Transplacental benzene exposure increases tumor incidence in mouse offspring: possible role of fetal benzene metabolism

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Childhood cancer is the leading cause of disease-related death in children aged 1–14 years in Canada and the USA and it has been hypothesized that transplacental exposure to environmental carcinogens such as benzene may contribute to the etiology of these cancers. Our objectives were to determine if transplacental benzene exposure increased tumor incidence in mouse offspring and assess fetal benzene metabolism capability. Pregnant CD-1 and C57Bl/6N mice were given intraperitoneal injections of corn oil, 200 mg/kg, or 400 mg/kg benzene on gestational days 8, 10, 12 and 14. A significant increase in tumor incidence was observed in CD-1, but not C57Bl/6N, 1-year-old offspring exposed transplacentally to 200 mg/kg benzene. Hepatic and hematopoietic tumors were predominantly observed in male and female CD-1 offspring, respectively. Female CD-1 offspring exposed transplacentally to 200 mg/kg benzene had significantly suppressed bone marrow CD11b+ cells 1 year after birth, correlating with reduced colony-forming unit granulocyte/macrophage numbers in 2-day-old pups. CD-1 and C57Bl/6N maternal blood benzene levels and fetal liver benzene, t, t-muconic acid, hydroquinone and catechol levels were analyzed by gas chromatography/mass spectrometry. Significant strain-, gender- and dose-related differences were observed. Male CD-1 fetuses had high hydroquinone levels, whereas females had high catechol levels after maternal exposure to 200 mg/kg benzene. This is the first demonstration that transplacental benzene exposure can induce hepatic and hematopoietic tumors in mice, which may be dependent on fetal benzene metabolism capability.

Introduction

Childhood cancer is the greatest cause of disease-related deaths in children aged 1–14 years in Canada (1) and the USA (2). The vast majority of childhood cancers are of unknown etiology; however, prenatal factors such as maternal exposure to carcinogens during fetal development may play a role (1). In humans, in utero exposure to diethylstilbestrol or ionizing radiation is associated with enhanced cancer rates in children and young adults (3). In addition, there is evidence that transplacental exposure to toxicants such as cigarette smoke, pesticides and benzene is correlated with a higher incidence of childhood cancer (4–9). In mice, transplacental carcinogenesis has been induced by environmental toxicants such as the tobacco-specific carcinogen 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butane (10), the food-derived mutagen 2-amino-1-methyl-6-phenylimidazo[4,5-b]-pyridine (11), arsenic (12) and the polycyclic aromatic hydrocarbons 3-methylcholanthrene (13–15) and dibenzo[a,l]pyrene (16).

Abbreviations: ANOVA, analysis of variance; AUC, area under the curve; BFU-E, blast-forming unit erythroid; BM, bone marrow; CFU-G, colony-forming unit granulocyte; CFU-M, colony-forming unit macrophage; GC, gas chromatography; GD, gestational day; i.p., intraperitoneal; MS, mass spectrometry; PD, postnatal day; TMS, trimethylsilyl.

Benzene is a ubiquitous pollutant with known carcinogenic and hematotoxic effects in humans and mice (17). Mechanisms of benzene-induced hematotoxicity are unclear; however, hepatic benzene metabolism is a crucial step. For example, both humans and mice with altered capacities to metabolize benzene have differing sensitivities to its toxic effects (18–20). Benzene metabolism (Figure 1) is primarily mediated by cytochrome P450 (CYP) 2E1 (CYP2E1), resulting in the formation of metabolites that have varying degrees of cell-damaging ability; for example, hydroquinone and catechol have much higher DNA-damaging capabilities compared with t,t-muconic acid (21–23).

In this study, we show that transplacental benzene exposure increases hepatic and hematopoietic tumor incidences in mice, which might be due to differences in fetal benzene metabolism capability.

