfere with pulmonary toluene transport mechanisms. Indeed, there are models of lung function that describe multiphasic decreases in pulmonary solvent uptake as solvent concentration increases (e.g., [39]).

These data show that abuse patterns of inhalation resulted in elevated levels of toluene in a number of maternal tissues, as well as in placenta and fetal brain. Toluene was also present in amniotic fluid, although at much lower levels compared to maternal blood and other maternal and fetal tissues. These results demonstrate that brief inhalations of high-concentration toluene readily reach a variety of potential sites of action throughout the dam and her fetuses, even attaining organ tissue levels that exceed the maximums reached in maternal blood. The present results are consistent with earlier investigations in pregnant rats using a variety of administration models demonstrating not only that toluene crosses the placenta [20,22], but that it is distributed and concentrated in both maternal and fetal compartments [20].

Current toluene levels in maternal liver, kidney, heart and cerebellum were similar, with general increases in toluene levels as a function of exposure duration. Relative toluene levels were greater in other tissues as compared to levels in maternal blood. There were only two significant

differences between the 8,000-ppm and 12,000-ppm groups: the maternal saphenous blood levels and levels in fetal brains were overall higher in the 12,000-ppm group. The differences in toluene profiles in fetal brain at 8,000 ppm compared to 12,000 ppm may be due to interference with placental transfer or maternal transport mechanisms following high concentrations of toluene. While there is no comparable study assessing blood or tissue toluene levels in pregnant rats after repeated binge exposure(s) to high concentrations of inhaled ambient toluene, there are two inhalation studies of perhaps limited relevance in 42-day-old male rats using different patterns of exposure. One study assessed BTLs in Fisher-344 rats during and after exposure accompanying 3 hours of restraint to 99 ppm, 549 ppm, or 1145 ppm of toluene [23] and another assessed BTLs in Wistar rats after a single 30-min inhalation of 2,000 ppm or 10,000 ppm of toluene [2]. Blood or serum toluene levels were assayed in separate rats at different times during the 3-hr exposure [23], or from the same rats (via femoral catheters; [2]), for the next 4 to 10 hours. Gospe and Al-Bayati [23] showed that serum toluene levels asymptoted at ~15 ppm between 1 hr and 2 hrs into the exposure period, similar to the ~14.4 ppm level achieved after 2,000 ppm by Ameno, et al. [2], and also comparable to the asymptotic levels at 30 min to 45 min in the current study.

As far as we are aware, the current study is the first to assess changes in maternal BTLs across the course of pregnancy with repeated exposures by any route of administration. Compared to earlier days, overall decreases in maternal blood toluene concentrations were observed on GD14 and GD20 following 30- and 45-min exposures – but not after 15-min exposures – to both 8,000 ppm and 12,000 ppm. Repeated 30- and 45-min exposures to 8,000 ppm or 12,000 ppm resulted in ~35% and ~70% decreases in BTLs, respectively, by GD20 compared to initial levels on GD8. Some other studies have exposed pregnant animals to toluene on a single day during gestation via intragastric intubation [24–26], systemic injection [13,14,38], or in drinking water [38], none of which produce pharmacokinetic functions that faithfully represent those following the inhalation route of administration that defines solvent abuse. Several studies have demonstrated that repeated toluene inhalations yield progressively lower blood and brain toluene levels consistent with tolerance due to up-regulation of hepatic catabolism by cytochrome P450 [37,44,58]. Ghantous and Danielsson [20] subjected separate groups of pregnant mice to single, acute, 10-min inhalation exposures to 2,000 ppm of ¹⁴C-radiolabeled toluene on GD11, GD17, or GD20, but did not report analyses of relative tissue levels of toluene among days. Such an analysis may have characterized differences in acute uptake at different stages of pregnancy but still would not have assessed changes due to chronic exposures across pregnancy within dams, as in the current study. The extent to which these potential differences in metabolism or other pharmacokinetic parameters with repeated exposures across pregnancy may contribute to the observed differences in the developmental effects of toluene has not been determined.

The current evidence is consistent with the development of maternal metabolic or dispositional tolerance over the course of repeated toluene exposures during gestation. This also depended upon the ambient toluene concentration inhaled and the duration of each daily exposure. Specifically, maternal saphenous BTLs after the 30- or 45-minute exposures decreased between GD8 and GD20 for both concentrations of toluene. In contrast, after the 15-minute exposure, maternal toluene levels actually increased slightly across gestation, suggesting that a threshold level of toluene is necessary to induce the enzymatic responses leading to metabolic tolerance [16]. It is also possible that the decrease in BTLs over time is secondary to an increase in maternal proportion of body fat during pregnancy since there is a preferential relative distribution of toluene into fat [16,20]. Toluene has high fat solubility, readily concentrates in lipid-rich tissues, and in the present study toluene levels were higher in relatively lipid-rich tissues like cerebellum as compared to blood and lung. In this case, fat could serve as a reserve depot concentrating toluene and possibly establishing a pool that is at an unbalanced equilibrium with a maternal blood pool and thereby indirectly limiting maternal circulating

