Regulatory Toxicology and Pharmacology 70 (2014) S58–S68

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

journal homepage: www.elsevier.com/locate/yrtph

Health assessment of gasoline and fuel oxygenate vapors: Developmental toxicity in mice

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A R T I C L E I N F O

Article history:
Available online 28 June 2014

Keywords:
Developmental toxicity
Gasoline vapor condensates
Methyl tertiary butyl ether (MTBE)
Fuel oxygenates
Evaporative emissions
Gastroschisis
Ectopia cordis

A B S T R A C T

CD-1 mice were exposed to baseline gasoline vapor condensate (BGVC) alone or to vapors of gasoline blended with methyl tertiary butyl ether (G/MTBE). Inhalation exposures were 6 h/d on GD 5–17 at levels of 0, 2000, 10,000, and 20,000 mg/m^3. Dams were evaluated for evidence of maternal toxicity, and fetuses were weighed, sexed, and evaluated for external, visceral, and skeletal anomalies. Exposure to 20,000 mg/m^3 of BGVC produced slight reductions in maternal body weight/gain and decreased fetal body weight. G/MTBE exposure did not produce statistically significant maternal or developmental effects; however, two uncommon ventral wall closure defects occurred: gastroschisis (1 fetus at 10,000 mg/m^3) and ectopia cordis (1 fetus at 2000 mg/m^3; 2 fetuses/1 litter at 10,000 mg/m^3). A second study (G/MTBE-2) evaluated similar exposure levels on GD 5–16 and an additional group exposed to 30,000 mg/m^3 from GD 5–10. An increased incidence of cleft palate was observed at 30,000 mg/m^3 G/MTBE. No ectopia cordis occurred in the replicate study, but a single observation of gastroschisis was observed at 30,000 mg/m^3. The no observed adverse effect levels for maternal/developmental toxicity in the BGVC study were 10,000/2000 mg/m^3, 20,000/20,000 for the G/MTBE study, and 10,000/20,000 for the G/MTBE-2 study.

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1. Introduction

The 1990 amendments to the Clean Air Act (CAA) mandated the use of oxygenates in motor gasoline. In 1994, the U.S. Environmental Protection Agency (EPA) issued a final rule under the Act which added new health effects information and testing requirements to the Agency's existing registration requirements. As described in more detail in a companion paper (Henley et al., 2014), requirements include inhalation exposures to evaporative emissions of the gasoline or gasoline blended with the additive in question. The health endpoints include assessments for standard subchronic toxicity, neurotoxicity, genotoxicity, immunotoxicity, developmental and reproductive toxicity, and chronic toxicity/carcinogenicity. The results of chronic toxicity testing of gasoline and gasoline combined with MTBE have already been reported (Benson et al., 2011) and reported elsewhere in this issue are the findings for subchronic toxicity testing (Clark et al., 2014), genotoxicity (Schreiner et al., 2014), neurotoxicity (O'Callaghan et al., 2014) and immunotoxicity (White et al., 2014). This paper describes the results of developmental toxicity testing in mice which have been submitted to EPA. Other papers in this issue report on the results of developmental and reproductive toxicity testing in rats (Roberts et al., 2014; Gray et al., 2014).

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http://dx.doi.org/10.1016/j.yrtph.2014.06.011
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2. Materials and methods

Two studies to meet the CAA requirements were conducted at ExxonMobil Biomedical Sciences, Inc. (EMBSI) Laboratory Operations, Mammalian Toxicology Laboratory, 1545 Route 22 East, P.O. Box 971, Annandale, New Jersey 08801. The test materials were a baseline gasoline vapor condensate (BGVC) and a vapor condensate of gasoline mixed with methyl-t-butyl ether (G/MTBE). Due to an unusual finding in the G/MTBE study, a repeat of the G/MTBE study was conducted at Huntingdon Life Sciences (HLS), Princeton Research Center, 100 Mettlers Road, East Millstone, NJ. The postmortem maternal and fetal evaluations and analyses were conducted by staff of RTI International (RTI), POB 12194, 3040 Cornwallis Road, Research Triangle Park, NC, 27709, at the HLS testing facility. All of the laboratories are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International).

2.1. CAA Studies

2.1.1. Test material preparation and characterization

Test articles included two different vapor condensates: one prepared from an EPA-described ‘‘baseline gasoline’’ (BGVC) (US EPA, 1994), and the other from BGVC blended with methyl-t-butyl ether (G/MTBE) prepared and supplied in 100 gal gas cylinders by Chevron Research and Technology Center (Richmond, CA). The methodology for preparation and analytical characterization of the samples is described in a companion paper (Henley, et al., 2014). The test substances, as received, were considered the ‘‘pure’’ substance for the purpose of dosing.

2.1.2. Animal selection and care

The test animals were Caesarean-originated Virus Antibody Free (VAF) Crl:CD-1® (ICR) BR outbred albino mice supplied by Charles River Laboratories, Inc.(Portage, MI). Sexually mature males were used for mating purposes only. Females were 12–13 weeks of age and weighed 26–35 g at the start of mating.

Certified Rodent Diet, No. 5002; (Meal) (PMI Nutrition International, St. Louis, Missouri) was available ad libitum. Analysis of each feed lot used during this study was performed by the manufacturer. Water was available without restriction via an automated watering system. There were no known contaminants in the feed or water to interfere with test results of these studies. Animals were without food and water while in the exposure chambers.

2.1.3. Housing and environmental conditions

Animals were housed individually in suspended stainless steel wire mesh cages. A twelve hour light/dark cycle was controlled by an automatic timer. Temperature and relative humidity were maintained within the specified range (64–72 degrees F, and 30–70%, respectively).

2.1.4. Experimental design

The experimental design is shown in Table 1. Untreated animals were mated (1 nulliparous female; 1 male) until sufficient plug positive presumed-pregnant females were identified by the presence of a copulatory plug in the vagina. Plug-positive female mice were distributed by body weight into four different exposure groups (25/group) on gestational day (GD) 0. Presumed pregnant females were exposed to 0 mg/m^3 (air control), 2000 mg/m^3, 10,000 mg/m^3 and 20,000 mg/m^3, 6 h/day from GD 5 through GD 17. The highest exposure level represented approximately 50% of the Lower Explosive Limit of each test material. On GD 18, animals were sacrificed and evaluated as described in Section 2.1.6.