Materials and methods

Animals and experimental design

Seven- to nine-week-old female C57Bl/6N and CD-1 mice were obtained from Taconic Farms (Germantown, NY). Animals were acclimated for 1 week prior to use and were housed in a temperature-controlled room with a 12 h light:dark cycle (lights on from 6 a.m. to 6 p.m.). Mice were given free access to autoclaved standard rodent chow (Purina Rodent Chow; Ralston Purina International, Strathroy, Ontario, Canada) and tap water. A maximum of three females were housed with one male overnight and the presence of a vaginal plug the following morning designated gestational day (GD) 1. Pregnant C57Bl/6N and CD-1 mice were exposed to either Mazola® corn oil (vehicle), 200 mg/kg benzene or 400 mg/kg benzene (Sigma–Aldrich Canada Ltd, Oakville, Ontario, Canada) on GDs 8, 10, 12 and 14 by intraperitoneal (i.p.) injection, which encompassed hematopoietic stem cell emergence (approximately GD 10 in the aorta–gonad–mesonephros) and fetal liver hematopoietic stem cell expansion (GD 11 to birth) (24). Figure 2 depicts the experimental design of this study. Offspring tissue was sampled at GD 14 for benzene and metabolite level analysis, GD 16 and postnatal day (PD) 2 for hematopoietic progenitor cell colony formation analysis, 6 months for spleen and bone marrow (BM) cell analysis and 1 year for spleen and BM analysis as well as tumor incidence analysis. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care. Experimental procedures were approved by the Queen’s University Animal Care Committee.

Flow cytometric analyses of 6-month and 1-year-old offspring spleen and BM

Blood was obtained by cardiac puncture at the time of killing. Spleen and BM tissue were collected upon necropsy and placed in Iscove’s modified Dulbecco’s medium (Invitrogen, Burlington, Ontario, Canada) on ice. Spleen tissue was loaded with 4′,6-diamidino-2-phenylindole (DAPI) to analyze DNA content by flow cytometry. Spleen and BM tissues were collected upon necropsy, GD 16 and postnatal day (PD) 2 for hematopoietic progenitor cell colony formation analysis, 6 months for spleen and bone marrow (BM) cell analysis and 1 year for spleen and BM analysis as well as tumor incidence analysis. All animals were treated in accordance with the guidelines of the Canadian Council on Animal Care. Experimental procedures were approved by the Queen’s University Animal Care Committee.

Histology

Tumor incidence was analyzed in 1-year-old offspring by histopathological analysis. For CD-1 mice, five offspring per gender from each of five litters per treatment group were analyzed. Due to smaller litter sizes, for C57Bl/6N mice, three to four offspring per gender from each of eight litters per treatment group were analyzed. The following tissues were collected from 1-year-old offspring at necropsy: spine, heart, thymus, lung, spleen, liver, stomach, small and large intestine, kidney and any abnormal tissue. Tissues were immediately fixed in 10% formalin and stored at room temperature for at least 24 h and then paraffin embedded and stained with hematoxylin and eosin. Blinded histopathological analysis of tissue sections was performed under a light microscope by a pathologist. Tissue abnormalities were identified based on histological morphology.
DNA was isolated from tissue using a QIAGEN DNeasy blood and tissue kit (QIAGEN, Mississauga, Ontario, Canada). The primers were purchased from Cortec (Kingston, Ontario, Canada) and the sequences were as follows: SRY9791: 5'-AGCTGTTTGTCTTGTGCTAGCC-3'; SRY8212: 5'-TTGTGTGTTGTTAGCCTACAGGC-3'; D7Mit164F: 5'-ACACAAATTGGAATTTGGGACC-3' and D7Mit164R: 5'-TTCCTACTGGAATTTCGTTGG-3'. The expected product size of SRY is 1.6 kb and D7Mit164 is 304 bp. Polymerase chain reaction was conducted using a QIAGEN multiplex PCR kit (QIAGEN). Briefly, 20 µl of the reaction mix (containing 0.25 µM of each primer) was added to 125 ng fetal DNA and reactions were run on a Techne Touchgene gradient thermal cycler using the following conditions: 95°C for 15 min (94°C for 30 s, 63°C for 90 s and 72°C for 90 s) for 35 cycles and 72°C for 10 min. Polymerase chain reaction products were electrophoresed through a 3% agarose gel and visualized using a UVP BioDoc-It™ System Ultraviolet transilluminator.

