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Binge Toluene Exposure in Pregnancy and Pre-weaning Developmental Consequences in Rats

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Abstract

The persistent rate of abuse of inhaled organic solvents, especially among women of child-bearing age, raises the risk for teratogenic effects of maternal toluene abuse. In this study, timed-pregnant Sprague Dawley rats were exposed from Gestation Day (GD) 8 to GD20 to 12,000 or 8,000 parts per million (ppm) toluene, or 0 ppm (controls) for 30 min twice daily, 60 min total daily exposure. Pups were assessed from postnatal day (PN) 4 to PN21 using a developmental battery measuring growth (i.e., body weight), maturational milestones (e.g., eye opening & incisor eruption), and biobehavioral development (e.g., negative geotaxis & surface righting). Pups exposed *in utero* to 12,000 ppm or 8,000 ppm toluene weighed significantly less than the non-exposed control pups beginning at PN4 and PN12 (respectively) until PN21. Toluene resulted in significant increases in an index of poor perinatal outcome, specifically a composite of malformations, defined “runting” and neonatal death. No significant delays were observed in reaching maturational milestones. The results reveal that brief, repeated, prenatal exposure to high concentrations of toluene can cause growth retardation and malformations in rats. A comparison of the present, conservative results with findings in previous studies implies that binge patterns of toluene exposure in pregnant rats modeling human solvent abuse can result in developmental and morphological deficits in offspring. These results do not exclude the possibility that maternal toxicity as well as teratogenic effects of toluene may contribute to outcomes. The results suggest that abuse of inhaled organic solvents like toluene may result in similar early developmental outcomes in humans.

1. Introduction

Volatile substance abuse is a dangerous behavior that has been documented throughout the world [13,43]. Within just the United States, a recent National Survey on Drug Use and Health estimates that more than 22 million Americans twelve years of age and older have abused volatile substances at least once in their lifetimes [48]. There is evidence that this

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abuse is not decreasing even while drug abuse rates overall have decreased over the past several years. In 2011, 482,000 individuals under 18 years old used inhalants and 719,000 individuals aged 12 or older reported using inhalants for the first time within the past 12 months [51]. The average age at first use among those aged 12 to 49 years was also relatively unchanged from 2009 and 2010 (16.9 and 16.3 years, respectively) with 68.4 percent being under age 18 years old when they first used [50]. Once seen almost exclusively in males, the abuse of volatile substances has been increasing in females such that females at certain ages abuse volatile substances more often than their male counterparts [44,48]. Since volatile substance abuse can extend also into adulthood, the frequency of volatile substance abuse by women in their prime reproductive years may be escalating [6,37,42], raising concern about negative impacts of intentionally inhaled organic solvents on the developing fetus [7,12,24,31].

Solvents containing toluene are among the most commonly inhaled psychoactive substances during pregnancy [36]. Toluene, an aromatic hydrocarbon (CAS #108-88-3; C_7H_8 ; methylbenzene; toluol), is commonly found in household products including cleaners, glues, paint thinners, gasoline and other petroleum distillates [24,31]. Like other drugs of abuse, toluene is abused for its intoxicating and rewarding effects in the brain [28]. Acute inhalation of high concentrations of toluene produces initial excitation/disinhibition followed by a more prolonged dose-dependent inhibition, dizziness, loss of coordination, disorientation and distorted perceptions [28]. Long term exposures to high concentrations of toluene can result in cerebellar damage, neuronal atrophy and severe white matter abnormalities [15,16,38,45]. Chronic high-level exposure can also lead to a number of neurological problems including ataxia, convulsions, peripheral neuropathy and encephalopathy, as well as accidental death [9,34,39,41].

Toluene abuse by pregnant women can result in developmental disorders and malformations in offspring [4,5,18,25,26]. Maternal exposure to very high levels of abused solvents like toluene, both clinically and in animal models, can result in fetal or infant death [5,55] and gross morphological teratogenicity in surviving neonates [7,24]. Infants born to mothers who abused toluene during their pregnancy were usually premature, retarded in their growth, and microcephalic with distinct dysmorphology including deep-set eyes, small face, low set ears, micrognathia, spatulate fingertips and hypoplastic fingernails [5,18,25,26,29,53]. As the abuse of toluene continues [46,47,49,51], the potential for adverse pregnancy outcomes, developmental delays and neurobehavioral problems in children born to inhalant abusers also continues [12,24].

