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Genotoxicity of intermittent co-exposure to benzene and toluene in male CD-1 mice

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ABSTRACT

Benzene is an important industrial chemical. At certain levels, benzene has been found to produce aplastic anemia, pancytopenia, myeloblastic anemia and genotoxic effects in humans. Metabolism by cytochrome P450 monooxygenases and myeloperoxidase to hydroquinone, phenol, and other metabolites contributes to benzene toxicity. Other xenobiotic substrates for cytochrome P450 can alter benzene metabolism. At high concentrations, toluene has been shown to inhibit benzene metabolism and benzene-induced toxicities. The present study investigated the genotoxicity of exposure to benzene and toluene at lower and intermittent co-exposures. Mice were exposed via whole-body inhalation for 6 h/day for 8 days (over a 15-day time period) to air, 50 ppm benzene, 100 ppm toluene, 50 ppm benzene and 50 ppm toluene, or 50 ppm benzene and 100 ppm toluene. Mice exposed to 50 ppm benzene exhibited an increased frequency (2.4-fold) of micronucleated polychromatic erythrocytes (PCE) and increased levels of urinary metabolites (*t*,*t*-muconic acid, hydroquinone, and s-phenylmercapturic acid) vs. air-exposed controls. Benzene co-exposure with 100 ppm toluene resulted in similar urinary metabolite levels but a 3.7-fold increase in frequency of micronucleated PCE. Benzene co-exposure with 50 ppm toluene resulted in a similar elevation of micronuclei frequency as with 100 ppm toluene which did not differ significantly from 50 ppm benzene exposure alone. Both co-exposures – 50 ppm benzene with 50 or 100 ppm toluene – resulted in significantly elevated CYP2E1 activities that did not occur following benzene or toluene exposure alone. Whole blood glutathione (GSH) levels were similarly decreased following exposure to 50 ppm benzene and/or 100 ppm toluene, while co-exposure to 50 ppm benzene and 100 ppm toluene significantly decreased GSSG levels and increased the GSH/GSSG ratio. The higher frequency of micronucleated PCE following benzene and toluene co-exposure when compared with mice exposed to benzene or toluene alone suggests that, at the doses used in this study, toluene can enhance benzene-induced

Abbreviations: MPO, myeloperoxidase; CYP, cytochrome P450; NQO1, nicotinamide adenine dinucleotide phosphate (reduced form): quinone oxidoreductase-1; HQ, hydroquinone; *t*,*t*-MA, *trans*,*trans*-muconic acid; s-PMA, s-phenylmercapturic acid; BSTFA, N,O-bis(trimethylsilyl)tri-fluroacetamide; TMCS, trimethylchlorosilane; MN, micronuclei; PCE, polychromatic erythrocytes; NCE, normochromatic erythrocytes; PNP, *p*-nitrophenol; 4-NCC, 4-nitrocatechol; GST, glutathione *S*-transferase; CDNB, 1-chloro 2,4-dinitrobenzene; GSH, glutathione; GSSG, oxidized glutathione.

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clastogenic or aneugenic bone marrow injury. These findings exemplify the importance of studying the effects of binary chemical interactions in animals exposed to lower exposure concentrations of benzene and toluene on benzene metabolism and clastogenicity. The relevance of these data on interactions for humans exposed at low benzene concentrations can be best assessed only when the mechanism of interaction is understood at a quantitative level and incorporated within a biologically based modeling framework.

1. Introduction

Benzene is a volatile aromatic hydrocarbon used in a variety of industrial applications. Occupational exposure to benzene occurs in the oil, shipping, automobile repair, shoe manufacture, and certain other industries. The American Conference of Governmental Industrial Hygienists set the recommended exposure limit for benzene at 0.5 ppm (threshold limit value; time-weighted average (TLV-TWA)) [1]. Benzene is also a common contaminant of outdoor and indoor air. Environmental sources of benzene include cigarette smoke, gasoline, and automobile emissions. Benzene concentrations in metropolitan outdoor air were estimated to average approximately 0.6 ppb [2]. Non-occupational personal exposure has been estimated to average approximately 5 ppb, likely due to passive exposure to cigarette smoke [3].

Epidemiologic studies of workers exposed to benzene in rubber hydrochloride manufacturing plants have revealed an association between chronic workplace exposure to benzene and incidence of aplastic anemia or acute myelogenous leukemia [4]. Rodent inhalation studies have shown that benzene causes cancer in multiple tissues, with strong evidence of hematopoietic neoplasms, leukemias, and lymphoid tumors in mice [5–12]. Further, rodents exposed to benzene develop cytogenetic changes in the bone marrow and the peripheral blood, micronuclei and sister chromatid exchanges in lymphocytes, and other genotoxic effects [13–16].

Several studies suggest that metabolism of benzene is required for hematotoxicity to occur [17–19] and further indicate that no single benzene metabolite fully replicates the toxicity of benzene [17,20,21]. Benzene undergoes hepatic oxidation by cytochrome P450 2E1 (CYP2E1) to benzene oxide and other reactive intermediates (Fig. 1) [16,22]. Benzene oxide can be oxidized to form catechol [23], undergo ring opening to produce *trans-trans*-muconaldehyde, or spontaneously rearrange to form phenol. Phenol is then hydroxylated, most likely by CYP2E1, to form hydroquinone. In the bone marrow, hydroquinone and catechol are converted by myeloperoxidase (MPO) to 1,4-benzoquinone and 1,2-benzoquinone, respectively, which can be detoxified by reduction via nicotinamide adenine dinucleotide phosphate (reduced form): quinone oxidoreductase-1 (NQO1) [24–28]. Genetic variations in CYP2E1, MPO, and NQO1 have been shown to influence the hematotoxicity of benzene in people [29] and rodents [15,30,31]. Additionally, genetic variation in microsomal epoxide hydrolase has been shown to alter susceptibility to benzene-induced hematotoxicity in male mice [32].

The metabolites most commonly studied with respect to benzene hematotoxicity are hydroquinone (HQ) and the benzoquinones [22,23]. *In vitro* studies have shown that 1,4-benzoquinone binds to DNA and other macromolecules, while 1,4-hydroquinone is largely responsible for the production of reactive oxygen species [33–35]. Phenol has been shown to stimulate the myeloperoxidase-dependent formation of benzoquinone from HQ [17]. When co-administered, phenol and HQ can reproduce the myelotoxicity observed following benzene administration [17]. Benzene metabolite-induced covalent and oxidative damage to DNA and proteins can result in apoptosis [36–38].

The involvement of numerous metabolic pathways indicates that benzene metabolism can be altered through several different mechanisms. Further, benzene metabolism is dose-dependent, with saturable metabolism observed at concentrations greater than 50 mg/kg (oral) or air concentrations between 130 and 900 ppm [21,39]. Benzene can induce or inhibit oxidation or conjugation enzymes and also compete with metabolites phenol, HQ, catechol, and other hydroxylated metabolites for available enzyme sites [21,40]. Further, HQ and the benzoquinones cause cytochrome P450 destruction in rat and human liver microsomes *in vitro* [41].