Y-chromosome-specific polymerase chain reaction for sex determination of GD 14, GD 16 and PD 2 pups

Hematopoietic progenitor cell colony formation assay on fetal and postnatal liver tissue

The progenitor cell types measured were colony-forming unit, colony-forming unit granulocyte/monocyte (CFU-GM), colony-forming unit granulocyte (CFU-G) and colony-forming unit monocyte (CFU-M). Isolated fetal and pup livers were immersed in Iscove's modified Dulbecco’s medium and single-cell suspensions were obtained by gently passing the tissue through a 21 gauge needle. Cells were at least 90% viable as tested by the trypan blue exclusion assay. Nucleated cells were counted using 3% acetic acid with methylene blue (Strain Cell Technologies, Vancouver, British Columbia, Canada) and were plated in 35 mm culture dishes at a concentration of 1 x 10^5 or 1 x 10^6 cells/ml in methylcellulose medium containing erythropoietin [to detect colony-forming unit erythroid and blast-forming unit erythroid (BFU-E) colonies] or stem cell factor, interleukin-3 and interleukin-6 (to detect myeloid CFU-G, CFU-M and CFU-GM colonies), respectively (MethoCult M3334 and M3534; Stem Cell Technologies). For PD 2 CFU-GM cultures, the number of cells plated was increased to 1 x 10^5 cells/ml due to low colony number yield. Cultures were placed in a humidified incubator with 5% CO_2 at 37°C. Erythroid colony-forming unit erythroid colonies (each containing 10–30 cells) and BFU-E colonies (each containing >30 cells) were counted 3 days after incubation. Myeloid CFU-G, CFU-M and CFU-GM colonies containing at least 30 cells were counted 12 days after incubation.

Gas chromatographs/mass spectrometry detection of benzene levels in maternal blood and GD 14 fetal liver tissue

Fetal liver tissue and maternal blood were collected 1, 2 or 4 h after maternal benzene administration and immediately frozen in liquid nitrogen. Subsequently, 30–80 mg (wet weight) of fetal liver tissue of the same sex were combined, weighed and homogenized manually and then centrifuged at 6000 g for 2 min before benzene extraction. Whole maternal blood (100 µl) and homogenized fetal liver tissue were added to 500 µl of 100% high-performance liquid chromatography grade methanol (Sigma–Aldrich Canada Ltd) and vortexed for 20 s. Samples were then centrifuged at 8000 g for 3 min to pellet debris. The supernatants were diluted to 5 ml and the internal standard, fluorobenzene, was added. The sample was immediately injected into the purge and trap unit of a Hewlett Packard 5890 gas chromatograph (GC) and analyzed with a 5972 mass selective detector [mass spectrometry (MS)]. The sample was purged with high-purity helium gas for 11 min and the injector temperature was 275°C. The trapped components were desorbed from the VOCARBTM trap (Supelco Analytical, Oakville, Ontario, Canada) in the unit by heating to 225°C and holding for 4 min. A VOCOL™ column (Supelco Analytical) (60 m x 0.32 mm x 2.0 µm) was used for the analysis. The GC oven temperature program was ramped from 65°C to a maximum of 200°C. The MS was run in selective ion monitoring mode (benzene: m/z 78; fluorobenzene: m/z 90). Retention times were 10.1 min for fluorobenzene and 9.8 min for benzene. Concentrations were calculated using a 40 p.p.b. BTEX (benzene, toluene, ethylbenzene and xylenes) aqueous standard prepared from a 2000 µg/ml stock (Supelco Analytical).