We employ animal models of prenatal toluene exposure that are helping define how factors specific to binge high-level toluene abuse, in contrast to long-term lower-level exposures that may occur in well-managed business or industrial setting. Some of these factors including route of administration, dosage and timing of exposure, influence the toluene developmental toxicity and impact on biobehavioral maturation of offspring. To our knowledge, relatively fewer studies have examined gross dysmorphology after repeated, high-dose binge patterns of prenatal toluene exposure [8,10,11,19,54]. In our previous study, prenatal binge exposure to repeated 15-min bouts of toluene (8,000 or 12,000 ppm) twice a day (30-min total daily exposure) from Gestation Day 8 (GD8) through GD20, resulted in significantly increased numbers of minor malformations, "runting" and fetal or postnatal death [8]. The present research expands this earlier study with a pattern of twice-daily 30-min exposures repeated high-dose toluene during gestation in rats modeling binge exposure patterns common in human solvent abuse [2,12,14,23,40]. The teratogenic impact of toluene on prenatal and early neonatal growth, perinatal outcome, and pre-weaning neurobehavioral development of offspring was assessed.

2. Materials and Methods

The Wayne State University Institutional Animal Care and Use Committee (IACUC) granted prior approval to all procedures which were in accordance with the NIH “Guide for the Care and Use of Laboratory Animals: Eighth edition” (National Academy of Sciences 2011, revised 2010). The procedures were the same as those detailed in Bowen, et al., 2005, except for exposure duration.

2.1 Prenatal Toluene Exposure

Briefly, 56 timed-pregnant female Sprague-Dawley rats arrived on gestational day (GD) 4 (date sperm-plug positive = GD0) from Charles River Laboratories (Portage, MI, USA). Each pregnant rat was housed individually in polypropylene cages (52 × 28 × 22 cm) with hardwood chip bedding. Dams had free access to water and Rodent Lab Diet 5001 (PMI, Nutrition International, Inc., Brentwood, MO) except during brief periods in the exposure chambers. The AAALAC-certified vivarium had a 12-hr light/dark cycle (lights on at 0600 hrs) with controlled temperature (20–22°C) and relative humidity (40%–70%). Dams were assigned to one of three gestational treatment groups, counter-balanced for maternal body weight on GD4. From GD8 to GD20, dams were weighed every other day and exposed daily via inhalation (see below) to 8,000 ppm or 12,000 ppm of toluene, or an “air-only” control group (0 ppm). Beginning on GD20 dams were inspected frequently between 0800 hrs and 2000 hrs for births; day of birth = PostNatal day 0 (PN0).

2.2 Inhalation Exposure Procedures

For each daily exposure, the rats were transported from the vivarium, in their home cages, to the lab and placed into an exposure chamber (detailed in Bowen, et al., 2005). Exposures were done twice a day between 0900 hrs and 1400 hrs, 2 hours apart, for 30 min each from GD8 through GD20 to expand assessments regarding the duration of high-dose “binge” exposures reported previously in human abusers [14,40]. The 60-mins of total daily exposures were double the vapor exposures given previously [8]. Toluene (#T324; Fisher Scientific, Fair Lawn, New Jersey) vapor concentrations were confirmed periodically by infrared spectrometry. Mean toluene concentrations were within 3% of nominal ~2.5 min after the toluene was added and remained within ~5% throughout the 30-min sessions. Changes in levels of waste gases (i.e., water vapor and CO₂) were negligible. Each animal was exposed to only one solvent concentration. At the conclusion of each exposure, dams were removed immediately and returned to their home cages to await the next exposure with the same procedure repeated 2 hrs later. After the two daily exposures, animals were returned to the vivarium in their home cages.