Epidemiologic studies examining benzene hematotoxicity are often confounded by multiple chemical exposures to toluene or other aromatic hydrocarbons. Toluene (Fig. 2) (and certain other xenobiotic substrates for cytochrome P450) may competitively and noncompetitively inhibit the metabolism of benzene [36,42]. Studies addressing co-exposure to benzene and toluene have demonstrated a reduction in benzene-induced hemopoietic toxicity, myeloclastogenicity, and genetic toxicity [43–45]. While these studies have all demonstrated a reduction in benzene-induced toxicities, they have all been conducted at high concentrations (300 and 900 ppm via inhalation; 440 mg/kg by ip and po). The present study was conducted to investigate the modulation of the genotoxic potential of benzene by lower and intermittent co-exposures to benzene and toluene and thereby improve the human health risk assessment for benzene. Modulation of phase I and phase II enzyme activities, glutathione (GSH) levels and urinary metabolite profiles were also investigated.

2. Materials and methods

2.1. Chemicals

Benzene (C_6H_6; CAS Number: 71-43-2; purity > 99%) and toluene ($C_6H_5CH_3$; CAS Number: 108-88-3; purity > 99%) were purchased from Sigma–Aldrich (St. Louis, MO). May-Gruenwald/Giemsa solution, sodium citrate, fetal bovine serum, PNP, CDNB, β-nicotinamide

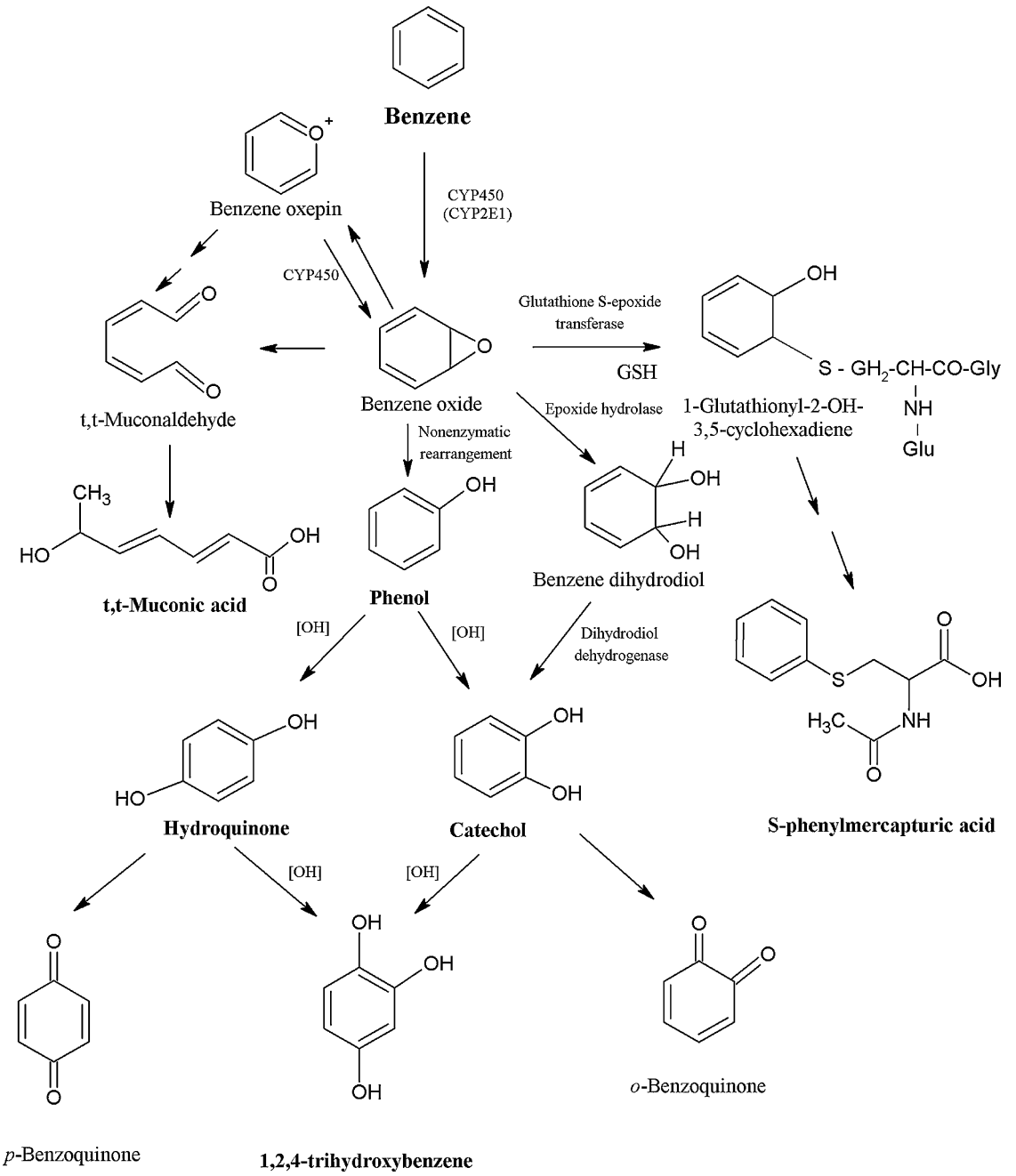

Fig. 1. Overview of CYP450-mediated metabolism of benzene (in bold). Urinary concentrations were determined for those metabolites displayed in bold.

adenine dinucleotide phosphate, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, perchloric acid, carboxyfluorescein, flavin adenine dinucleotide, GSH, MTT, dicoumarol, menadione, acetonitrile and additional reagents were obtained from Sigma–Aldrich (St. Louis, MO). Neutral buffered formalin was obtained from Fisher. N,O-bis(trimethylsilyl)tri-fluoroacetamide (BSTFA), trimethylchlorosilane (TMCS) and the bicinchoninic acid (BCA) protein assay kit were obtained from Pierce Chemical (Rockford, IL). Testosterone and 6 β-hydroxytestosterone were obtained from Steraloids (Newport, RI). The HPLC column (Prodigy) was obtained from Phenomenex (Torrance, CA). The GSH/GSSG kit was obtained from Oxis Research (Bioxytech GSH/GSSG-412 kit; Portland, OR).

2.2. Animals

Five-week-old male CD-1 mice were purchased from Charles River Laboratories (Raleigh, NC) and allowed to acclimate to stainless steel wire mesh cages in a 1 m^3 stainless steel and glass inhalation chamber (Hazelton H1000, Lab Products, Seaford, DE) for 2 weeks prior to the start