2.1.5. Administration of test substance and exposure schedule

The experimental and control animals were placed into whole-body inhalation chambers operated under dynamic conditions for at least 6 h per day for GD 5–16 at 2000, 10,000, and 20,000 mg/m^3, or for GD 5–10 at 30,000 mg/m^3 after target exposure levels were reached. The animals remained in the chambers for at least an additional 23 min (theoretical equilibration time) while the test atmosphere cleared. Females were exposed in 1.0 m^3 stainless steel and glass chambers operated at a flow rate of approximately 12–15 air changes/hour. During exposure periods, animals were individually housed in stainless steel, wire mesh cages. Flow rate and slightly negative pressure was monitored continuously and recorded approximately every 30 min. Light (ca. 30–40 foot-candles 1.0 m above the floor) and noise levels (<85 db) in the exposure room were measured pretest and at the beginning, middle and end of the study. Oxygen levels in the exposure chambers were maintained between 20.6 and 20.7%.

The control group was exposed to clean filtered air under conditions identical to those used for groups exposed to the test substance. The test substance was administered fully vaporized in the breathing air of the animals. The chamber concentrations were measured in the breathing zone of the mice by on-line gas chromatography (GC). The chromatographic analyses were used to assess the stability of the test substance over the duration of the study. Additionally, sorbent tube samples were collected once weekly and stored in a freezer for analysis by a detailed capillary GC method to compare component proportions of the test material atmosphere with the liquid test material. Homogeneity of the exposure system was validated prior to the start of each study. Particle size determination confirmed that exposures were to vapor only (see Section 3.1).

2.1.6. Experimental evaluation

Animals were examined for viability at least twice daily during the study. Body weights were recorded prior to selection and on GD 0, 5, 8, 11, 14, 17, and 18. Food consumption was measured for mated females on GD 5, 8, 11, 14, 17, and 18. A clinical examination of each female occurred prior to selection and daily during gestation. Additionally, group observations of the animals for mortality and obvious toxic signs while in the chambers were recorded at 15, 30, 45, and 60 min after initiation of the exposure and then hourly during each exposure.

Body weights were recorded on GD 18, the day of necropsy. Dams were sacrificed by CO_2 asphyxiation followed by exsanguination. A gross necropsy was performed on all confirmed-mated females. Uterine weights with ovaries attached were recorded at the time of necropsy. Uterine contents were examined and the numbers and locations of implantation sites, early and late resorptions, and live and dead (live or dead *in utero*) fetuses were counted. Ovarian corpora lutea also were counted. The uteri of all apparently non-pregnant females were stained with 10% ammonium sulfide to confirm non-pregnancy status. Evaluations of dams during cesarean section and subsequent fetal evaluations were conducted without knowledge of treatment group in order to minimize bias. Each fetus was weighed and examined externally for gross malformations and variations. Fetal sex was determined by external examination and confirmed internally only on those fetuses receiving visceral examinations. Fetuses were euthanized by hypothermia after the external examination and weighing.

The viscera of approximately one-half of the fetuses of each litter were examined by fresh dissection (Staples, 1974; Stuckhardt and Poppe, 1984) prior to decapitation of the fetus. The heads were preserved in Bouin's solution for at least two weeks, then rinsed and subsequently stored in 70% ethanol. Free-hand razor blade sections of the Bouin's-fixed fetal heads were examined for the presence of abnormalities. The remaining fetuses were eviscerated,

Table 1
Experimental Design.

Study	BGVC, G/MTBE-1				G/MTBE-2				
Laboratory	EMBSI (NJ)				HLS – RTI (NJ – NC)				
Species/strain/source	VAF Crl:CD-1®(ICR)BR; Charles River MI				VAF Crl:CD-1®(ICR)BR; Charles River NC				
Test substance	BGVC		G/MTBE		G/MTBE				
Wt./Age at mating	25–33 g;11–12 wk		26–35 g; 12–13 wk		20–35 g; 9–11 wk				
Exposure levels [mg/m^3]	0	2,000	10,000	20,000	0	2,000	10,000	20,000	30,000
Number of females	25	25	25	25	23	23	23	23	38
Exposure days [6 h/d]	GD 5–17				GD 5–16				GD 5–10
Necropsy	GD 18				GD 17				
Body weights	GD 0, 5, 8, 11, 14, 17 and 18				GD 0 and daily GD 5–17				
Food consumption	GD 0, 5, 8, 11, 14, 17 and 18				GD 0 and daily GD 5–17				
Maternal organ weights					Liver, paired kidney and adrenal weights				
Fetal weight/external observations	100%				100%				
Skeletal/visceral observations	~50% examined				Not done				

processed by double staining with Alizarin red and Alcian blue, and examined for bone and cartilage development and any abnormalities. The fetal skeletons were preserved in glycerine with thymol after they were processed and stained.

2.1.7. Statistical analysis

Statistical methods included evaluation of equality of means by appropriate one-way analysis of variance and a test for ordered response in the dose groups. Bartlett's Test was performed to determine if the dose groups had equal variance followed by standard one-way analysis of variance (Snedecor and Cochran, 1989) after which parametric methods were employed when variances were equal. For continuous data, percentages were calculated and transformed by Cochran's transformation, followed by the arc sine transformation (Snedecor and Cochran, 1989). The raw percentages and the transformed percentages were both tested for statistical significance.

For the parametric procedures, a standard one-way ANOVA using the F distribution to assess significance was used (Snedecor and Cochran, 1989) followed by Dunnett's Test if significant differences in ANOVA were found (Dunnett, 1964). A standard regression analysis for linear response in the dose groups was performed, which also tested for linear lack of fit in the model.

For the nonparametric procedures, equality of means was assessed by the Kruskal–Wallis Test (Hollander and Wolfe, 1973) followed by Dunn's Summed Rank Test (Hollander and Wolfe, 1973), if needed. Jonckheere's Test for monotonic trend in the dose response was also performed (Hollander and Wolfe, 1973). Bartlett's Test for equal variance was conducted at the 1% level of significance. All other tests were conducted at the 5% and 1% level of significance. Data for non-pregnant females, dams delivering before GD 18, or from pups which were delivered early in those litters were not included in analyses.