2.3 Birth Measures

All assessments were done “blind” to the prenatal exposure status. Pups were examined on PN1 for obvious morphological anomalies (e.g., missing digits, facial malformations, etc.), sexed by relative anogenital distance, and culled pseudo-randomly to 10 pups, that is, “random” except for culling “runts” first (defined below) and keeping an equal number of male and female pups per litter when possible. Gestation length was calculated at birth and the following litter data were collected on PN1: litter size, sex ratio (as percent males), body weight for each pup, and the number of malformed offspring. Postnatal deaths prior to weaning on PN21 were noted. Litters remained with their dams. Postnatal biobehavioral maturation of the pups was assessed until they were weaned on PN21 when the dams were euthanized and the uterine horns excised, washed in saline, and stored in 15% ammonium sulfate to aid later visualization of implantation sites.

2.4 Developmental and Behavioral Assessment

On PN1, the pups were uniquely identified within each litter with a small tattoo on one or more of the paws. Whole litters were assessed in three outcome categories: 1) reflex development; 2) motor coordination; and 3) strength. Tests were selected from standard neurobehavioral developmental test batteries [cf., [3]]. Pups from each litter were weighed on PN1, PN4, PN8, PN12, PN16, and PN21, and the emergence of physical maturation landmarks were noted each day, including pinnae detachment (typically between PN3 – PN5), incisor eruption (PN8 – PN10), and eye opening (PN12 – PN16). The dam was first removed from the home cage and the individual tests within each testing category proceeded in the following order: 1) reflex development – righting reflex, 2) motor coordination – the negative geotaxis test, and 3) muscle strength tests. After all tests were administered, the dam was returned to the litter in the home cage. The order of testing litters was randomized on each day. All testing was conducted between 0900 hrs and 1500 hrs.

2.4.1 Reflex development—For righting reflex, the pup was placed on its back and the latency (secs) to right itself recorded. Each pup received one trial per day from PN4 – PN12. The criterion for successful righting was the animal's turning itself over to a position in which all four paws were in direct contact with the floor and the limbs were in a position so that walking was possible. Pups that did not successfully right themselves were assigned the maximum of 60 secs.

2.4.2 Coordination—Coordination was examined using the negative geotaxis test. The pup was placed head down, 20 cm from the bottom of a 37-cm-wide wire mesh screen tilted 30°. The latency (60-sec max) to turn 180° to face up the screen was recorded. Pups were given one trial each day on PN6, PN8, and PN10.

2.4.3 Strength—Forelimb grip strength was assessed on PN12 and PN16 with a modified “push-pull” strain gauge (Exttech #475040). Animals were held by the base of the tail and the forepaws placed on a 2.4-mm-dia. horizontal bar supported 5 cm above the apparatus. The animal was then pulled gently and the maximum grasp strength (grams) was measured with the strain gauge at the point the animal released the bar. The peak strength of two determinations at each age was averaged and used in the analysis. Strength was also assessed using a hanging grip test measuring how long the pup could hang from a 2.2-mm-dia. bar suspended 12 cm from a surface covered with woodchip bedding. The hang time was assessed in one trial (60-sec max) each on PN12 and PN16 immediately following the second trial in the forelimb grip test. The pups were held by the nape of the neck and their forepaws placed on the bar. The time from placement on the bar until the animal let go was measured (secs). Pups that fell off immediately were deemed not to have been correctly positioned on the apparatus and were given another chance.

2.5 Statistical Analyses

Maternal body weight was analyzed using a 3×8 two-way, repeated-measures ANOVA with prenatal Treatment (i.e., toluene concentration – 0 ppm, 8,000 ppm or 12,000 ppm) and Gestational Day (GD4, GD8, GD10, GD12, GD14, GD16, GD18 & GD20) as factors. Litter size, gestation length and sex ratio were analyzed by one-factor ANOVAs with prenatal Treatment as the factor. Offspring development variables, including pup weight, were analyzed using litter as the unit of measure. The means of all male and female pups, respectively, within a litter were calculated for each outcome and used in the analyses [cf., [1]]. Two- and three-way ANOVAs were used with prenatal Treatment and Sex as between-subjects factors. For those variables assessed multiple times over the neonatal period, Age was also used as a repeated factor. An *alpha* level of $p < 0.05$ was used to determine statistical significance. The Greenhouse-Geisser correction procedure was used for repeated measures