GC/MS detection of benzene metabolites in GD 14 fetal liver

Fetal liver tissue was collected 1, 2 or 4 h after maternal benzene administration and immediately frozen in liquid nitrogen. The protocol used to detect...
**Results**

Treatment of pregnant mice with oil, 200 mg/kg, or 400 mg/kg benzene on GDs 8, 10, 12 and 14 did not elicit acute fetal or maternal toxicities as measured by significant alterations in litter size, resorption number, fetal weight or birth weight or abnormal maternal weight change during pregnancy (data not shown).

Transplacental benzene exposure causes long-term effects in the offspring

Flow cytometric analysis of mouse spleen and BM cells did not show any differences in CD4⁺ (cytotoxic T cells), CD8a⁺ (T-helper cells), CD45R/B220⁺ (B cells) cells or hematocrit values in CD-1- or C57Bl/6N-exposed offspring 6 months or 1 year after birth compared with controls (data not shown). However, female CD-1 mice exposed in utero to 200 mg/kg benzene had significantly decreased numbers of BM CD11b⁺ (myeloid cells) cells compared with oil-treated controls 1 year after birth (P < 0.05; n = 24) (Figure 3A). This effect was not seen in female or male C57Bl/6N mice or male CD-1 mice (data not shown).

Tumor incidence was evaluated in mice 1 year after birth (Table I). All tumors observed originated from lung, liver or hematopoietic tissue. Significantly increased numbers of total tumors (including lung, liver and hematopoietic) and liver tumors (primarily adenomas) were observed in male CD-1 mice exposed in utero to 200 mg/kg benzene compared with oil-treated controls (total tumor incidence P = 0.0329; relative risk: 2.3; odds ratio: 4.7; liver tumor incidence P = 0.0452; relative risk: 3.3; odds ratios: 5.3). Likewise, female CD-1 mice exposed in utero to 200 mg/kg benzene also had a significantly increased incidence of total tumors compared with oil-treated controls (P = 0.0019; relative risk: 10.4; odds ratio: 17.1), although female mice had an increased tendency to develop hematopoietic tumors (including hyperplasias, myeloproliferative disorders and myeloid/lymphoid neoplasias; P = 0.0232; relative risk: 7.3; odds ratio: 9.9).

**Statistical analysis**

Fisher’s exact test was used to assess tumor incidence versus treatment. Data from flow cytometric analysis of blood cell parameters and maternal blood and fetal liver area under the curve (AUC) values of benzene levels were analyzed by a two-way analysis of variance (ANOVA) and Bonferroni post hoc test. Data from colony assays were analyzed by a one-way ANOVA (for each colony type) followed by a Dunnet’s post hoc test. Data from GC/MS analysis of fetal liver AUC values of metabolite concentrations were analyzed by a three-way ANOVA followed by two-way ANOVA and a Bonferroni post hoc test if an interaction was identified between variables. P < 0.05 was considered statistically significant in all cases.

**Fig. 3.** (A) BM CD11⁺ parameters in 6-month and 1-year-old female CD-1 offspring transplacentally exposed to corn oil, 200 mg/kg or 400 mg/kg benzene (BZ). Values statistically different from controls are indicated with an asterisk. (B) Mean number of CFU-GM colonies formed in the colony formation assay of GD 16 and PD 2 CD-1 liver tissue after transplacental exposure to corn oil, 200 mg/kg or 400 mg/kg benzene. Data were separated by gender. Values statistically different from controls are designated with an asterisk.
Transplacental benzene carcinogenesis in mice

Table 1. The effect of in utero benzene exposure on tumor incidence in CD-1 and C57Bl/6N offspring 1 year after birth

<table>
<thead>
<tr>
<th>Treatment</th>
<th>CD-1</th>
<th>C57Bl/6N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Corn oil</td>
<td>200 mg/kg BZ</td>
</tr>
<tr>
<td>Gender/F</td>
<td>M/22</td>
<td>F/25</td>
</tr>
<tr>
<td>Number of animals</td>
<td>(27%)</td>
<td>(4%)</td>
</tr>
<tr>
<td>with any tumor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals</td>
<td></td>
<td></td>
</tr>
<tr>
<td>with tumors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lung tumors</td>
<td>1 (4%)</td>
<td>0</td>
</tr>
<tr>
<td>Liver tumors</td>
<td>3 (14%)</td>
<td>0</td>
</tr>
<tr>
<td>Hematopoietic tumors</td>
<td>2 (9%)</td>
<td>1 (4%)</td>
</tr>
</tbody>
</table>