Means and standard deviations were calculated for animal, exposure and chamber environmental data. Body weight change and food consumption were evaluated for individual intervals and also for the pre-exposure, exposure, and post-exposure periods. The coefficient of variation was calculated when considered relevant. Fetal body weight was analyzed by a mixed model analysis of variance using the litter as the basis for analysis and the litter size as a covariate. The analysis of anomalies (malformations or variations) was based on a generalized estimating equation (GEE) application of the linearized model, Ryan (1992). The model used the litter as the basis for analysis and considered correlation among littermates by incorporating an estimated constant correlation and the litter size as a covariate. If the overall effect of dose, or the dose by sex effect was statistically significant, the dose group means were tested pairwise against the control group using least squares means which accounted for differences in litter size and sex. The mathematical model was based on a method by Chen et. al (1996). The analysis was run using SAS (Little, et. al, 1997).

In addition to the specific anomalies, a series of combined analyses were performed within each category as applicable. These combined categories were: Combined Malformations and Variations for All Fetuses; for Live Fetuses; for Dead Fetuses; Malformations for All Fetuses; for Live Fetuses; for Dead Fetuses; and Variations for Live Fetuses; and for Dead Fetuses.

2.2. Second G/MTBE study

2.2.1. Experimental design

The repeat of the G/MTBE study at HLS-RTI was similar to the studies conducted at EMBSI with the exceptions summarized in Table 1. The same strain of mouse was used but was obtained from Charles River Laboratories in Raleigh, NC. An additional 30,000 mg/m^3 exposure group of 38 females was added to the study design. The exposure period for the first four exposure groups was 6 h/day from GD 5–16. The 30,000 mg/m^3 group was exposed from GD 5–10 since the specific malformations observed in the original G/MTBE study (ventral wall defects) are formed early in the gestation period (GD 7–9; Rugh, 1968). On GD 11–16, females in the 30,000 mg/m^3 exposure group were removed from their home cage and placed in another suspended cage without feed to match as closely as possible the housing conditions of Group 1–4 females for the 6-h exposure period. They were then returned to their home cage at the same time as the exposed females. At sacrifice, the liver, paired adrenal glands, and paired kidneys of each plug-positive female were weighed. Fetal body weights were measured and all fetuses were examined for external anomalies, but neither visceral nor skeletal observations were conducted (Table 1).

2.2.2. Second study statistics

The unit of comparison was the pregnant female or litter. Quantitative continuous data (e.g., body weights, etc.) were compared among the 4 treatment groups and 1 vehicle control group using either parametric ANOVA under the standard assumptions or robust regression methods (Zeger and Liang, 1986; Royall, 1986; Huber, 1967), which do not assume homogeneity of variance or normality. If the ANOVA test was statistically significant, then statistical pairwise comparisons were made (see below). The homogeneity of variance assumption was examined via Levene's Test (Levene, 1960). If Levene's Test indicated lack of homogeneity of variance ($p < 0.05$), robust regression methods (heterogeneous variance methods) were used to test all treatment effects. Overall treatment group differences were tested by Wald Chi-Square Tests followed by individual tests for exposed vs. control group comparisons when the overall treatment effect was significant. The presence of linear trends was analyzed by robust regression methods

for non-homogenous data in the REGRESS procedure of SUDAAN® Release 8. (RTI, 2001).

Analytical methods when variances were homogeneous were similar to those employed in the EMBSI studies. For the litter-derived percentage data, the ANOVA was weighted according to litter size. The presence of linear trends was analyzed by GLM procedures for homogenous data (SAS Institute Inc., 1999a,b,c,d,e, 2000, 2001).

Nominal scale measures were analyzed by Chi-Square Test for Independence for differences among treatment groups (Snedecor and Cochran, 1989) and by the Cochran–Armitage Test for Linear Trend on Proportions (Cochran, 1954; Armitage, 1955; Agresti, 1990), followed by a Fisher's Exact Probability Test, with appropriate adjustments for multiple comparisons, if Chi Square results were significant.

A test for statistical outliers (SAS Institute Inc., 1999b) was performed on female body weights, feed consumption (in g/day), and selected organ weights. Body weight change and food consumption were evaluated for individual intervals and also for the pre-exposure, exposure, and post-exposure periods. If examination of pertinent study data did not provide a plausible, biologically sound reason for inclusion of the data flagged as "outlier," then the data were excluded from summarization and analysis and designated as outliers. Unless otherwise specified, the level of significance used for the various tests was $p < 0.05$.

2.3. Compliance

All studies were conducted to meet/exceed compliance with the EPA's guidelines and standards for the conduct of inhalation exposures and developmental toxicity (US EPA 1994a, 1994b, 1996, 1998), with OECD guidelines for chemical testing (OECD, 2001), and with provisions of the Animal Welfare Act (Code of Federal Regulations, 1966) and the National Research Council's Guide for the Care and Use of Laboratory Animals (NRC, 1996).

3. Results

3.1. Exposure monitoring

Chamber distribution analyses conducted by the laboratory during the conduct of the studies showed that the test substances were evenly distributed within the exposure chambers. The mean measured concentrations (analytical and nominal) were reasonably close to the targeted exposure levels for each of the gasoline oxygenate studies Table 2.

The concentrations of the major components in the test materials supplied for the studies as reported by Henley et al. (2014) are shown in Table 3. The values are expressed as area percent to facilitate comparison between studies. Analysis of the major components in the exposure chamber samples measured by the lab showed a close comparison between the neat test substance and the vaporized test substance. The average molecular weights of the hydrocarbons in the condensate test materials shown in Table 2 can be used to calculate the approximate part-per-million concentrations.

Table 2
Total hydrocarbon concentrations measured in exposure chambers.

Chamber concentrations (mg/m^3)[a]				
Target	Actual (mean ± SD)			
		BGVC	G/MTBE-1	G/MTBE-2
2000	Analytical	2086 ± 175	2122 ± 57	2074 ± 248
10,000	Analytical	10,625 ± 358	10,788 ± 322	9899 ± 700
20,000	Analytical	20,903 ± 600	20,741 ± 923	20,297 ± 1815
30,000	Analytical	n/a	n/a	29,259 ± 1480
Average MW[b]		73.8	73.7	73.7

[a] Total hydrocarbon concentrations as determined by gas chromatography (infrared spectroscopy for G/MTBE-2).
[b] Average molecular weight of hydrocarbons in condensate samples.

Table 3
Concentration of major hydrocarbons measured in test substance supplied to laboratories.