with more than 4 levels in the factor to mitigate potentially inflated estimates of significance [cf., [33]]. Planned comparisons among the concentrations of toluene were also used, as well as Tukey's B *post hoc* analyses, as appropriate ($p < 0.05$). Frequency data for malformations were analyzed with Kruskal-Wallis one-way ANOVA by ranks [56].

3.0 Results

3.1 Maternal and Litter Results

Of 65 rats received, 2 were not pregnant as determined by lack of uterine implantation sites (1 from 8,000-ppm group and 1 from 12,000-ppm toluene group). Three dams gave birth to live litters containing < 4 pups (2 dams from 8,000-ppm group & 1 from 12,000-ppm toluene group). One 8,000-ppm dam died during the last repeated exposure period on GD20. Of the 59 confirmed pregnant dams, 56 gave birth to live litters. Two animals from the 0-ppm group suffered from dystocia and were unable to give birth. One 8,000-ppm dam delivered only stillborn pups. Maternal and litter data for the 56 remaining dams included in the three groups are presented in Table 1. There were no significant differences among groups for weight of dams on GD4 before toluene exposures began (mean = 220.03 g; $F(2,62) = 0.09$, $p = 0.91$). Maternal weight gain and percent maternal weight gain during gestation were significantly different among prenatal Treatment groups by GD20 ($F(2,62) = 9.03$, $p < 0.001$; $F(2,62) = 7.59$, $p < 0.001$, respectively). Further, there were significant effects of Treatment ($F(2,60) = 3.16$, $p < 0.05$), and gestational Day ($F(7,420) = 711.71$, $p < 0.001$), with both the 8,000- and 12,000-ppm groups weighing less overall and gaining less weight as compared to the air-control group. There was also a significant Treatment $\times$ Day interaction ($F(14,420) = 5.79$, $p < 0.001$) in that day-to-day changes in maternal weight during gestation by both the 8,000- and 12,000-ppm groups were significantly slower than for the 0-ppm controls. No significant prenatal Treatment group differences were found in total litter size or mean gestation length, although the maximum resolution on gestation length was only one half day.

Table 1 presents the frequencies of gross malformations and external soft tissue malformations, pups classified as "runts" (PN1 weight > 2 SD's below the pup's own litter mean with the "runt" included), and neonatal mortality. Two pups in two litters in the 0 ppm-group were "runts" and one pup in another 0-ppm litter had a malformation, being born without a tail. Three pups in one litter in the 8,000-ppm toluene group were "runts" and "bottle-nosed", and three different litters had pups that were "runts" (two pups were euthanized and one died prior to weaning). Within the 12,000-ppm toluene group, 20 pups in 15 different litters were variously affected, including one "runt" and the remainder born with major and minor malformations. These malformations included missing digits, missing limbs, and missing eyes (unilateral anophthalmia). In addition, there was one neonatal death at PN21 in the 8,000-ppm group. Considering the exclusive combined cumulative frequencies of malformations plus "runts" and neonatal deaths, 15.8% of the 0-ppm litters, 33.33% of the 8,000-ppm litters, and 63.16% of the 12,000-ppm toluene litters were adversely affected. The percentage of litters affected by these three kinds of negative outcomes in the higher toluene-exposure concentration group was nearly four times the rate in the non-exposed, 0-ppm group (63.2% vs 15.8%). The percentage of litters so affected was also significantly higher in the 12,000-ppm toluene-exposed group than in the 0-ppm controls ($p < 0.05$). The rate of combined malformations, plus "runting," and neonatal deaths between litters was also significantly higher in the 12,000-ppm group than in the untreated controls ($p < 0.05$), but the 8,000-ppm group was not significantly different from either of the other two groups. There were no significant differences or interactions in the rate of these outcomes between the sexes.