M. male; F, female; BZ, benzene.
aCompared with the oil-treated controls (P = 0.0329; relative risk: 2.3; odds ratio: 4.7).
bCompared with the oil-treated controls (P = 0.0019; relative risk: 10.4; odds ratio: 17.1).
cCompared with the oil-treated controls (P = 0.0452; relative risk: 3.3; odds ratio: 5.3).
dCompared with female offspring in the same treatment group (P = 0.0014; relative risk: 10.9; odds ratio: 19.2).
eCompared with male offspring in the same treatment group (P = 0.0376; relative risk: 4.1; odds ratio: 6.0).

Liver tumor incidence in CD-1 mice exposed in utero to 200 mg/kg benzene was significantly higher in males compared with females (P = 0.0014; relative risk: 10.9; odds ratio: 19.2). However, the incidence of hematopoietic tumors in CD-1 mice exposed to 200 mg/kg benzene in utero was significantly higher in females compared with males (P = 0.0376; relative risk: 4.1; odds ratio: 6.0). No statistical difference was detected in total tumor incidence in CD-1 mice exposed to 400 mg/kg benzene in utero. Interestingly, there was no evidence of altered tumor incidences in C57Bl/6N mice after in utero benzene exposure in any of the treatment groups. It should be noted that some mice had to be euthanized before the end of the study due to wounds or illnesses; however, there was no significant association between treatment and early termination.

In utero exposure to benzene alters fetal liver hematopoietic colony numbers

Previous studies have shown that in utero exposure to benzene in mice alters the growth of myeloid and erythroid progenitor cells in fetal tissue, which correlates with sustained hematopoietic alterations up to at least 6 weeks after birth (26). Therefore, one of our objectives was to determine if benzene-induced alterations in fetal hematopoietic progenitor cell colony numbers are associated with increased tumor incidence and could therefore be used as an early marker of transplacental benzene carcinogenesis. We have recently reported colony number changes at GD 16 in C57Bl/6N fetal liver tissue (27) in response to in utero benzene exposure; however, these changes appear to be transient as no benzene-induced changes were detected at PD 2 in this strain for any of the colony types tested (colony-forming unit erythroid, BFU-E, CFU-G, CFU-M and CFU-GM; data not shown).

In contrast, female CD-1 offspring exposed to 200 mg/kg benzene in utero showed a significant decrease in CFU-GM colonies at PD 2 (P < 0.05; n = 6) compared with controls, despite no evidence of altered colony numbers at GD 16 (Figure 3B). No significant benzene-induced changes were detected in BFU-E, CFU-G or CFU-M colonies in CD-1 GD 16 and PD 2 offspring liver tissue (data not shown).

Benzene levels in maternal blood and fetal liver

Benzene levels 1, 2 and 4 h after exposure in maternal blood and fetal liver (supplementary Figure 1 is available at Carcinogenesis Online) were used to generate AUC values for each strain in each treatment group. AUC values were used to represent total maternal and fetal bioavailability of benzene over time. In both strains, maternal blood benzene levels showed significantly higher AUC values in the 400 mg/kg benzene treatment group compared with the 200 mg/kg treatment group (CD-1: P < 0.001; C57Bl/6N; P < 0.01; n = 4) (Figure 4A). In addition, CD-1 maternal blood benzene AUC values were significantly higher than benzene levels in C57Bl/6N in both the 200 and the 400 mg/kg exposure groups (200 mg/kg: P < 0.05; 400 mg/kg: P < 0.01; n = 4) (Figure 4A). In both strains, benzene levels were approaching non-detectable levels (limit of detection: 2 ng/ml) in maternal blood by 4 h post exposure to either 200 or 400 mg/kg benzene (data not shown). There were no benzene levels above the limit of detection in any of the oil-treated control samples.