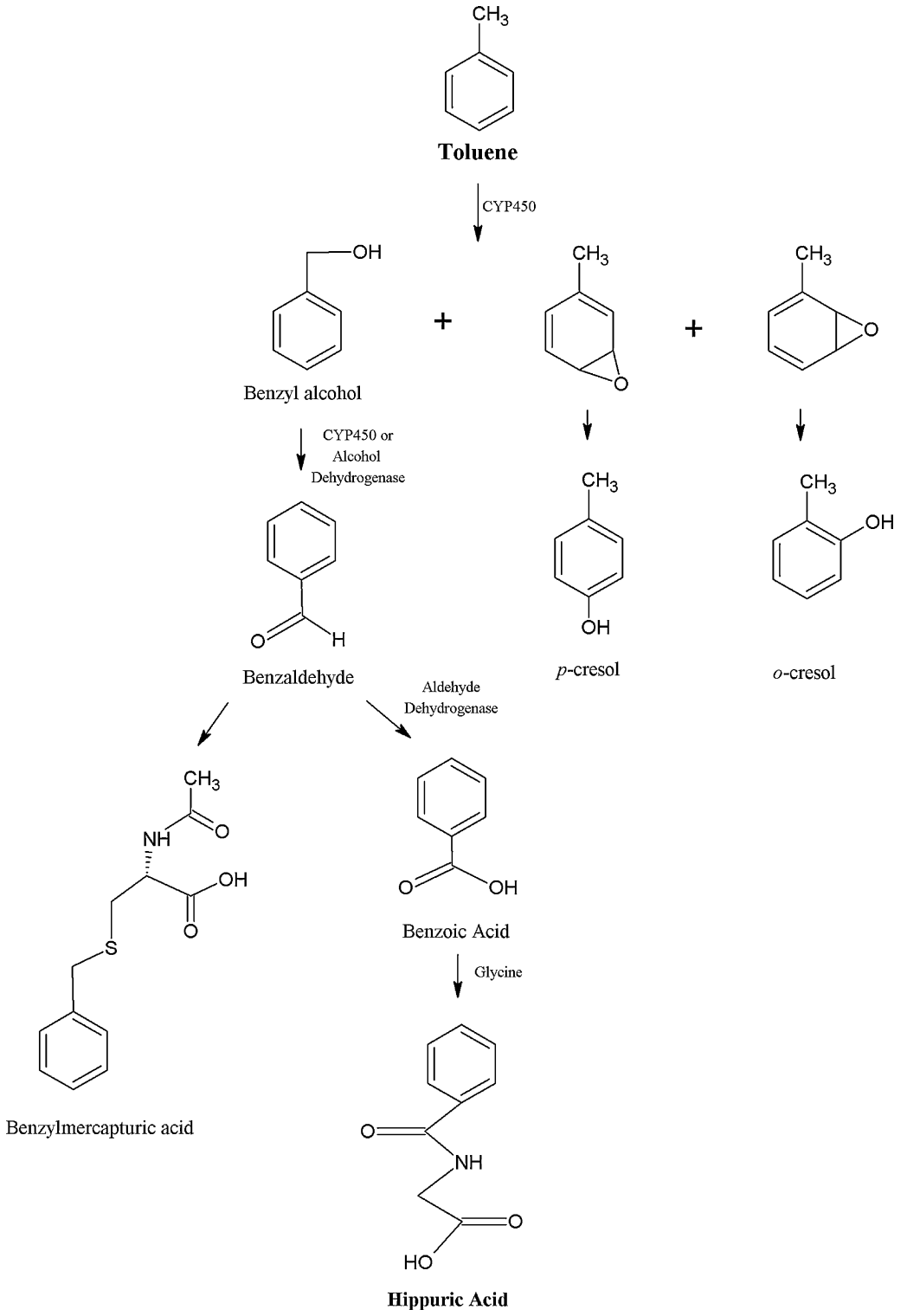

Fig. 2. Overview of CYP450-mediated metabolism of toluene.

of the exposures. Animal room temperatures were maintained between 18 and 26 °C while relative humidity was kept between 30 and 70%. Automatic light controls were set to provide fluorescent lighting for an approximate 12 h photo period (approximately 07:00–19:00 h for light phase). Pelleted, NIH-07 rodent chow (Zeigler Bros., Gardners, PA) and reverse osmosis water (HydroService and Supplies, Research Triangle Park, NC) were available *ad libi-*

tum. All animal use was approved by the Hamner Institutes for Health Sciences Institutional Animal Care and Use Committee and complied with all federal guidelines for the care and use of laboratory animals. All animal use was completed within the Hamner Institutes for Health Sciences research facility, which is accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care, International. Animals were examined for morbidity and mortality at least twice daily on all exposure days and at least once on all other days. Cage side observations were performed immediately after each inhalation exposure and prior to necropsy on study day 16. Body weight and clinical observation data were collected weekly and at necropsy.

2.3. Experimental design

There is biologic evidence based on animal studies that the timing, duration and concentration of exposure are important factors in the hematotoxicity and genotoxicity of benzene. Toft et al. [46] reported several examples of dose rate effects for cellularity, granulopoietic stem cells and micronuclei in polychromatic erythrocytes (PCEs) while Luke et al. [47] showed that mice exposed for 3 consecutive days per week for 13 weeks exhibited a greater suppression of polychromatic erythrocyte counts than those exposed for 5 consecutive days over this 13-week period. Cox [48] extended these observations through modeling to predict that benzene metabolite effects on stem cells undergoing this compensatory proliferation could be a possible mechanism of benzene-induced carcinogenesis and leukemogenesis.

In consideration of this, a discontinuous exposure protocol was selected for this study to provide exposure to stem cells undergoing cell division and proliferation, rather than the cell suppression resulting from a more continuous exposure regimen. It was also recognized that many occupational operations involve intermittent exposure. The exposure concentrations were chosen to be below the higher concentrations associated with competitive inhibition but yet still result in micronuclei induction.

This study was comprised of an air-exposed control group and the following exposure groups: 50 ppm benzene; 100 ppm toluene; 50 ppm benzene and 50 ppm toluene; and 50 ppm benzene and 100 ppm toluene. Animals were randomly assigned to one of three exposure cohorts. The inhalation exposures for each cohort were run independently of each other and each cohort included animals from each of the five treatment groups. Two cohorts were comprised of 3 animals per treatment group (15 animals per cohort) and 1 was comprised of 4 animals per group (20 animals per cohort) for a grand total of 10 animals per treatment group. Exposure atmospheres were monitored for each of the three cohorts to ensure consistent exposures were administered to each treatment group within each cohort. Seven-week old mice were exposed whole body for 6 h/day over a 15-day time period for eight total exposures (exposures occurred on study days 1, 2, 5, 7, 9, 12, 13, and 15). Animals were killed 18 h after the last exposure.

2.4. Whole-body inhalation exposure

Exposure atmospheres were generated by metering a carrier gas with a mass flow controller (MKS Instruments Inc., Andover, MD) through a pressure vessel of concentrated benzene and/or toluene and mixing it with HEPA-filtered air upstream of the exposure chamber inlet. The supply airflow through the chambers was controlled by the Continuum Building Automation System (Andover Controls Corp., Andover, MD). Benzene and/or toluene exposure atmospheres in the inhalation chambers were analyzed at least six times during the exposure period by a gas chromatograph equipped with a flame ionization detector and 12-port sampling valve.