3.2 Pup Body Weights and Biobehavioral Development

No significant main effects were found for prenatal Treatment on PN1 body weight for all pups, $p > 0.50$ (Table 1 & Fig. 1). A significant main effect was found for Sex, $F(1,56) = 4.06$, $p < 0.05$: female pups weighed less than male counterparts. There was a large overall main effect of Age as all animals gained significant weight by weaning, $F(5,265) = 4,222.14$, $p < 0.001$. There was a significant Age $\times$ Treatment interaction, $F(10,265) = 7.95$, $p < 0.001$. From PN1 to PN21, the 8,000- (602%) and the 12,000- (615%) ppm-exposed pups gained relatively less weight than the 0 ppm-exposed group (643%; see Table 1).

No delays or accelerations in indices of postnatal maturation were observed for incisor eruption, pinnae unfolding, or age of eye opening for toluene-exposed animals relative to sham-exposed animals. There was a significant main effect of Age as all animals improved (i.e., decreased) their surface righting times between PN4 and PN12, $F(8,400) = 17.24$, $p < 0.0001$ (data not shown). Although there was a trend for the 12,000-ppm exposed pups to take longer to right themselves, the main effect for prenatal Treatment on surface righting did not reach significance ($p = 0.08$), nor were there significant interactions among Treatment or Age, or Sex. There was an overall significant main effect of Age as male and female animals in all groups increased their mean forelimb grip strength between PN12 and PN16, $F(1,50) = 1169.98$, $p < 0.0001$ (Fig. 3). There was also a significant main effect for Sex, $F(1,50) = 3.94$, $p = 0.05$, and prenatal Treatment on grip strength, $F(2,50) = 3.41$, $p < 0.05$, but no significant interactions. There was a significant overall main effect of Age, $F(2,100) = 160.11$, $p < 0.0001$, in the negative geotaxis test with no effect of prenatal toluene Treatment, $p = 0.26$, and no significant interactions (data not shown). Between PN6 and PN10, all animals decreased their latencies to turn 180°.

4.0 Discussion

The present study evaluated developmental and behavioral effects in offspring of rats exposed repeatedly (twice a day for 13 days) during pregnancy (from GD8 through GD20) to very high concentrations of toluene (8,000 ppm or 12,000 ppm) for 30 min. This binge pattern of high-level prenatal toluene exposure significantly decreased maternal weight gain and percent maternal weight gain during gestation with both the 8,000- and 12,000-ppm groups weighing less overall and gaining less weight compared to the air-control group. In offspring, this pattern of exposure increased the rates of minor malformations, “runting” and fetal and early postnatal death, while also decreasing postnatal growth and reducing grip strength. Conversely, no significant effects were observed for gestation length, litter size, or litter sex ratio. These results extend our prior findings in rats with a shorter exposure period of twice-daily exposures from 15 min [8] to 30 min. We had also reported previously significant increases in the rates of fetal soft tissue anomalies and skeletal abnormalities after 30-min prenatal exposures to 8,000, 12,000 or 16,000 ppm toluene in mice following the same regime of 2 exposures per day from GD8 through GD20 [elaborated below; [11]]. The current results show that morphological and marginal neurodevelopmental outcomes in early postnatal rats also arise with 30-min exposures.

The current research is important because the patterns of inhalant use among solvent abusers similarly occur in episodic bursts with varying durations to very high concentrations of toluene levels, exceeding 10,000 ppm [2,12,23]. These rodent models of high-level prenatal toluene exposure showing teratological and developmental consequences (e.g., [8,11]) stand in contrast to models using gavage or intraperitoneal injections that are not able to mimic the pharmacodynamics of inhalation (e.g., [19–22]). The present model has high fidelity to clinically relevant characteristics which can determine the shape of “dose/response” and “time/concentration” curves for toluene, such as diffusion and absorption, susceptibility to gastric metabolism, and kinetics of repeated administration [8,35].