BTLs. In fact, the highest relative levels of toluene were reported in fat of pregnant rodents [20] and toluene has been shown to be sequestered in fat with a long half life [$\sim$ 80 hours; [16]]. A single, acute 10-min inhalation of 2,000 ppm of ^{14}C radio labeled toluene by pregnant mice lead to rapid accumulation in lipid-rich maternal, placental and fetal tissues, including heart and brain, and other highly perfused organs, such as kidney and liver, peaking about 20 mins after the exposure [20].

These results are also consistent with the interpretation that the patterns of BTLs in the current study reflect equilibrium with other depots – organs and fat – that concentrate toluene at levels higher than in maternal blood. Since volatile toluene is cleared within one hour [16,20], the persistent levels of toluene in blood or serum [2,23] four to ten hours after removal from the toluene vapor also suggest transfer from other pools. There were also clear concentration-dependent differences in toluene levels [2,23] not seen in the current study, suggesting that it will be important to include lower concentration exposures in future studies to aid in interpretation of the impact of the much higher concentrations. In particular, the highest toluene level of $\sim$ 200 ppm, measured 30 mins after the 10,000-ppm exposure reported by the Ameno et al. study [2] was substantially higher in blood and brain than even after two 45-min exposures at 12,000 ppm in the current study. The design differences between Ameno et al. [2] and the current study include the exact concentration and exposure duration, the strain, age and sex of the animals, pregnancy, and the repeated versus single acute toluene exposures, all suggesting the importance of clearly characterizing the potential roles of maternal tolerance (i.e., clearance rates), lower concentration exposures, and differential tissue distribution of toluene (esp. into fat) in future studies. Brain levels of toluene in Ameno et al. [2] were also reported but only as a proportion of blood levels. Yet similar to our results in pregnant dams, toluene also concentrated in young male rat brains at levels ranging from $\sim$ 140% (hippocampus) to $\sim$ 315% (pons) of blood levels [2]. Also as in the current results, there were apparently no significant differences between the lower (2,000 ppm) and higher (10,000 ppm) toluene exposure groups in brain levels of toluene [2].

We confirmed with chromatography earlier work using autoradiography and liquid scintillation assessments of toluene distribution in mice after a single 10-min inhalation of approximately 2,000 ppm of radio labeled toluene at one of three stages of gestation [20]. Exposure to inhaled toluene resulted in higher toluene levels in maternal cerebellum, liver, kidney, and heart than in maternal blood, but interestingly, there was relatively less toluene in the fetal-placental unit in the Ghanous and Danielsson report [20] than in the current study, with levels only 2% to 8% of the maternal blood levels for fetus and placenta, respectively (cf., [20]; Table 1, p.99), compared to the 88% and 148% for fetal brain and placenta, respectively, in the current study. However, to our knowledge, the present study is the first to show that prenatal exposure to high concentrations of toluene can result in significant levels of toluene being achieved in both placenta and fetal brain. It is possible that the differences between these and earlier results [20] can be attributable to different species and/or the abuse pattern of exposure. The higher tissue levels of toluene in the present study may also be due to the higher exposure concentrations perhaps overwhelming the maternal cytochrome P450 system. Pregnancy has been demonstrated to decrease cytochrome P450 content compared to non-pregnant animals, as a result decreasing the overall metabolism of toluene [44,45]. Another important difference was that the prior work used only a single 10-min administration [20] while we had 13 days of exposure throughout pregnancy which likely lead to the development of maternal – but not fetal – tolerance.

The current study demonstrates that toluene abuse, modeled by brief, repeated inhalations of high concentrations of toluene, results in significant levels of toluene in blood and organs of pregnant rats, and in placenta and fetus. Toluene also reaches amniotic fluid. Blood toluene levels depend upon both the ambient concentration and duration of exposure and reach an

apparent maximum after about 30 minutes. The results also indicate that toluene concentrates in certain compartments, including maternal liver and brain, placenta, and fetal brain, at levels substantially higher than the apparent asymptotic level in maternal blood. These very high maternal solvent exposures typical of abuse can lead to perinatal death [3], morphological teratogenicity (e.g., [7]), prematurity and/or growth restriction, microcephaly, digital and/or facial dysmorphism, and the possibility of long-term detrimental outcomes [3,49,59-61]. Using this same pattern of exposure in our preclinical model, we have shown that prenatal toluene exposure delays postnatal development, restricts growth, and increases likelihood of morphological and neurobehavioral teratogenic outcome [5,8].