2.5. Urine collection and chemical analysis

Urine was collected overnight prior to the animals' scheduled necropsy. Nine randomly chosen mice per exposure group were placed individually into polycarbonate metabolism cages for approximately 16 h. Urine was kept cold during the collection period. At the end of the collection period, total urine volume was determined and the urine samples were flash frozen in liquid nitrogen. Urine samples were stored at −70 °C until chemical analysis. Chemical analyses were performed on samples with an adequate urine volume ($\geq$0.4 ml; n = 7–9 mice/exposure group). Urinary creatinine levels were determined by a commercial laboratory (Antech Diagnostics, Cary, NC). All other chemical analyses were performed by the analytical chemistry laboratory of ExxonMobil Biomedical Sciences Inc. Six benzene urinary metabolites – phenol, CAT (pyrocatechol or 1,2-dihydroxybenzene), HQ (hydroquinone or 1,4-dihydroxybenzene), 1,2,4-trihydroxybenzene (pyrogallol), *trans*,*trans*-muconic acid (*t*,*t*-MA), and s-phenylmercapturic acid (s-PMA) – were extracted and analyzed based on the method of Waidyanatha et al. [49]. The toluene metabolite, hippuric acid, was also analyzed.

Portions of thawed, centrifuged mouse urine samples were diluted with water, acidified and hydrolyzed, then extracted with ethyl acetate. The ethyl acetate extracts were evaporated to dryness and the residue derivatized with BSTFA and 10% TMCS. Derivatized sample extracts were analyzed by gas chromatography with mass selective detection operated in the selective ion monitoring mode (Agilent HP6890 GC with a 5975 MSD). A 30 m × 0.25 mm i.d. 1.0 μ Rtx-5MS capillary column with 10 m Integra guard column (Restek) was used for the chromatographic separation. A single ion was monitored for quantitation and a secondary ion was used as a qualifier. All metabolite concentrations were normalized to urinary creatinine levels (μg metabolite/mg creatinine).

2.6. Necropsy procedures

Mice were euthanized with carbon dioxide (CO_2) inhalation. The following samples were collected from each animal: femurs (for bone marrow collection for micronucleus assay), sternum (for histopathologic evaluation), whole blood (for determination of glutathione concentration) and liver (for enzyme activity assays and

determination of glutathione concentration). Blood was collected by cardiac puncture and blood samples were transferred to microtainer® tubes (Becton Dickinson, San Jose, CA) containing EDTA. A blood smear was also prepared. Complete blood counts were conducted by Antech Diagnostics (Cary, NC) using a Bayer Advia 120.

2.7. Bone marrow micronucleus assay

The genotoxicity of intermittent benzene and toluene co-exposure in male CD-1 mice was investigated in a short-term mutagenicity assay, the mammalian erythrocyte micronucleus (MN) test. Bone marrow cells were collected by flushing the femur with a 1 ml volume of 1% sodium citrate and fetal bovine serum (70:30). After centrifugation at $50 \times g$ for 5 min at room temperature, the pellet was resuspended in a minimal volume of the supernatant and bone marrow cell smears were made on slides and stained by May-Gruenwald/Giemsa solution. A minimum of 200 erythrocytes were randomly counted manually and the number of polychromatic erythrocytes and normochromatic erythrocytes (NCEs) were recorded separately. The ratio of PCE to erythrocytes (PCE + NCE) was used as an indicator of toxicity. Micronuclei in 2000 PCEs were observed randomly and recorded [50–52]. The number of PCEs, NCEs and miconucleated PCEs were recorded in a pre-designed spreadsheet (Excel) and scored blindly. Micronuclei usually appear as densely stained small round bodies in the cytoplasm of the PCEs. The general size of the micronuclei is approximately (1/20)–(1/5) of diameter of the PCEs. The results were expressed as percent of micronucleated PCEs. In accordance with the OECD guidelines [52], the number of micronucleated NCEs, an indicator of chronic genotoxicity, was not evaluated since the exposures in this study were for less than 4 weeks.

2.8. Sternum pathology

The sternum was removed from the mouse, placed in 10% neutral buffered formalin phosphate (Fisher, Fair Lawn, NJ) for 48 h, and then transferred to 70% ethanol. Sternums underwent decalcification in 7.5% formic acid for 5 days before further processing. Samples were embedded in paraffin, and at least two sections per tissue were prepared and stained with hematoxylin and eosin. Bone marrow cellularity was assessed by evaluating five 40× fields (0.069 mm^2) per sternum. Bone marrow cellularity was recorded without making a distinction between erythrocytic, granulocytic, lymphocytic, or thrombocytic components. The cellularity of the marrow was determined by estimating the percentage of the total volume of the medullary cavity that was filled with cellular elements. Bone marrow cellularity was graded on a scale of 1–5 where grade 1 had 0–10% of the medullary cavity filled; grade 2, >10–30%; grade 3, >30–60%; grade 4, >60–90%; and grade 5, >90%.

2.9. Subcellular fractionation

Cytosolic and microsomal fractions of mouse liver were prepared as previously described [53]. Briefly, immediately after sacrifice, mouse liver tissue was homogenized in ice-cold homogenization buffer (50 mM potassium phosphate, 0.1 mM EDTA, pH 7.5, with 1.15% KCl; four volumes of buffer per gram wet weight). The homogenate was centrifuged at $9000 \times g$ for 30 min at 4 °C. The supernatant was then centrifuged at $100{,}000 \times g$ for 60 min at 4 °C. The supernatant or cytosolic fraction was stored at −80 °C until use. The microsomal pellet was resuspended in half the original volume of homogenization buffer and spun again at $100{,}000 \times g$ for 60 min at 4 °C. The pellet was resuspended in storage buffer (50 mM potassium phosphate, 0.1 mM EDTA, pH 7.5, with 0.25 M sucrose). Aliquots were stored at −80 °C until use. Protein content was determined using the bicinchoninic acid method (Pierce; Rockford IL).

2.10. Hepatic enzyme activity analyses

CYP2E1 enzyme activity was determined by measuring the hydroxylation of *p*-nitrophenol (PNP) to 4-nitrocatechol (4-NCC) as described by Koop [54] and Reinke and Moyer [55]. Ninety percent of PNP hydroxylation reflects CYP2E1 activity; consequently, this assay was used as a measure of CYP2E1 activity [56]. Reaction mixtures containing 100 mM potassium phosphate buffer with $MgCl_2$ (pH 6.8), NADPH regenerating system and mouse liver microsomes were preincubated at 37 °C for 2 min. Reactions were started with the addition of PNP. After 10 min of shaking in a 37 °C water bath, the reactions were terminated by the addition of 0.6N perchloric acid. Proteins were precipitated by centrifugation ($1800 \times g$ for 4 min) and the supernatant was mixed with 10N NaOH for the measurement of 4-NCC at 546 nm. Activity of PNP hydroxylase was determined as rate of 4-NCC formation per mg microsomal protein (nmol 4-NCC/(min × mg microsomal protein)). Two independent experiments were conducted in triplicate.

CYP3A enzyme activity was determined through the analysis of 6 β-hydroxytestosterone formation from testosterone [57,58] as described in Usmani et al. [59]. Briefly, following pre-incubation at 37 °C, mouse liver microsomes were added to 100 mM potassium phosphate buffer containing 100 μM testosterone (final concentration; Steraloids Inc., Newport, RI) and NADPH regenerating system (components obtained from Sigma–Aldrich, St. Louis, MO). Samples were incubated for 10 min at 37 °C after which MeOH was added to terminate the reaction and precipitate protein. Supernatants were transferred to an HPLC vial for testosterone metabolite analysis by reversed-phase HPLC. Products were detected by their absorbance at 247 nm. Formation of 6 β-hydroxytestosterone (Steraloids Inc.) was determined by extrapolating peak heights from standard curves. Samples were analyzed in duplicate. Testosterone and its metabolites were separated on a Prodigy column (Prodigy 3 μ, 150 mm × 4.6 mm, ODS (3), 100A; Phenomenex, Torrance, CA). The mobile phase for pump A was 5% tetrahydrofuran/95% H_2O; for pump B 100% methanol. The following gradient system was employed: 0–1 min (30% B); 1–10 min (30–60% B); 10–22 min (60–65% B); 22–28 min (65–80% B); 28–30 min (80–90% B); 30–32 min (90% B); 32–34 min

(90–30% B); 34–36 min (30% B). The flow rate was 0.5 ml/min.