In our current study, growth deficits were more persistent after our repeated 30-min binge exposures to high concentrations than were seen with shorter 15-min binge exposures [8] or with much longer exposures (6 hrs) to lower concentrations (1,800 ppm or less) in the few research reports that have used similar prenatal inhalation exposures [27,32,52]. In the only other study we are aware of in rats that used identical exposure patterns, all levels of toluene resulted in significant reductions in fetal growth (i.e., placental weight, fetal weight, and crown-rump length) without affecting maternal weight gain [11]. Prenatal toluene exposure also produced a number of gross morphological anomalies including short and/or missing digits and missing limbs. Skeletal examinations revealed reduced ossification of the extremities as well as misshapen scapula, missing and supernumerary vertebrae and ribs, and fused digits. Soft tissue anomalies were also reported (e.g., cryptorchidism, displaced abdominal organs, gastromegaly, distended/hypoplastic bladder, and delayed cardiac development). Bowen et al. [8] exposed pregnant dams to high-level toluene inhalations (8,000 ppm or 12,000 ppm) for 15 min twice a day from GD8–GD20 which generated a high “load” of 6,000 ppm/hours/day, equal to one-half the calculated “load” with the highest dose in the present investigation (2×30 -min exposures with 12,000 ppm). In this study, pups exposed to 12,000 ppm toluene *in utero* weighed significantly less than the control pups at all ages before PN16. In addition, *in utero* toluene exposure resulted in significant increases in poor perinatal outcome (i.e., a combination of malformations, “runting” and neonatal death). As with the current investigation, there were no significant delays in reaching maturational milestones.

In further support of the dose-dependency of teratogenic and developmental effects, exposures to lower levels of toluene inhalations (200 ppm, 400 ppm, or 2,000 ppm) for 3 hrs a day (3×60 -min exposures with 2,000 ppm) limited to GD12–GD17, resulted in no significant increases in neonatal deaths or minor malformations [32]. Compared to the effects of the abuse pattern of exposure in the current study, the longer exposures in the Jones and Balster study were also associated with reliable decreases in pup weight gain and in grip strength, with the effects on initial offspring body weight being more modest. In yet another study, pregnant Wistar rat dams exposed to toluene inhalations no greater than 1,200 ppm for 6 hrs/day from GD9 to GD21 produced offspring with lower body weights (highest doses only) with only one minor indication of maturational effects [52]. Although there are differences among these studies (e.g., number of days exposed; strain of rat), the comparisons of results among these studies is consistent with the hypothesis that repeated binge patterns of exposure to higher concentrations of toluene found in intentional solvent misuse/abuse pose a greater maternal and/or embryonic/fetal risk than continuous exposures to lower concentration, for at least some outcomes.

Although body weights of the toluene-exposed pups did not differ from non-exposed pups at birth (i.e., PN1), toluene-exposed pups failed to gain weight at the same rate as their non-exposed counterparts and from PN4 until PN21 weighed significantly less than the 0-ppm-exposed pups. These results are different from our previous investigation using 15-min exposures as well as earlier reports of prenatal exposure to lower concentrations of toluene (2,000 ppm) in mice [32]. The lower relative weight gain over the first 3 weeks of postnatal life for both toluene-exposed groups may be secondary to the reduced gestational body weight and possible under-nutrition of the mother. These results differ from our previous report with 15-min exposures [8] where no significant differences in maternal weight or weight gain were observed during gestation, suggesting that differences in length of toluene exposure (30 min *vs* 15 min) led to some general toluene maternal toxicity as an additional factor in pup outcomes. However, it should be noted in this context that no significant differences were observed for pup weights on PN1. Others have reported that prenatal exposure to toluene decreases fetal and placental weights regardless of maternal food consumption [19,21]. It is also possible that secondary nutritional reasons may account for

the decreased weight gains in the present study including altered nutrient absorption and/or metabolism [30], as well as altered pup suckling and/or maternal behavior and should be the focus of future investigations. It is possible that toluene-induced maternal toxicity during pregnancy may have had persistent effects on lactation, nursing or other maternal behaviors that contributed to the growth restrictions noted in the pups. Further studies with cross fostering may be useful in addressing a possible maternally mediated, secondary postnatal reduction in pup weight gain.