Hydrocarbon concentration (area percent)[a]		
	BGVC	G/MTBE
Isobutane	3.6	2.2
n-butane	15.2	11.1
Isopentane	35.1	31.0
n-pentane	13.2	9.1
trans-2-pentene	2.5	2.0
2-methyl-2-butene	3.8	2.9
2,3-dimethylbutane	1.6	0.9
2-methylpentane	6.3	4.5
3-methylpentane	3.6	2.6
n-hexane	3.0	2.1
Methylcyclopentane	1.5	1.1
2,4-dimethylpentane	1.0	0.9
Benzene	2.1	1.5
2-methylhexane	1.1	1.0
2,3-dimethylpentane	1.1	1.0
3-methylhexane	1.3	1.1
Isooctane	1.3	1.2
Toluene	3.0	2.5
MTBE	0.0	21.3

[a] Values for these 18 reference hydrocarbons were derived pre-study (Henley et al., 2014). A total of 131 peaks were separated and identified for the BGVC study. The reference hydrocarbons comprised over 81% of the total mass but are normalized to 100% to enable comparisons across the three labs.

In the BGVC study, particles sized at 0.26 mg/m^3 in the control chamber and 0.20 mg/m^3 in the 20,000 mg/m^3 chamber were measured. These particles were judged to be ambient background particles (e.g., animal dander) and not from the generation of the test substance. No particles were detected in the G/MTBE or G/MTBE-2 study chambers.

3.2. Clinical in-life observations and survival

All BGVC- and G/MTBE-exposed dams were free of clinical or postmortem effects attributable to treatment. One control, one 10,000 mg/m^3 target, and two 20,000 mg/m^3 target dams in the BGVC study were determined at the scheduled terminal sacrifice to be not pregnant, and one control and one 20,000 mg/m^3 target dam delivered their litters prior to their scheduled sacrifice. In the G/MTBE study, one control dam, one dam exposed at 2,000 mg/m^3 and three exposed at 20,000 mg/m^3 were not pregnant at the scheduled terminal sacrifice. Additionally, one control dam, one dam exposed at 2,000 mg/m^3 and two exposed at 20,000 mg/m^3 delivered all or part of their litters prior to the scheduled sacrifice. In the repeat G/MTBE study (G/MTBE-2), one female was removed from study due to a pre-existing condition. There were no clinical signs indicative of exposure-induced maternal toxicity during the studies, except for slight emaciation noted in one dam at 20,000 mg/m^3 BGVC dam (19% body weight loss, GD 8–11). In addition, labored breathing was observed in the G/MTBE-2 study on GD 9 post exposure in one dam at 20,000 mg/m^3 and on GD 10 post exposure for one dam at 30,000 mg/m^3.

3.3. Gestational body weight

In the BGVC study, maternal toxicity at 20,000 mg/m^3 was evident as statistically significant decreases in mean gestational body weight from GD 11 onward, and mean gestational body weight

Table 4
Effects of BGVC and G/MTBE on maternal body weight.

	BGVC		G/MTBE	
Concentration (mg/m^3)	0	20,000	0	20,000
N	23	21	24	20
Gestation day	Mean gestation body weight (g) ± SD			
0	30.7 ± 2.3	30.3 ± 1.7	28.9 ± 1.4	28.7 ± 1.3
5	33.3 ± 1.8	33.0 ± 1.7	31.6 ± 1.9	31.3 ± 1.4
8	35.2 ± 2.3	34.2 ± 1.6	33.5 ± 2.0	33.1 ± 1.3
11	39.5 ± 2.4	37.2 ± 2.4*	37.8 ± 2.4	37.1 ± 1.5
14	46.7 ± 3.2	43.5 ± 3.2*	44.5 ± 2.9	43.7 ± 2.1
17	56.5 ± 4.9	52.1 ± 4.5*	53.0 ± 3.5	52.8 ± 2.7
18	59.0 ± 5.5	54.3 ± 4.9*	55.5 ± 4.0	54.9 ± 3.1
18C	36.0 ± 2.7	35.2 ± 1.7	34.7 ± 2.1	34.5 ± 2.3
Uterus weight	23.0 ± 3.3	19.1 ± 4.0**	20.8 ± 2.8	20.4 ± 2.2
	Mean gestation body weight change (g) ± SD			
0–5	2.7 ± 1.5	2.7 ± 1.3	2.7 ± 1.0	2.6 ± 1.2
5–8	1.9 ± 0.9	1.2 ± 0.7	1.9 ± 1.0	1.9 ± 0.7
8–11	4.3 ± 0.9	3.0 ± 1.3**	4.2 ± 1.3	4.0 ± 1.0
11–14	7.2 ± 1.3	6.3 ± 1.3	6.8 ± 1.3	6.6 ± 1.4
14–17	9.8 ± 2.0	8.6 ± 2.0	8.5 ± 1.4	9.1 ± 1.0
17–18	2.5 ± 1.3	2.2 ± 1.0	2.5 ± 1.0	2.2 ± 0.8
5–18	25.7 ± 4.4	21.4 ± 3.8**	23.8 ± 3.0	23.7 ± 2.8
0–18	28.4 ± 4.8	24.0 ± 4.1**	26.5 ± 3.4	26.3 ± 2.8
0–18C	5.3 ± 2.6	5.0 ± 2.0	5.8 ± 1.9	5.9 ± 1.9

C = terminal body weight minus gravid uterine weight at necropsy.
* $P < 0.05$ Dunnett's test.
** $P < 0.01$ Dunnett's test.

Table 5a
Effects on maternal body weight and food consumption in G/MTBE-2 study.