Since it is possible that these very high concentrations may actually limit toluene absorption due to irritation of pulmonary membranes and interference with transport [16], and since other studies in young male rats with lower concentration exposures report higher blood toluene levels, further studies of abuse exposures to lower concentrations of toluene in pregnant dams are necessary. The results are consistent with the hypothesis that maternal metabolic tolerance (e.g., induction of cytochrome P450 enzymes) develops over the course of exposures during pregnancy, but it is also possible that the relative decrease in maternal blood levels between GD8 and GD20 is a function of altered body mass composition, specifically in increase in the “relative fat depot” or percentage of body fat, which preferentially absorbs toluene [1]. Body fat may also serve as a reservoir for toluene, reaching a dynamic, relative equilibrium with levels in maternal blood. Further studies are also necessary to evaluate to what degree and in what way neurochemical and metabolic changes in animals exposed to toluene may be related to maternal and/or fetal tissue toluene levels. It is also important to understand how those relations may play a role in the enduring effects of prenatal toluene on behavior and brain development, particularly since the levels and activity of cytochrome P450 in metabolizing toluene vary as a complex interactive function of sex, age, pregnancy and toluene concentration [45]. Finally, these results and differences in blood toluene levels from findings assessing inhalation exposure to relatively constant, low-concentration toluene in occupational models, stress the importance of using models of repeated high-dose “binge” inhalation exposures to model patterns of exposure seen in abuse. High fidelity to actual abuse exposure patterns is necessary to understand both pharmacokinetics in the maternal-placental-fetal unit, and effects in the offspring following that exposure.

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Figure 1.

Calibration curves (0.025-250 ppm; mV $\pm$ SEM) of toluene standards in water/acetone (open symbols) and in blood/acetone (filled symbols). Slopes for standard in water (0.7192 ± 0.00702 sem; 95% C.L.: 0.7035-0.7348) and in blood (0.6904 ± 0.0082 sem; 95% C.L.: 0.6721-0.7088). Y-intercepts (when $X=0$) for standard in water (-0.4586 ± 0.7203 sem) and in blood (0.2937 ± 0.8450 sem).

Figure 2.
Total chromatogram of toluene at a concentration of 250 $\mu\text{g/ml}$.

Figure 3.

Concentrations of toluene ($\pm$ SEM) in saphenous blood of pregnant rats following 15-, 30-, or 45-min toluene exposure. Toluene concentrations in inspired air were 8,000 ppm (left panel) or 12,000 ppm (right panel) with 12,000 ppm producing higher overall blood concentrations ($p < 0.05$). The '*' indicates significantly different from the 15 min exposed group. The '+' indicates where toluene-levels tended to be higher than the last day measured.

Figure 4. Concentrations of toluene ($\pm$ SEM) in maternal organs of pregnant rats following 15-, 30-, or 45-min toluene exposure. Toluene concentrations in inspired air were 8,000 ppm (left panel) or 12,000 ppm (right panel). The '*' indicates significantly different from the 15 min exposed group.

Figure 5. Concentrations of toluene ($\pm$ SEM) in placenta and fetal brain following 15-, 30-, or 45-min toluene exposure. Toluene concentrations in inspired air were 8,000 ppm (left panel) or 12,000 ppm (right panel). The '*' indicates significantly different from the 15 min exposed group.

TABLE 1

Maternal Characteristics (means $\pm$ sem)

Level of Toluene:	8,000 ppm	12,000 ppm
15-minute exposures		
Number of dams ($N=29$)	15	14
Maternal weight on GD8 (g)	247.26 $\pm$ 4.97	251.20 $\pm$ 3.96
Maternal weight gain to GD20 (g)	83.83 $\pm$ 3.95	81.78 $\pm$ 3.21
Percent weight gain	34.12 $\pm$ 1.84	32.64 $\pm$ 1.36
30-minute exposures		
Number of dams ($N=18$)	9	10
Maternal weight on GD8 (g)	253.48 $\pm$ 3.50	244.31 $\pm$ 5.51
Maternal weight gain to GD20 (g)	73.13 $\pm$ 3.17	72.80 $\pm$ 2.96
Percent weight gain	28.90 $\pm$ 1.33	29.95 $\pm$ 1.46
45-minute exposures		
Number of dams ($N=15$)	8	9
Maternal weight on GD8 (g)	261.15 $\pm$ 13.46	238.03 $\pm$ 6.19
Maternal weight gain to GD20 (g)	43.97 $\pm$ 14.64	61.78 $\pm$ 5.18
Percent weight gain	17.96 $\pm$ 5.51	26.04 $\pm$ 2.33