The clinical literature has little information on the effects of occupational or environmental toluene exposure on prenatal and/or postnatal development, and there are fewer articles describing the impact of abuse patterns of either toluene exposure alone, or of many other solvents and combinations of solvents (e.g., 1,1,1-trichloroethane & gasoline), with or without toluene, that are intentionally abused during pregnancy [7,24]. From these few articles, we know that there are individual cases of malformations [4,5,18,25,26,44,53] and growth deficiencies [17,44] reported in children born to women disclosing that they abused toluene during their pregnancies. Additionally, infants exposed *in utero* to toluene have been shown to have a number of behavioral, developmental, and physical problems (termed “Fetal Solvent Syndrome”) including hyperactivity, language impairment, cerebellar dysfunction, growth retardation, and facial dysmorphology [5,18,25,26,29,53]. Later assessments of toluene-exposed children up to 3 years of age, uncovered cerebellar dysfunction, cerebral palsy, developmental delays, language impairment, hyperactivity, mental retardation, postnatal growth retardation, and seizures [5,25,26,44]. Finally, concentrations of maternal solvent exposure reported as “heavy” or “very high” have been reported to result in perinatal death [5,55].

In the current study, rat offspring exposed *in utero* to high concentrations of toluene displayed significant deficits compared to non-exposed control animals. While there was no significant toluene-induced shift in physical maturation as assessed by developmental “milestones” (i.e., pinnae detachment & incisor eruption), there was significant reduction in postnatal growth up to weaning. Unlike our previous study, the changes that occurred did so in the presence of obvious maternal effects, assessed as dam gestational weight gain. However, abuse patterns of prenatal toluene exposure significantly increased the cumulative rate of poor perinatal outcome assessed as an index combining minor morphological anomalies, “runting,” and early neonatal mortality. The developmental effects of prenatal toluene were assessed conservatively by culling “runts” and excluding small litters ($n's < 6$), both of which were more frequent after toluene exposure, so that the developmental impact of toluene may be underestimated. As a whole, these results, in combination with our previous work [8,10], suggest that an inhaled, high-concentration, binge pattern of exposure to toluene is capable of producing developmental and morphological deficits in rats, even when the daily exposures are very brief in comparison to occupational or environmental exposures. This study extends our animal model of gestational exposure to abuse patterns of inhaled toluene by characterizing relatively brief, repeated exposures to very high concentrations of toluene via inhalation for 30 min, an abuse-like binge pattern of exposure seen in pregnant women and women of childbearing age that can have serious injurious impact on fetal growth, survival, and early postnatal development.

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Figure 1. Effects of 0 ppm, 8,000 ppm, or 12,000 ppm toluene exposure on percent weight gain in pregnant rats. Shown are means $\pm$ SEM of percent weight gain from GD4 through GD20. Inset graph shows mean $\pm$ SEM of dam weights (grams) on PN20. Toluene 8,000 (#) and 12,000 (*) ppm groups significantly different from sham exposure group ($p < 0.05$).

Figure 2. Effects of 0 ppm, 8,000 ppm, or 12,000 ppm toluene exposure on pup weight gain. Shown are means $\pm$ SEM of percent weight gain from PN1 through PN21. Inset graph shows mean $\pm$ SEM of pup weights (grams) on PN1. Toluene 8,000 (#) and 12,000 (*) ppm groups significantly different from sham exposure group ($p < 0.05$).

Figure 3. Effects of prenatal 0 ppm, 8,000 ppm, or 12,000 ppm toluene exposure on forelimb grip strength. Shown are means $\pm$ SEM of pup strength (grams) for PN12 (left) and PN16 (right). The inset depicts effects of prenatal 0 ppm, 8,000 ppm, or 12,000 ppm toluene exposure collapsed across Day.