GC/MS analysis of fetal liver benzene levels showed no significant gender differences in either strain; therefore, sexes were pooled for analysis of strain differences. There was no significant difference in fetal liver benzene AUC values in C57Bl/6N fetuses exposed transplacentally to 200 mg/kg benzene compared with fetuses exposed to 400 mg/kg benzene. However, AUC values for CD-1 fetuses exposed to 400 mg/kg benzene were significantly higher than values from...
fetuses exposed to 200 mg/kg benzene ($P < 0.01$; $n = 8$) (Figure 4B). Comparing strain differences in fetal liver benzene AUC values, our analysis found that CD-1 fetal liver tissue had significantly higher levels compared with C57Bl/6N fetal liver tissue in both the 200 mg/kg benzene ($P < 0.001$; $n = 8$) and the 400 mg/kg benzene ($P < 0.001$; $n = 8$) treatment groups (Figure 4B). By 4 h after exposure, benzene was essentially non-detectable in fetal liver tissue in all fetuses tested (data not shown). There were no benzene levels detected in any of the oil-treated control samples.

**Benzene metabolite levels in fetal liver tissue**

In cord oil-treated controls, levels of t,t-muconic acid or catechol were not detected in fetal liver tissue; however, background levels of hydroquinone were evident in male fetuses, but not female fetuses, from both strains (mean background levels: 0.020 ng/mg ± 0.009 SD). Levels of t,t-muconic acid, hydroquinone and catechol measured at 1, 2 and 4 h after maternal exposure to 200 or 400 mg/kg benzene (supplementary Figure 2 is available at Carcinogenesis Online) were used to generate AUC values for both male and female fetuses in each strain and in each treatment group. A significant effect of gender ($P < 0.01$), an effect of treatment ($P = 0.06$) and a significant strain–gender interaction ($P < 0.05$) were detected in a three-way ANOVA evaluating hydroquinone AUC values. Therefore, subsequent two-way ANOVA revealed that male CD-1 fetuses exposed to 200 mg/kg benzene had significantly higher hydroquinone AUC values compared with male CD-1 fetuses exposed to 400 mg/kg benzene ($P < 0.05$; $n = 4$), female CD-1 fetuses exposed to 200 mg/kg benzene ($P < 0.05$; $n = 4$) and male C57Bl/6N fetuses exposed to 200 mg/kg benzene (Figure 5). A significant treatment–gender interaction ($P < 0.05$) and a treatment–strain–gender interaction ($P = 0.05$) were detected in a three-way ANOVA evaluating catechol AUC values. Therefore, subsequent two-way ANOVA revealed that female CD-1 fetuses exposed to 200 mg/kg benzene had significantly elevated catechol AUC values compared with female CD-1 fetuses exposed to 400 mg/kg benzene ($P < 0.001$; $n = 4$), male CD-1 fetuses exposed to 200 mg/kg benzene ($P > 0.05$; $n = 4$) and C57Bl/6N female fetuses exposed to 200 mg/kg benzene ($P < 0.01$; $n = 4$) (Figure 5). A three-way ANOVA of t,t-muconic acid levels showed a significant effect of strain ($P < 0.005$) where C57Bl/6N fetuses had higher AUC values for t,t-muconic acid than CD-1 fetuses (Figure 5).

**Discussion**

The impact of in utero exposure to environmental carcinogens on the incidence of childhood cancers is unclear, but there is accumulating evidence that it plays a major role (6–9). Benzene is a ubiquitous toxicant that many people are exposed to through both occupational and environmental (i.e. smoking and automobile exhaust) means (17,28). Benzene is a suspected transplacental carcinogen as maternal occupational exposure, smoking and residence proximity to high traffic density or automobile repair garages have all been associated with an increased incidence of childhood leukemia (8,9,29,30). Therefore, understanding the effects of benzene exposure during pregnancy should be regarded as a high research priority.