Concentration (mg/m^3)	0	20,000	30,000
N	23	23	36
Gestation day	Mean gestation body weight (g)		
0	26.7 ± 0.4	27.3 ± 0.3	27.5 ± 0.2
5	27.6 ± 0.4	28.5 ± 0.3	27.5 ± 0.2
8	29.2 ± 0.4	30.0 ± 0.3	29.8 ± 0.3
11	32.8 ± 0.5	33.2 ± 0.5	33.0 ± 0.5
14	39.2 ± 0.6	38.7 ± 0.9	39.7 ± 0.7
17	48.4 ± 0.7	46.7 ± 1.4	48.3 ± 1.2
	Mean gestation body weight change (g)		
0–5	0.8 ± 0.2	1.2 ± 0.2	0.8 ± 0.2
7–8	0.5 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
12–13	2.1 ± 0.1	1.5 ± 0.2**	2.0 ± 0.1
16–17	3.2 ± 0.2	2.7 ± 0.2	3.1 ± 0.1
5–10	3.3 ± 0.2	NC	3.0 ± 0.2
5–16	17.6 ± 0.4	15.5 ± 1.2	NC
10–17	17.5 ± 0.4	NC	17.0 ± 0.9
Gestation change	20.4 ± 0.6	18.6 ± 1.5	20.0 ± 1.1
Uterus weight (g)	17.3 ± 0.5	15.7 ± 1.2	16.8 ± 0.9
Gestation change corrected	3.1 ± 0.3	2.9 ± 0.4	3.2 ± 0.3
	Mean gestation food consumption (g)		
0–5	6.1 ± 0.2	5.3 ± 0.2	6.8 ± 0.4
7–8	7.0 ± 0.4	6.2 ± 0.2	6.4 ± 0.2
12–13	7.1 ± 0.2	6.9 ± 0.2	7.8 ± 0.2
16–17	7.6 ± 0.2	7.3 ± 0.2	7.5 ± 0.2
5–10	6.9 ± 0.4	NC	6.2 ± 0.1
5–16	6.9 ± 0.2	6.8 ± 0.3	NC
10–17	7.3 ± 0.2	NC	7.6 ± 0.2
0–17	6.5 ± 0.1	6.1 ± 0.2	6.9 ± 0.2

NC = not calculated due to differences in exposure duration between 30,000 mg/m^3 and other exposure groups. Gestation change = total body weight change during gestation. Gestation corrected = body weight change during gestation minus gravid uterine weight.
** $P < 0.01$ Dunnett's test.

change for GD 5–8, GD 5–18, and GD 0–18 (Table 4). Statistically significant differences were not evident at 2,000 or 10,000 mg/m^3. In the G/MTBE study, there were no statistically significant differences in the mean gestational body weight, mean gestational body weight change, or mean uterine weights between treated and control dams at any interval during gestation (Table 4).

In the G/MTBE-2 study, there was a decreased daily maternal body weight change only for GD 12–13 interval at the 2000 and 20,000 mg/m^3 dose levels (Table 5). These decreases were considered incidental since they only occurred once and did not display a dose–response pattern. Maternal gestational weight change (gestational body weight gain minus gravid uterine weight) was unaffected across all groups.

In the G/MTBE-2 study, maternal absolute gravid uterine weight, paired adrenal gland weight, and paired kidney weight were unaffected across all groups (Table 5b). Maternal paired adrenal gland and paired kidney weights (relative to terminal body weights) were equivalent across all groups (not shown). Maternal absolute liver weights were equivalent at 0, 2000, 20,000, and 30,000 mg/m^3 but were significantly increased (11%; $p < 0.05$) at 10,000 mg/m^3. Relative (to terminal body weight) maternal liver weights were significantly increased in a concentration-related manner at 2000 (6%; $p < 0.01$), 10,000 (11%; $p < 0.001$), and 20,000 (11%; $p < 0.001$) mg/m^3, in dams exposed through GD-16; relative liver weight was unaffected at 30,000 mg/m^3, where exposures ended on GD 10.

3.4. Gestational food consumption

Exposure to BGVC produced no statistically significant differences in mean gestational food consumption between treated and control dams at any interval during the gestation period. Similarly, exposure to G/MTBE produced no effects upon food consumption in either the original or repeated studies.

3.5. Gross postmortem observations

All BGVC-exposed dams were free of grossly observable abnormalities at the gross postmortem examination. In G/MTBE exposed dams, the only gross observation was red contents in the stomach of a dam that had delivered her litter. Additionally, the fetus closest to the cervix in one of the uterine horns of a control dam was found partially in the body of the uterus. This was apparently the beginning of her delivery. As the fetus had not delivered, the dam and litter were treated as a terminal sacrifice and not a delivered litter. All other dams exposed to G/MTBE and G/MTBE-2 had no grossly observed abnormalities.

3.6. Uterine implantation data

Table 6 presents the number of ovarian corpora lutea, uterine implantation sites, live fetuses per litter, and resorption data for dams exposed in the three studies. The uterine implantation data for BGVC exposed dams revealed a statistically significant decrease in the mean number of live fetuses in the 20,000 mg/m^3 target group (11.0 per litter vs. 13.6 in the control group). Live litter size is a consequence of the number of eggs ovulated (measured by the corpora lutea in the ovaries), implantation success (assessed as pre-implantation loss), and viability of the embryo/fetus (post-implantation loss, identified as early or late resorptions or dead fetuses). The statistically significant decrease in mean live litter size was due to both a lower value for ovarian corpora lutea, which occurred before exposure, and a higher incidence of implantation site resorptions, which occurred after exposure was initiated. The mean number of corpora lutea per litter at 20,000 mg/m^3 was 1.7 less in the 20,000 mg/m^3 BGVC group than that observed in the control group. During pregnancy, the numbers of ovarian corpora lutea are established when the eggs are ovulated from the ovary, prior to the day of pregnancy when exposures to the test material began. Based upon the ratio of implantations to ovarian corpora lutea, the overall implantation success rate was greater than 95% for both groups. The difference in the mean number of corpora

Table 5b
Absolute and relative organ weights of mice in G/MTBE-2 study.

	Gasoline/MTBE vapor condensate (mg/m^3 inhaled)				
	0[a]	2,000[a]	10,000[a]	20,000[a]	30,000[b]
No. of mice	23	22	19	22	36
Terminal body wt (g) at GD 17	47.1 ± 0.7	46.7 ± 1.5	47.5 ± 0.8	45.9 ± 1.4	47.4 ± 1.2
Gravid uterine Wt (g)	17.3 ± 0.5	15.6 ± 1.1	16.6 ± 0.4	15.7 ± 1.2	16.8 ± 0.9
Liver wt[c]					
Absolute (g)	2.45 ± 0.05	2.58 ± 0.09	2.72 ± 0.05*	2.63 ± 0.07	2.43 ± 0.06
Relative[d]	5.20 ± 0.06	5.53 ± 0.09***	5.74 ± 0.09	5.76 ± 0.07***	5.15 ± 0.09
Paired adrenal wt:[c]					
Absolute (g)	0.014 ± 0.001	0.014 ± 0.001	0.013 ± 0.00[e]	0.014 ± 0.001	0.014 ± 0.00
Relative[d]	0.029 ± 0.001	0.031 ± 0.002	0.028 ± 0.001[e]	0.031 ± 0.002	0.030 ± 0.002
Paired kidney wt[c]					
Absolute (g)	0.428 ± 0.009	0.445 ± 0.011	0.439 ± 0.005	0.438 ± 0.009	0.431 ± 0.007
Relative[d]	0.907 ± 0.011	0.978 ± 0.045	0.928 ± 0.016	0.982 ± 0.050	0.935 ± 0.034