TABLE 1

MATERNAL AND LITTER CHARACTERISTICS (means $\pm$ sem)

Level of Toluene:	0 ppm	8,000 ppm	12,000 ppm	p
Number of dams ($N=65$) ^a	21	23	21	--
Number of live litters ($N=56$) ^b	19	18	19	--
Maternal weight on GD4 (g)	219.43 $\pm$ 3.94	219.49 $\pm$ 3.12	221.24 $\pm$ 2.63	ns
Maternal weight gain to GD20 (g) ^c	118.91 $\pm$ 5.15	94.65 $\pm$ 4.78	95.70 $\pm$ 3.48	0.001
Percent maternal weight gain ^d	54.83 $\pm$ 2.87	43.48 $\pm$ 2.48	43.29 $\pm$ 1.57	0.001
Gestational age at birth (days)	22.16 $\pm$ 0.08	22.35 $\pm$ 0.11	22.42 $\pm$ 0.12	ns
Mean number of implant sites	12.37 $\pm$ 0.56	11.48 $\pm$ 0.57	11.70 $\pm$ 0.42	ns
Litter size (range; live pups)	12.26 $\pm$ 0.54 (8–16)	11.00 $\pm$ 0.76 (3–15)	11.45 $\pm$ 0.66 (3–16)	ns
Number males	6.16 $\pm$ 0.62	6.17 $\pm$ 0.38	4.95 $\pm$ 0.45	ns
Number females	6.10 $\pm$ 0.54	6.06 $\pm$ 0.41	6.95 $\pm$ 0.55	ns
Sex ratio (% males)	49.86 $\pm$ 4.32	51.79 $\pm$ 2.62	43.16 $\pm$ 4.52	ns
Mean overall weight on PN1 (g)	7.06 $\pm$ 0.20	6.84 $\pm$ 0.16	6.80 $\pm$ 0.17	ns
Male weight on PN1 (g)	7.27 $\pm$ 0.31	7.03 $\pm$ 0.23	7.02 $\pm$ 0.24	ns
Female weight on PN1 (g)	6.84 $\pm$ 0.26	6.65 $\pm$ 0.21	6.58 $\pm$ 0.24	ns
Mean overall weight on PN21 (g)	52.43 $\pm$ 1.25	48.09 $\pm$ 1.08	47.54 $\pm$ 0.61	0.01
Male weight on PN21 (g)	53.50 $\pm$ 1.81	48.91 $\pm$ 1.54	48.13 $\pm$ 0.84	0.05
Female weight on PN21 (g)	51.36 $\pm$ 1.74	47.28 $\pm$ 1.55	46.95 $\pm$ 0.88	0.06
Litters w/malformed, "runted," dead pups	3	6	12	0.01 ^d
Percent affected litters	15.79%	33.33%	63.16%	0.05 ^d
Total number of affected pups	3	11	20	0.01 ^d
Number of affected pups per litter	0.16 $\pm$ 0.08	0.52 $\pm$ 0.23	1.00 $\pm$ 0.23	0.05 ^d
Percent affected pups per litter	1.33%	10.98%	9.05%	0.01
Affected pups per affected litter	8.44 $\pm$ 0.65	38.43 $\pm$ 19.47	15.08 $\pm$ 2.25	ns

^aOf the total of 65 dams received into the experiment, 2 were not pregnant (1 from 8,000-ppm; 1 from 12,000-ppm toluene exposure groups). One dam in the 8,000-ppm toluene exposure group died on GD20 and is not included.

^bOne dam in the 0-ppm, 2 dams in the 8,000-ppm and 1 dam in the 12,000-ppm toluene exposure groups had litters with < 6 pups and were not used for birth outcomes (e.g., body weight) or for postnatal developmental analyses. One 8,000-ppm dam delivered only stillborn pups. All *p* values are from the overall analyses.

^cMean $\pm$ sem maternal weight gain from GD4–GD20. Maternal weight before exposure on GD4 subtracted from maternal weight before exposure on day 20. The 8,000 and 12,000-ppm toluene exposure groups are significantly lower than 0-ppm controls.

^dThe 12,000-ppm toluene exposure group is significantly higher than 0-ppm controls.