Epoxide hydrolase activity was determined fluorometrically as described in Doderer and Schmid [60]. Briefly, epoxide hydrolases metabolize epoxides (e.g., styrene oxide) to vicinal diols, which are cleaved in the presence of periodate. Periodate is in turn reduced to iodate. Residual periodate will react with carboxyfluorescein and reduce its fluorescence. Consequently the higher the epoxide hydrolase activity, the lower the amount of residual periodate, leading to a greater emission of fluorescence. The measurement of fluorescence is thus used to calculate the epoxide hydrolase activity in the samples. Mouse liver microsomes (75 μg in 20 μl) were mixed with 80 μl styrene oxide solution (31.25 mM (25 mM final concentration) in 50 mM sodium phosphate buffer, pH 8, with 5% (v/v) EtOH) in a black polystyrene microtiter plate and incubated at room temperature for 9 h. Sixty microliters of sodium periodate (90 mM in 0.1 M sodium acetate buffer, pH 4.5) was added to the plate and incubated at *RT* for 10 min. Following carboxyfluorescein (100 μl of a 0.425 mM solution) addition, plate was sealed and incubated at 70 °C for 2 h. After excitation at 480 nm, fluorescence at 515 nm was measured in a spectrofluorimeter, correcting background through the use of negative (no enzyme) controls. Two independent experiments were conducted in triplicate.

GST activity was measured using a method adopted from Habig et al. [61] and modified for use in a 96-well plate [62]. A reaction mixture containing a potassium phosphate buffer (0.1 M with 1 mM EDTA; prewarmed to 37 °C), 20 mM GSH (Sigma–Aldrich, St. Louis, MO) and 20 mM CDNB was prepared. Mouse liver cytosol was added in triplicate to each well of a 96-well plate. The reaction mixture was then added to start the reaction. The plate was scanned at 340 nm in a plate reader every minute for up to 5 min. The potassium phosphate buffer was used as a blank control. The GST specific activity was determined for each sample as units/mg protein using an extinction coefficient of 9.6 mM^{-1} cm^{-1} for CDNB. Three independent experiments were conducted in triplicate.

The *NQO1 enzyme activity* was measured by a method adopted from Prochaska and Santamaria [63] and Sharma et al. [64]. Reaction mixtures containing (final concentrations) 25 mM Tris–HCl (pH 7.4), 0.07% bovine serum albumin, 0.01% (v/v) Tween 20, 5 μM FAD, 30 μM NADP, 1 mM G6P, 0.4 U G6PDH, 0.06 ng MTT, 0 or 10 μM dicoumarol (a specific and potent NQO1 inhibitor) and liver cytosolic enzyme were prepared. Menadione (a quinone that serves as an electron acceptor for NQO1) (40 μM in acetonitrile) was added to initiate the reaction, which was carried out at 25 °C. The reaction was stopped after 5 min by the addition of 0.050 ml dicoumarol (0.3 mM) and the plate was scanned at 610 nm. Background subtraction of negative control samples lacking menadione was conducted prior to calculation of percent induction over controls. Three independent experiments were conducted in triplicate.

2.11. Liver and blood GSH measurement

Total glutathione and oxidized glutathione (GSSG) in liver and whole blood samples were determined via an enzymatic recycling method developed by Griffith [65] as employed in the Bioxytech GSH/GSSG-412 kit (Oxis-Research, Portland, OR). The reduced form (GSH) was reacted with 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) to form a colorimetric product that is directly proportional to the concentration of GSH in the sample. The oxidized form (GSSG) was measured following derivatization of GSH with 1-methyl-2-vinyl-pyridium trifluoromethane sulfonate (M2VP) to remove it from the reaction. Total protein was determined with the BCA Protein assay (Pierce, Rockland, IL). At necropsy, whole blood or liver homogenate were each added to two tubes, with one tube (for analysis of GSSG levels) containing scavenger M2VP. Samples were mixed prior to storage at −80 °C. Prior to analysis, the GSSG and GSH samples were thawed, mixed and incubated at *RT* for 5 min. Metaphosphoric acid (MPA; at 5%) was added and the tubes were vortexed for 15 s prior to centrifugation at 1000 × *g* for 10 min. Fifty microliters of the MPA extract was added to tubes containing sodium phosphate buffer with EDTA and placed on ice. Reaction mixtures containing equal volumes of samples or standards (GSSG, 0.05–1.5 μM; GSH, 0.1–3.0 μM), chromogen DTNB and enzyme glutathione reductase (GR) were added to cuvettes and incubated at *RT* for 5 min. NADPH was added to start the reaction and the change in absorbance at 412 nm was monitored for 3 min. GSH and GSSG concentrations were determined based on the GSH and GSSG standard curves. Results are reported as the GSH/GSSG ratio with values normalized to total protein.

2.12. Analysis of data

The continuous variables and the MN or PCE counts were tested for homogeneity of variance by Levene's test. If they were homogeneous a standard analysis of variance (ANOVA) followed by a Tukey–Kramer comparison procedure for significant ANOVA was used. In the event the Levene's test was significant, then the data were transformed by a natural log (ln) transformation. If the Levene's test remained significant, then the data were analyzed by nonparametric statistics (Wilcoxon/Kruskal–Wallis). Statistical analyses were performed with JMP Statistical Software (SAS Institute Inc., Cary, NC). A significance level value of <0.01 was used for Levene's test, and a significance level of <0.05 was used as the critical level of significance for all other statistical tests.

3. Results

3.1. Exposure atmospheres

The mean atmospheric concentrations for each exposure cohort within each treatment are provided in Table 1. The atmospheric concentrations generated for benzene were within 6% of the desired concentration for all treatment groups. The atmospheric concentrations generated for toluene were within 4% of the desired concentration for all treatment groups. Neither benzene nor toluene was detected in the control chamber.

Table 1
Inhalation exposure atmospheres

Treatment		Solvent	Cohort		
Benzene (ppm)	Toluene (ppm)		1	2	3
0	100	100 ppm Toluene	95.84 ± 0.57	99.35 ± 0.22	97.38 ± 0.63
50	0	50 ppm Benzene	48.85 ± 0.45	51.86 ± 0.11	50.29 ± 0.70
50	50	50 ppm Benzene	47.74 ± 0.47	49.99 ± 0.20	49.08 ± 0.51
		50 ppm Toluene	47.11 ± 1.14	48.89 ± 0.17	49.16 ± 0.13
50	100	50 ppm Benzene	48.11 ± 0.29	49.66 ± 0.13	48.78 ± 0.37
		100 ppm Toluene	103.19 ± 0.45	103.34 ± 0.67	103.76 ± 0.86

Mean ± S.E.M. (n = 8 readings/exposure cohort). Measurements were taken on each exposure day (n = 8, over the 15-day study period) for each cohort.