*$p < 0.05$ Dunnett's test.
**$p < 01$ and ***$p < 0.001$ Individual *t*-test in robust regression model.
[a] Exposure GD 5–16.
[b] Exposure GD 5–10.
[c] Includes all pregnant dams until terminal sacrifice on GD 17. Reported as mean ± SEM.
[d] Relative = % sacrifice body weight.
[e] Paired adrenals 10,000 mg/m^3; $N = 18$ due to exclusion of one mouse as a statistical outlier.

Table 6
Uterine data.

Uterine data (mean + SD)					
Endpoint	Exposure group mg/m^3				
No. of litters	0	2,000	10,000	20,000	30,000
BGVC	23[a]	25	24	22[a]	n/a
G/MTBE	24[a]	20[a]	25	20[b]	n/a
G/MTBE-2	23	22	19	22	36
Corpora lutea per dam					
BGVC	14.6 ± 2.4	14.6 ± 2.6	14.7 ± 2.4	12.9 ± 2.6	n/a
G/MTBE	14.0 ± 1.7	13.4 ± 2.2	13.5 ± 3.0	13.5 ± 2.5	n/a
G/MTBE-2	13.0 ± 0.4	12.2 ± 0.6	12.8 ± 0.4	13.2 ± 0.6	13.2 ± 0.5
Implantation sites per litter					
BGVC	14.1 ± 2.1	14.2 ± 2.7	14.0 ± 2.9	12.4 ± 2.6	n/a
G/MTBE	13.5 ± 1.5	13.2 ± 2.4	12.9 ± 3.4	13.3 ± 2.0	n/a
G/MTBE-2	12.7 ± 0.4	12.0 ± 0.6	12.7 ± 0.3	13.2 ± 0.5	12.9 ± 0.4
Total live fetuses per litter					
BGVC	13.6 ± 2.1	13.5 ± 2.7	13.3 ± 3.0	11.0* ± 3.7	n/a
G/MTBE	12.4 ± 2.0	12.6 ± 2.3	12.1 ± 3.2	12.8 ± 1.9	n/a
G/MTBE-2	12.0 ± 0.4	11.2 ± 0.7	11.8 ± 0.3	12.6 ± 0.5	12.3 ± 0.3
Resorptions per litter					
BGVC	0.4 ± 0.8	0.6 + 0.7	0.5 ± 1.1	1.3*[,c,d] ± 3.1	n/a
G/MTBE	1.1 ± 1.1	0.6 ± 1.0	0.6 ± 1.0	0.6 ± 0.6	n/a
G/MTBE-2	0.6 ± 0.2	1.3 ± 0.5	0.7 ± 0.2	1.7 ± 0.9	1.6 ± 0.5
Fully resorbed litters					
BGVC	0/24	0/25	0/24	1/23	n/a
G/MTBE	0/24	0/21	0/25	0/22	n/a
G/MTBE-2	0/23	1/22	0/19	2/22	3/36

BGVC – baseline gasoline vapor condensate exposure.
G/MTBE – gasoline combined with MTBE vapor condensate exposure.
G/MTBE-2 – repeat of G/MTBE study.
* $p < 0.05$.
[a] 1 additional litter delivered prior to scheduled necropsy.
[b] 2 additional litters delivered prior to scheduled necropsy.
[c] contained 1 female with unexplained emaciation, GD 11.
[d] statistically significant as resorptions/implantation following arc sine transformation.

lutea per litter contributes to an explanation for the difference in the mean number of live fetuses per litter (due to the decreased numbers of eggs ovulated, and subsequently the number of ovarian corpora lutea present). Additionally, the incidence of resorptions, which was statistically significantly higher than in the control group (1.3 per litter vs. 0.4 in the control group), also contributed to the decrease in live litter size. The ratio of live fetuses per implantation sites was not statistically different between the control and high-exposure groups. The statistically significant decrease in the number of live fetuses per litter was considered to be partly treatment-related.

Exposure to BGVC appeared to cause an increase in resorptions at 20,000 mg/m^3. The difference was not statistically significant as a mean value per litter, but reached statistical significance ($p < 0.05$) when analyzed as a transformed ratio of resorptions/implantations. This parameter was strongly influenced by a single animal in the high-exposure group with complete litter resorption. This animal was noted to be emaciated during mid-gestation, losing a total of 9 g of body weight (25% of her weight) between GD 5–11. This weight loss was greater than could be accounted for by loss of the developing embryos. Although this animal was not considered an outlier, the data were reanalyzed to determine the potential impact of excluding this litter from the statistical analysis. When her data were removed from the data set, statistical significance disappeared for resorption parameters, but live litter size remained statistically different than control.

There were no other statistically significant differences between the control group and BGVC treated dams for the other implantation parameters. In both the G/MTBE and G/MTBE-2 studies, there were no statistically significant differences in the implantation parameters between the control and treated dams (Table 6), although 8.3% and 9.1% of litters were fully resorbed in the 30,000 and 20,000 mg/m^3 exposure groups, respectively.

There were 0, 1 (4.5%), 0, 2 (9.1%), and 3 (8.3%) fully resorbed litters (100% resorptions) in the G/MTBE-2 study at 0, 2000, 10,000, 20,000, and 30,000 mg/m^3, respectively (Table 6). These findings did not differ statistically across groups but the slightly higher numbers of fully resorbed litters may be an indication of maternal stress in some of the pregnant animals at 20,000 and 30,000 mg/m^3.

3.7. Fetal body weight

The mean fetal weights and litter weights are presented in Table 7. There was a statistically significant difference in the mean fetal body weight among the BGVC dose groups based on a mixed model analysis of variance that considered dose group, litter size, and fetal sex as explanatory values. The mean fetal body weight of the 20,000 mg/m^3 target group and the 10,000 mg/m^3 target group were statistically significantly lower than the control fetal weight based on a pair-wise comparison using least square means. There were no statistically significant differences in the mean fetal

Table 7
Fetal body weights.