Our results showed that transplacental benzene exposure increased tumor incidence in CD-1 offspring. This trend was also evident in C57Bl/6N offspring; however, the incidences were too low to generate sufficient statistical power. Strain differences in susceptibility to transplacental 3-methylcholanthrene carcinogenesis have also been reported and are associated with maternal xenobiotic metabolism capability, where low maternal metabolism results in elevated xenobiotic exposure to the fetus ultimately leading to a higher incidence of tumors in the offspring (31). This is a likely hypothesis to apply to maternal benzene exposure as we detected lower benzene levels in C57Bl/6N compared with CD-1 fetuses after the same maternal dose.

Interestingly, there was a marked sex difference in the types of tumors elicited by transplacental benzene exposure in CD-1 offspring. Male and female offsprings had a higher propensity for developing liver and hematological tumors, respectively. Other studies evaluating tumor incidence after transplacental polycyclic aromatic hydrocarbons or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone exposure have also noted a higher incidence of liver tumors in male offspring (10,16,32). In humans, liver cancer is also more prevalent in males with childhood incidences rising annually, and studies suggest that this trend is due to transplacental toxicant exposure (33–38). Regarding hematological tumors in humans, female infants have a higher incidence of leukemia (39), although the precise role of maternal benzene exposure in mediating this gender effect is not determined. In our study, CD-1 female offspring transplacentally exposed to 200 mg/kg benzene displayed a depression of CFU-GM numbers in PD 2 puer liver tissue correlating with BM myeloid cell suppression at 1 year of age. Considering that myelodysplasias are risk factors for acute myeloid leukemia (40,41), our results may underestimate the incidence of hematological tumors in female CD-1 offspring as our study design was perhaps not long enough to detect tumors that develop only after a long latency period. Overall, our findings that in utero exposure to benzene increases the development of hepatic and hematopoietic tumors in murine offspring provide more evidence that maternal exposure to benzene may be a risk factor for childhood cancers in humans.

AUC values for maternal blood benzene levels were significantly higher in CD-1 compared with C57Bl/6N mice suggesting that C57Bl/6N dams have a higher rate of benzene metabolism. This resulted in CD-1 fetuses being exposed to significantly higher benzene levels compared with C57Bl/6N fetuses, which introduces a potential confounder when evaluating fetal benzene metabolite levels between strains subject to the same maternal benzene dose. Therefore, we cannot conclusively say that strain differences in fetal benzene metabolite level are due to differences in fetal benzene metabolism.
capability. Further studies are required to clarify this issue. Interestingly, there were no differences in C57Bl/6N fetal liver benzene levels between the 200 and 400 mg/kg benzene exposure groups. It is possible that higher levels of benzene in maternal blood induce metabolizing enzymes in the placenta limiting fetal exposure. Whether placental CYPs can be induced after benzene exposure is not known; however, it is probably since placental enzymes are highly inducible after maternal xenobiotic administration (42).

In CD-1 mice, transplacental exposure to 200 mg/kg benzene resulted in high fetal exposure to hydroquinone and catechol in male and female fetuses, respectively. This gender difference may explain the gender differences we observed in tumor origin in 1-year-old offspring. Perhaps, fetal hepatic and hematopoietic cells are more prone to hydroquinone- and catechol-induced damage, respectively. In addition, our results suggest the presence of different metabolizing enzyme systems in male and female fetuses as early as GD 14, which is an important finding considering that many developmental studies in mice do not take fetal gender into account, which may dull or mask gender-specific responses. In humans, benzene has been detected in fetal cord blood at levels equal to or greater than levels found in maternal blood, indicating that benzene can cross the placenta and may accumulate in the conceptus (43). Considering that human embryos express CYP-metabolizing enzymes as early as 11 weeks of gestation (44), it is probably that human embryos have the capacity to metabolize benzene warranting further investigation into the risks maternal benzene exposure poses to the fetus.