Table 2
Percent of polychromatic erythrocytes (PCEs) in mouse bone marrow after exposure to benzene and/or toluene

Treatment		Number of PCEs[a]	Number of NCEs[b]	Total number of erythrocytes	% of PCE[a] in total erythrocytes
Benzene (ppm)	Toluene (ppm)				
0	0	121.00 ± 6.63	136.40 ± 9.83	257.40 ± 5.98	47.37 ± 3.02
0	100	117.40 ± 5.51	125.10 ± 8.08	242.50 ± 6.24	48.66 ± 2.44
50	0	127.40 ± 10.03	126.50 ± 5.45	253.90 ± 10.54	49.72. ± 2.46
50	50	114.10 ± 7.63	122.50 ± 9.40	236.60 ± 5.51	48.45 ± 3.32
50	100	125.70 ± 6.71	133.10 ± 11.15	258.80 ± 7.24	49.11 ± 3.28

No statistically significant differences were noted. Mean ± S.E.M. (n = 10 mice/exposure group).
[a] Polychromatic erythrocytes (PCEs).
[b] Normochromatic erythrocytes (NCEs).

3.2. Body weights, clinical signs, clinical chemistry, and sternum histopathology

No animals died or became moribund during the course of the study. No treatment related effect on body weight gain, terminal body weight, or clinical signs incidence were seen for any of the exposure groups (data not shown). Mice in all treatment groups exhibited total red blood cell and platelet counts within the normal ranges (data not shown). No other treatment related changes in any other clinical chemistry parameter was observed (data not shown). Histopathologic examination of animals exposed to benzene, either alone or in combination with toluene, revealed no significant changes in sternum bone marrow cellularity.

3.3. MN induction

Benzene and toluene exposure, either individually or in combination, did not alter the ratio of PCE in total erythrocytes in the bone marrow when compared to air-exposed control mice (Table 2). The results of the effect of benzene and toluene on the bone marrow cell MN induction are presented in Table 3. The number of PCE micronuclei were statistically significantly increased for benzene alone (50 ppm) and for both combinations of 50 ppm benzene with toluene (50 and 100 ppm) when compared to air exposed controls or to toluene alone (100 ppm). Exposure of mice to 50 ppm benzene resulted in a 2.4-fold statistically significant increase in the frequency of MN-PCE when compared to the air-exposed controls. Exposure to 100 ppm toluene resulted in a nonsignificant increase in the frequency of MN-PCEs (up by 1.5-fold) over air-exposed controls. Animals exposed to a combination of benzene (50 ppm) and toluene (50 or 100 ppm) exhibited a statistically significant 3.6–3.7-fold increase in the frequency of MN-PCE when compared to air-exposed controls. Mice exposed to a combination of 50 ppm benzene and 50 ppm or 100 ppm toluene exhibited a 1.50–1.54-fold increase, respectively, in MN-PCE frequency over mice exposed to benzene alone. Mice exposed to a combination of 50 ppm benzene and 50 or 100 ppm toluene exhibited a statisti-

Table 3
Percent of erythrocyte micronuclei (MN) in mouse bone marrow after exposure to benzene and/or toluene

Treatment		Number of MN[a]	Number of PCEs[b]	% of MN in PCE
Benzene (ppm)	Toluene (ppm)			
0	0	4.20 ± 0.33	2121.00 ± 6.63	0.20 ± 0.02
0	100	6.20 ± 0.71	2117.40 ± 5.51	0.29 ± 0.03
50	0	**10.30 ± 1.13**[c,d]	2127.40 ± 10.03	**0.48 ± 0.05**[c,d]
50	50	**15.20 ± 1.45**[c,d]	2114.10 ± 7.63	**0.72 ± 0.07**[c,d]
50	100	**15.80 ± 1.62**[c,d]	2125.70 ± 6.71	**0.74 ± 0.08**[c,d]

Mean ± S.E.M. (n = 10 mice/exposure group). Significantly different results are displayed in bold.
[a] Erythrocyte micronuclei (MN).
[b] Polychromatic erythrocytes (PCEs).
[c] $p < 0.05$ vs. air exposed controls (Tukey–Kramer test).
[d] $p < 0.05$ vs. 100 ppm toluene (Tukey–Kramer test).

Table 4
Hepatic enzyme activity levels following benzene and/or toluene exposure

Treatment		Enzymatic activity[a]			
Benzene (ppm)	Toluene (ppm)	NQO1 (nmol/mg prot/min)	GST (units/mg prot)	CYP2E1 (nmol 4NCC/mg prot/min)	EH (Fluorescent units[b])
0	0	75.28 ± 5.51	2.89 ± 0.40	1.55 ± 0.12	17001.14 ± 1403.44
0	100	73.60 ± 6.61	3.24 ± 0.23	1.53 ± 0.12	15394.19 ± 1608.83
50	0	90.21 ± 11.67	3.52 ± 0.24	1.40 ± 0.13	14629.75 ± 1679.24
50	50	80.73 ± 4.53	3.61 ± 0.54	**2.40 ± 0.13**[c,d,e]	15518.17 ± 1157.99
50	100	83.51 ± 3.25	2.53 ± 0.20	**2.33 ± 0.08**[c,d,e]	18733.01 ± 1606.43

[a] Mean (±S.E.M.) activity of NADPH:quinone reductase (NQO1), glutathione *S*-transferase (GST), cytochrome P4502E1 (CYP2E1), and epoxide hydrolase (EH) for $n = 9–10$ mice/exposure group. Significantly different results are displayed in bold.
[b] See Section 2 for details.
[c] $p < 0.05$ vs. air exposed controls (Tukey–Kramer test).
[d] $p < 0.05$ vs. 100 ppm toluene (Tukey–Kramer test).
[e] $p < 0.05$ vs. 50 ppm benzene (Tukey–Kramer test).

cally significant increase (2.48–2.55-fold) in the frequency of MN-PCE over mice exposed to 100 ppm toluene alone.

3.4. Hepatic enzyme activity

Table 4 presents hepatic enzyme activity levels following benzene, toluene, or benzene and toluene co-exposure. Hepatic CYP2E1 activity levels were significantly increased ≥1.5-fold following co-exposure to either 50 ppm benzene and 50 or 100 ppm toluene compared to air-exposed controls, or to 50 ppm benzene alone or to 100 ppm toluene alone. No increase in CYP2E1 activity was observed following exposure to 50 ppm benzene alone or 100 ppm toluene alone compared to air-exposed controls. Mice exposed to 50 ppm benzene alone exhibited a nonsignificant 1.2-fold increase in hepatic NQO1 activities when compared to air-exposed controls. Mice exposed to 50 ppm benzene alone or in combination with 50 ppm toluene exhibited a nonsignificant 1.2-fold increase in hepatic GST activities when compared to air-exposed controls. Hepatic EH activities exhibited no significant changes following benzene and/or toluene exposure when compared to air-exposed controls. Exposure to benzene or toluene alone or in combination with each other did not affect hepatic CYP3A activity (data not shown).