Average fetal body weight/litter (g)					
	Exposure concentration (mg/m^3)				
Study	0	2,000	10,000	20,000	30,000[a]
EMBSI[b]	Mean and least squares mean, males and females combined				
BGVC mean	1.32	1.29	1.25	1.26	N/A
Least squares mean	1.32	1.30	1.26[*]	1.23[**]	N/A
G/MTBE	Mean ± standard deviation				
Males	1.33 ± 0.10	1.33 ± 0.11	1.34 ± 0.12	1.27 ± 0.12	N/A
Females	1.29 ± 0.09	1.29 ± 0.11	1.29 ± 0.13	1.22 ± 0.11	N/A
HLS-RTI[c]	Mean ± standard deviation				
G/MTBE-2	1.00 ± 0.02	1.01 ± 0.02	0.95 ± 0.02	0.95 ± 0.03	1.02 ± 0.02

[a] Exposures conducted GD 5–7.
[b] Exposure conducted GD 5–17; weights on GD-18.
[c] All but 30,000 mg/m^3 exposures conducted at GD 5–16; weights on GD-17.
[*] $p < 0.05$.
[**] $p < 0.01$.

Table 8
Incidence of total variations and malformations in control and high dose groups.

Variations and malformations[a]	Test substances and concentration (mg/m^3)						
	BGVC[b]		G/MTBE[b]		G/MTBE-2[c]		
	0	20,000	0	20,000	0	20,000	30,000
External variations							
Fetuses	0/313	0/242	0/297	0/255	3/276	3/252	0/407
Litters	0/23	0/21	0/24	0/20	3/23	3/20	0/33
External malformations							
Fetuses	2/313	2/242	6/297	6/255	2/276	1/252	7/407
Litters	2/23	2/21	5/24	6/20	2/23	1/20	4/33
Visceral variations							
Fetuses	4/155	11/122	4/150	4/129	Not Determined		
Litters	3/23	8/21	4/24	3/20			
Visceral malformations							
Fetuses	2/155	0/122	0/150	0/129			
Litters	2/23	0/21	0/24	0/20			
Skeletal variations							
Fetuses	80/158	83/120	84/147	67/126			
Litters	21/23	20/21	21/24	17/20			
Skeletal malformations							
Fetuses	0/158	1/120	2/147	1/126			
Litters	0/23	1/21	2/24	1/20			

[a] Fetuses: number of observations per number of fetuses examined; Litters: number of litters with observations.
[b] Exposures were from gestation day 5–17.
[c] Exposures were from gestation day 5–16 for 20,000 mg/m^3 group and 5–10 for 30,000 mg/m^3 group.

body weights or litter weights between the control versus treated animals in either the G/MTBE or G/MTBE-2 studies.

3.8. Fetal external observations

The summary incidences of fetuses, for fetal variations, and for litters with malformations or structural variations are shown in Table 8. External observations were minimal in the BGVC exposed animals and there were no statistically significant differences with control treated animals for either the total numbers of litters with malformed fetuses, fetal variations, or for individual defects (Table 8). One dead fetus in the 20,000 mg/m^3 target concentration group had the following observations: open eye, spina bifida, curled tail, exencephaly, cranioschisis, and polyhydramnios (red). The observations for fetal neural tube closure defects for this fetus (exencephaly, cranioschisis, spina bifida) were combined for statistical analysis, and there were no statistical differences between groups.

In the G/MTBE study, there also were no statistically significant differences in external variations or malformations between the control and treated groups. Ectopia cordis and gastroschisis were observed in two fetuses of the same litter in the 10,000 mg/m^3 group (Table 9). Microstomia and misshapen nose were also observed in one dead fetus in the 10,000 mg/m^3 group, and micromelia and syndactyly were seen in one dead fetus in the 10,000 mg/m^3 group.

In the G/MTBE-2 study, there were no statistically significant differences across groups (Table 8). The fetal external malformations included encephalocele in one fetus at 2000 mg/m^3 and gastroschisis in one fetus at 30,000 mg/m^3. Cleft palate was observed in two fetuses (2 litters) in the control group, one fetus each in the 2000, 10,000, 20,000 mg/m^3 groups and in 7 fetuses (4 litters) in the 30,000 mg/m^3 (Table 9). This apparent (non-statistically significant) increase in the fetal (and litter) incidence of cleft palate at 30,000 mg/m^3 may be indirect evidence of maternal stress during exposures at this high concentration. Physical stressors such as restraint as well as corticosteroid stress hormones are well-established inducers of cleft palate in mice (Fraser and Fainstat, 1951; Lee et al., 2008).

3.9. Fetal visceral observations

There were no statistically significant differences in the incidence of visceral observations between the control and BGVC

treated groups (Table 8). Observations included umbilical artery arising from the left side of the urinary bladder, dilated renal pelvis, retinal fold, and misshapen cerebra (indentation in the cerebra). There were no fetuses with multiple visceral observations. There were also no statistically significant differences in the incidence of visceral observations between the control and G/MTBE treated groups. In the 10,000 mg/m^3 group, the two fetuses with ectopia cordis also had elongated pulmonary trunks with either a narrowing of the pulmonary trunk or an enlarged atrium.

3.10. Fetal skeletal observations

There were no statistically significant differences in the incidence of skeletal observations between the control and BGVC-treated groups (Table 8). Hypoplastic supraoccipital bones, hypoplastic sternebrae, rudimentary ossification site in the sternum, and ossified calcaneus were the most frequently noted skeletal observations. Ossification of the calcaneus, which was observed in the majority of the fetuses is an indication of slightly advanced development and is considered normal. The skeletal variations observed are common in mice and are considered unrelated to exposures. There were also no statistically significant differences between groups when related skeletal observations were combined for analysis.

There were no statistically significant differences in the incidence of skeletal observations between the control and G/MTBE treated groups.

4. Discussion

Maternal toxicity was observed after exposure to the baseline gasoline vapor condensates (BGVC). BGVC exposures resulted in slightly but statistically significant reduced gestational body weights and gestational body weight changes in the 20,000 mg/m^3 target group. BGVC exposure at similar concentrations did not affect gestational body weights in a companion developmental toxicity study in rats (Roberts et al., 2014); however a two-generation reproductive toxicity study in rats demonstrated decreased body weight gains in P0 and F1 males exposed to 20,000 mg/m^3 for a longer duration (Gray et al., 2014). Labored breathing was observed in animals of the 20,000 mg/m^3 and 30,000 mg/m^3 exposure group in the G/MTBE-2 study.