Surprisingly, our results did not follow a typical dose–response curve as we detected a greater tumor response and higher levels of fetal hydroquinone and catechol after maternal exposure to 200 versus 400 mg/kg benzene in CD-1 mice. However, this is not the first study to observe a more pronounced response to lower compared with higher concentrations of benzene. For example, transplacental exposure to 200 mg/kg, but not 400 mg/kg, benzene caused an increase in fetal liver reactive oxygen species levels and altered hematopoietic colony numbers in C57Bl/6N mice (27). In addition, 200 mg/kg, but not 400 mg/kg, benzene exposure leads to higher levels of nitrotyrosine products in mouse BM (45). This abnormal dose–response to benzene toxicity has been noted since 1989 when it was first suggested that for benzene metabolism, detoxification pathways may be low affinity and high capacity, whereas toxification pathways may be high affinity and low capacity (46). However, another possible explanation is that higher levels of benzene in the fetal compartment may induce detoxifying enzymes that would shunt benzene metabolism away from toxification pathways that produce hydroquinone and catechol. This is a plausible hypothesis considering that rat fetal liver tissue has been shown to upregulate a number of detoxifying enzymes such as glutathione S-transferase and uridine 5′-diphospho-glucuronosyltransferase in response to maternal exposure to phenobarbitol (47). Another likely explanation is that high levels of benzene cause substrate inhibition of metabolizing enzymes in the fetus. Many studies have shown substrate inhibition of a number of CYPs (48) including CYP2E1 (49), although whether high levels of benzene can cause substrate inhibition of metabolizing enzymes is unknown. Our study supports the later two hypotheses as we have shown a higher amount of fetal hydroquinone and catechol levels after maternal exposure to 200 versus 400 mg/kg benzene. Our results, along with others, highlight the need for further investigation of drug metabolism in pregnant animals and humans as this may be a risk factor for transplacental carcinogenesis.

Although the doses of 200 and 400 mg/kg benzene are relatively high, they were selected based on the pharmacokinetic pattern of non-inhalation exposure to benzene (50). In order to model the human pathology, adult mice develop leukemia after exposure to 300 p.p.m. inhaled benzene 6 h/day, 5 days/week for 16 weeks (51,52). Our maternal blood benzene levels peaked 1 h after i.p. injection of 200 or 400 mg/kg benzene with a mean concentration of 11.6 or 22.7 µg/ml, respectively. Therefore, our dose and route of exposure produces blood benzene levels that are very similar to that of adult mice that develop benzene-induced leukemia. It is important to note that in humans, benzene exposure usually occurs via the inhalation route, whereas the route of exposure in this study was through i.p. injection, which allows for the administration of precise dosages at desired time points. Therefore, although we anticipate that the effects reported in this study would also occur after in utero exposure to benzene via the inhalation route, further studies are required to confirm this. In addition, human environmental exposure to benzene is typically lower than the doses of benzene used in this study. In Canada and the USA, the current acceptable occupational exposure limit is 0.5 p.p.m. in an 8 h day, 40 h/week. Reports have shown that humans living near roadways or near industrial sources can be environmentally exposed to benzene concentrations ranging from 1.28 to 4.1 p.p.b. (53). The largest source of non-occupational exposure to benzene is through cigarette smoking. The inhaled dose of benzene from cigarettes has been reported to be in the range of 16–75 µg per cigarette (54). Blood concentrations of benzene in urban smokers have been reported to be 435 ng/l with 221 ng/l in urban non-smokers (55). Therefore, although our study has created a useful model of high-dose benzene-induced transplacental carcinogenesis in mice through an i.p. route, it is important to keep in mind that most human exposures to benzene occur through the inhalation route and to lower concentrations.

In summary, transplacental benzene exposure causes an increased incidence of tumors in mouse offspring that is strain and gender specific, which may reflect strain and gender differences in fetal ability to metabolize benzene into toxic metabolites. Overall, transplacental induction of carcinogenesis in mice after maternal benzene exposure raises concerns about the risks to humans from in utero exposure to this environmental toxicant.

Supplementary material

Supplementary Figures 1 and 2 can be found at http://carcin.oxfordjournals.org/

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References


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