3.5. Liver and blood GSH concentrations

Levels of total glutathione, oxidized glutathione and the GSH/GSSG ratio were determined in whole blood (Table 5) and liver samples. Mice exposed to 50 ppm benzene alone or co-exposed to 50 ppm benzene and 100 ppm toluene had a statistically significant decrease in GSH levels, while a similar but nonsignificant decrease was observed following exposure to 100 ppm toluene alone. Mice co-exposed to 50 ppm benzene and 100 ppm toluene exhibited statistically significant decreases in blood GSSG levels while nonsignificant decreases were observed following exposure to 50 ppm benzene alone or 100 ppm toluene alone when compared to air-exposed controls. Mice co-exposed to 50 ppm benzene and 100 ppm toluene exhibited statistically significant increases in GSH/GSSG ratio while nonsignificant increases were observed following exposure to 50 ppm benzene or 100 ppm toluene alone when compared to air-exposed controls. Exposure to benzene, toluene, or a combination of benzene and toluene did not affect liver GSH or GSSG concentrations or alter the GSH/GSSG ratio when compared to air-exposed controls (data not shown).

3.6. Urinary metabolite measurement

Urinary metabolite concentrations are presented in Table 6. Mice exposed to 50 ppm benzene or co-exposed to 50 ppm benzene and 50 or 100 ppm toluene exhibited non-statistically significant increases in urinary phenol, *t,t*-MA and s-PMA and HQ concentrations when compared to air-exposed controls. It should be noted that a U-shaped response was observed in urinary *t,t*-MA, HQ, and s-PMA levels, in that a decrease in metabolite concentrations was observed following co-exposure to 50 ppm benzene and 50 ppm toluene when compared to the 50 ppm benzene group. Upon co-exposure to 50 ppm benzene and 100 ppm toluene, urinary metabolite concen-

Table 5
Whole blood levels of glutathione (GSH) and oxidized glutathione (GSSG) in mouse after benzene and/or toluene exposure

Treatment		GSH (μmol)	GSSG (μmol)	GSH/GSSG
Benzene (ppm)	Toluene (ppm)			
0	0	1608.00 ± 149.20	194.50 ± 55.99	11.09 ± 1.25
0	100	1204.00 ± 53.50	86.60 ± 10.77	17.31 ± 3.73
50	0	**1202.22 ± 115.38**[a]	78.56 ± 10.50	20.41 ± 5.08
50	50	1385.00 ± 38.94	120.20 ± 19.17	13.57 ± 1.56
50	100	**1223.00 ± 53.98**[a]	**63.00 ± 11.56**[a]	**30.52 ± 8.31**[a]

Mean ± S.E.M., $n = 9–10$ mice/exposure group. Significantly different results are displayed in bold.
[a] $p < 0.05$ vs. air exposed controls (Tukey–Kramer test).

Table 6
Urinary benzene metabolite concentration in male mice exposed to benzene and/or toluene

Treatment		Urine metabolite concentration (μg/mg creatinine)						
Benzene (ppm)	Toluene (ppm)	Phenol	*t,t*-MA[a]	HQ[b]	CAT[c]	Trihydroxy-benzene	s-PMA[d]	Hippuric acid[e]
0	0	40.00 ± .[f]	nd	0.45 ± 0.17	4.63 ± 0.61	0.86 ± 0.08	nd	313.82 ± 93.71
0	100	68.73 ± 32.61	nd	0.40 ± 0.03	3.90 ± 0.41	0.85 ± 0.21	nd	486.89 ± 161.39
50	0	65.68 ± 17.00	17.13 ± 2.55[g]	**3.29 ± 0.70**[h]	5.76 ± 0.41	0.98 ± 0.12	4.50 ± 1.39[g]	128.37 ± 26.25
50	50	89.68 ± 39.77	9.71 ± 1.85[g]	1.41 ± 0.25	**5.87 ± 0.53**[h]	1.32 ± 0.35	3.17 ± 1.05[g]	287.73 ± 23.30
50	100	143.21 ± 41.37	15.29 ± 5.97[g]	2.58 ± 1.10	5.06 ± 0.50	0.96 ± 0.22	7.03 ± 3.55[g]	857.60 ± 528.33

Mean ± S.E.M., $n = 7$–9 mice/exposure group. Significantly different results are displayed in bold. Data below limits of detection for the metabolites were set to missing.

[a] *trans*,*trans*-Muconic acid.
[b] Hydroquinone (or 1,4-dihydroxybenzene).
[c] Catechol (pyrocatechol or 1,2-dihydroxybenzene).
[d] s-Phenylmercapturic acid.
[e] Hippuric acid is a toluene metabolite.
[f] Only one sample was above the limit of detection.
[g] Different from air exposed and 100 ppm toluene groups, which exhibited all values below the limits of detection.
[h] $p < 0.05$ vs. 100 ppm toluene (Tukey–Kramer test).

trations rebounded and were increased close to or above concentrations observed following exposure to 50 ppm benzene. Urinary CAT was significantly increased in the 50 ppm benzene/50 ppm toluene group when compared to 100 ppm toluene alone. Further, exposure to 50 ppm benzene alone significantly increased HQ concentrations when compared to 100 ppm toluene alone. Urinary trihydroxybenzene and hippuric acid concentrations were unaffected by exposure to benzene alone, toluene alone, or benzene and toluene combined.

4. Discussion

Prolonged exposure to benzene at sufficiently high levels is well known to result in bone marrow hypoplasia, leading to leukopenia, lymphopenia, anemia, thrombocytopenia, and an increased risk for leukemia in humans [4]. Exposure to benzene is also associated with genetic damage to bone marrow cells. Analysis of the frequency of MN in PCE detects clastogenic chemical-induced chromosomal or mitotic apparatus damage [66]. MN represent chromosomes or chromosome fragments that remain during anaphase. After telophase, some of these fragments are not included in the nuclei of the daughter cells and instead form single or multiple MN in the cytoplasm of the immature red blood cell. MN induction in the bone marrow and blood following inhalation of benzene has been examined in several mouse models including parental wild-type mice (B6C3F1, C57BL/6, FVB/N, 129/Sv and DBA/2) and transgenic mice (p53+/−, Tg.AC (v-Ha-ras), NQO1−/−, epoxide hydrolase −/−) [15,30,32,47,67–70]. These studies found that repeated exposure to 50, 100, 200 or 300 ppm benzene can result in a significant increase in MN formation. In a study addressing exposure to benzene at 10 ppm, one of the three mouse strains tested (129/Sv, males only) exhibited a significant increase in MN-PCE (69). Work by Luke et al. further addressed the effect of exposure regimen and duration on MN formation in different sexes and strains of mice [47,68,71]. The increased MN formation following exposure to 50 ppm benzene that was observed in this study is in general agreement with these studies. Interestingly, exposure to 100 ppm toluene also resulted in an increase (albeit nonsignificant) in micronuclei frequency. Mortazavi et al. [72] reported that a 2 weeks exposure to 500 ppm toluene resulted in increased splenic MN formation. Likewise, exposure of male NMRI mice to high intraperitoneal doses of toluene resulted in increased MN formation [73,74].

In the current co-exposure study, animals treated with benzene and either 50 or 100 ppm toluene exhibited an increase in MN-PCE frequency compared to mice exposed to benzene alone, indicating that a combination of benzene and toluene appears to increase the genotoxicity of benzene. This is contrary to the suppression of genotoxicity reported in other co-exposure studies in which higher concentrations were administered [44]. These disparate results suggest that different mechanisms are in effect in the different dose ranges, and is consistent with previous studies demonstrating dose-dependency of the metabolism of benzene after exposure to benzene alone and co-exposure to benzene and toluene. Oral doses of 50 mg/kg or higher saturated the capacity for benzene metabolism in both rats and mice, resulting in an increased proportion of the administered dose being exhaled as benzene [39]. Further, Sabourin et al. demonstrated that, as the exposure concentration of benzene increased, the pathways leading to the formation of toxic metabolites benzoquinone and muconaldehyde were saturated, leading to a shift in the metabolism of benzene from putative toxification pathways to detoxification pathways [75]. Dose–response studies addressing co-exposure to benzene and toluene have demonstrated similar effects. Sato and Nakajima noted a dose-dependent delay in the metabolism of benzene when toluene was present at sufficiently high concentrations [42]. Urinary *t,t*-MA excretion following benzene and toluene co-exposure also exhibited a concentration-dependent decrease [76]. When considered together, the findings of this report and previous studies suggest that the doses used in the current study may be in the linear range of hepatic metabolism. The significant induction in CYP2E1 activity observed following benzene and toluene co-exposure in this study further suggests that metabolism is not saturated at the co-exposure concentrations used. In this range, where there is no inhibitory effect of toluene on benzene metabolism,

micronuclei induction and an increase in activity of CYP2E1, the enzyme shown to be directly related micronuclei induction and genotoxicity [31], is clearly evident.

Benzene exposure, and various unrelated hematological disorders, have been associated with alterations in the numbers of polychromatic erythrocytes associated with ineffective erythropoiesis. PCEs are immature red blood cells that, upon release from the bone marrow, circulate in the blood for approximately 24 h before becoming mature red blood cells. In the current study, intermittent exposure to 50 ppm benzene with or without toluene co-exposure had no effect on the numbers of normochromatic or polychromatic erythrocytes or in the ratio of PCE in total erythrocytes. Previous studies demonstrating such alterations following benzene exposure used higher concentrations. Farris et al. [67] reported that male B6C3F1 mice exposed to ≥100 ppm benzene for 6 h/day for 5 days/week developed a >50% reduction in PCE counts after only 1 week of benzene exposure, while no adverse effects were observed following exposure to 10 ppm benzene. Healy et al. [15] likewise reported decreased PCE counts in male FVB/N mice after several weeks of exposure to 100 ppm benzene for 6 h/day for 5 days/week. On the other hand, intermittent (Monday, Wednesday, and Friday) exposure of C57BL6 mice to 100 ppm benzene for 6 h/day was associated with increased PCE counts. Work by Luke et al. [68] observed a rebound in PCE counts following a similar depression after exposure to 300 ppm benzene in DBA/2 mice; a rebound that was dependent on sex and exposure regimen (6 h/day for 3 or 5 days/week) [68]. These results as well as others [69,77,78] demonstrate that benzene-induced effects on PCE count and erythropoiesis in general are influenced by exposure concentration, inhalation regimen, mouse or rat strain, and gender of experimental animal.

The major site of benzene metabolism is the liver; consequently, exposure to benzene can potentially alter hepatic metabolism and subsequent urinary metabolite formation. While urinary metabolite analysis revealed increases in levels of phenol, *t*,*t*-MA, HQ, and s-PMA following benzene inhalation exposure, co-exposure to 50 ppm benzene and 50 or 100 ppm toluene did not significantly affect levels of *t*,*t*-MA, HQ, and s-PMA when compared to benzene exposure alone. This is consistent with the observations of Sato and Nakajima, who saw a dose-dependent inhibition in urinary phenol excretion with benzene and toluene co-administration, with no change in excretion when the two chemicals were co-administered at the lowest equimolar concentration tested (0.3 mmol/kg) [42]. Also, a concentration-dependent inhibition in urinary *t*,*t*-MA formation was observed following a single, 4 h co-exposure to 20 ppm benzene and toluene at concentrations ≥100 ppm [76]. While our findings contradict the results of a previous co-exposure study where a decrease in the presence of ^{3}H-labeled benzene metabolites was observed following toluene co-exposure in mice, the earlier study utilized much higher concentrations of both solvents [43]. Further, these three studies utilized one- or two-time administrations of benzene and/or toluene rather than the intermittent exposure scenario used in the current study. Combined, these results provide support for the existence of at least two distinct, dose-dependent mechanisms in the metabolism of benzene following benzene and toluene co-exposure.

In the present study, we observed that mice that were co-exposed to benzene and toluene had a statistically significant increase in hepatic CYP2E1 activity when compared to either air-exposed controls or mice treated with benzene or toluene alone. Interestingly, the changes in urinary metabolite levels do not track with the increase in MN frequency observed in this study nor with the enhanced levels of CYP2E1 induction observed following benzene and toluene co-exposure but not following benzene exposure alone. This may be due to the fact that the putative genotoxic metabolite was not measured directly. Further, target tissue levels of the metabolites may correlate better with toxicity than the urinary metabolites, which may be more representative of integrated exposure.

Each of the urinary metabolites analyzed in this study has been used as a biomarker of benzene exposure [79–82]. In those studies using urinary biomarkers to assess the impact of toluene on benzene metabolism, conflicting results have been reported. Qu et al. reported no significant effects on levels of benzene urinary metabolites following co-exposure to benzene (median concentration of 3.2 ppm) and toluene (median concentration of 12.6 ppm) [82]. However, Inoue's study revealed reduced urinary concentrations of phenol and HQ, but not CAT, in workers with co-exposure to benzene (median concentration of 17.9 ppm) and toluene (median concentration of 20.5 ppm) vs. workers that were solely exposed to benzene [81].

In general, associations between benzene exposure concentrations and urinary levels of some of these metabolites are greatest for benzene exposure concentrations greater than 1 ppm [82,83]. Conflicting results have been observed in several studies assessing urinary s-PMA concentrations with benzene in the 100 ppb range [84,85], and a number of studies have observed no correlation in urinary *t*,*t*-MA or s-PMA concentrations when exposure concentrations are in the ppb range (e.g., 2–20 ppb) associated with non-occupational exposures [86–89]. While there is poor correlation between benzene urinary metabolite formation and lower non-occupational exposures, their use holds more promise in elucidating the impact of exposures, and co-exposures, at higher levels relevant to an occupational setting. Also, in laboratory studies, urinary metabolite formation is more informative than blood analyses of benzene in assessing the impact of co-exposures [42,43,90].

The present study exemplifies the importance of studying the effects of binary chemical interactions in animals exposed to lower exposure concentrations of benzene and toluene on benzene metabolism, hematotoxicity and clastogenicity. The relevance of these data on interactions for humans exposed at low benzene concentrations can be best assessed only when the mechanism of interaction is understood at a quantitative level and incorporated within a biologically based modeling framework.

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