Statistically significant reduced fetal body weights, compared with the control fetal weights, were also noted in the 10,000 and 20,000 mg/m^3 BGVC exposure groups. This reduction in average fetal body weight was similar at both dose levels, at approximately 95% of control values. The reduction of these fetal weights occurred concurrently with decreased maternal body weight at 20,000 mg/m^3 but there were no statistically significant reductions in maternal body weight and body weight changes in the 10,000 mg/m^3 target concentration group. In the G/MTBE studies, in which BGVC comprised 80% of the test material, fetal body weights at 20,000 mg/m^3 were also 95–96% of control values but not statistically significantly lower. A 5% decrease in fetal body weight is at the margin of the statistical power to detect a decrease in a study of this size in mice. In companion developmental and two-generation reproductive toxicity studies in rats, exposure to BGVC did not affect fetal body weights (Roberts et al., 2014; Gray et al., 2014).

The number of live fetuses per litter was statistically significantly reduced in the 20,000 mg/m^3 BGVC target group. The numbers of implantation sites and ovarian corpora lutea were substantially lower in this group than in the control and the other treatment groups, but statistical analysis of the number of live fetuses normalized for the number of implantation sites and also transformed to further normalize the data revealed no statistically significant differences for these two indices. Although there was no decrease in the proportion of live fetuses per implantation sites there was a statistically significant increase in resorptions. Therefore, the reduced number of live fetuses appeared to be a function of both a reduction in the number of corpora lutea, due to the biological variation inherent in rodent ovulation, and an increase in resorptions. Resorptions in the high exposure group were influenced by total litter loss in one dam with unexplained weight loss. Exposure to BGVC at similar concentrations in rat developmental and two-generation toxicity studies did not affect the number of live fetuses per litter, estrous cyclicity, or ovarian pathology (Roberts et al., 2014; Gray et al., 2014). A range-finding study conducted with unleaded gasoline vapor exposures up to 9000 ppm (23,900 mg/m^3) reported no maternal or developmental effects in mice (n = 10/group), in the pilot test conducted with both mice and rats prior to this reported study (Roberts et al., 2001).

The BGVC 20,000 mg/m^3 target group displayed a transformed ratio of resorptions to implantations that was statistically significantly increased when compared with the control group. One animal had a strong effect on these data. The litter of this animal was completely resorbed and therefore only implantation sites were present in the uterus. This dam also lost 19% of her weight at the GD 8–11 (26% of her weight from GD 5) interval for no explainable reason. Since no other losses in maternal body weight were observed for any other dam in this group, it is unlikely that the weight loss in this dam was treatment related. However, there are no data that explains this weight loss, so this dam and her litter were not excluded from the statistical analysis of the data. Nevertheless, it is useful to note that when this litter was removed from the statistical analysis of all uterine implantation data to determine its effect on the implantation data (analysis not reported), the transformed ratio of resorptions to implantations in the 20,000 mg/m^3 target group was no longer statistically significant. In mice, weight loss during gestation has been associated with increased resorption rates (Chapin et. al., 1993).

In the first G/MTBE study there was no evidence of maternal toxicity, nor any statistically significant differences between the control and treated groups for any of the fetal parameters or observations. However, a malformation that is rarely observed in control fetuses, ectopia cordis, was observed in one dead fetus in the 2000 mg/m^3 group and in two viable fetuses from a single litter in the 10,000 mg/m^3 group. This malformation was not considered treatment related for the following reasons: there was no dose–response demonstrated (the malformation did not occur at the high dose, 20,000 mg/m^3). In addition, fatal malformations are often associated with increased incidences of early and late resorptions which may cause an observation to be overlooked, but the 20,000 mg/m^3 group had a lower incidence of early and late resorptions than the control group, and thus malformations were not likely missed due to prenatal deaths at this concentration. Finally, the two components of G/MTBE (gasoline vapor condensate and methyl t-butyl ether) have both been tested separately in mice and this malformation was not observed in either study (Benson et al., 2011; Bevan et al., 1997). In the previous MTBE study (Bevan et al., 1997), the highest dose evaluated was over-sixfold higher than the concentration of MTBE in this study. It is interesting to note that ectopia cordis was reported in mice of the same strain and supplier in a study conducted at a similar time with a chemically dissimilar ionic liquid, 1-butyl-3-methylimidazolium chloride (CAS 79917-90-1) (Rasco et al., 2008; Bailey et al., 2010), but did not occur in the researchers' earlier study at the same highest dosage level (Townsend et al., 2006). Genetic drift may occur in rodent species, particularly mice, but it is difficult to ascertain possible influence on developmental toxicity outcomes.

Table 9
Incidence of selected external malformations in the three studies.

Exposure group (mg/m^3)	Incidence (No. of fetuses/fetuses examined)				
	0	2,000	10,000	20,000	30,000
Ectopia cordis					
BGVC – fetuses	0/155	0/167	0/159	0/122	N/A
Litters	0/23	0/25	0/24	0/21	N/A
G/MTBE – fetuses	0/297	0/252	2/303	0/255	N/A
Litters	0/24	0/20	1/25	0/20	N/A
G/MTBE-2 fetuses	0/276	0/336	0/225	0/252	0/407
Litters	0/23	0/21	0/19	0/20	0/33
Gastroschisis					
BGVC – fetuses	0/155	0/167	0/159	0/122	N/A
Litters	0/23	0/25	0/24	0/21	N/A
G/MTBE – fetuses	0/297	0/252	1/303	0/255	N/A
Litters	0/24	0/20	1/25	0/20	N/A
G/MTBE-2 fetuses	0/276	0/336	0/225	0/252	1/407
Litters	0/23	0/21	0/19	0/20	1/33
Cleft palate					
BGVC – fetuses	0/155	0/167	0/159	0/122	N/A
Litters	0/23	0/25	0/24	0/21	N/A
G/MTBE – fetuses	0/297	0/252	0/303	1/255	N/A
Litters	0/24	0/20	0/25	1/20	N/A
G/MTBE-2 Fetuses	2/276	1/336	1/225	1/252	7/407
Litters	2/23	1/21	1/19	1/20	4/33
Malrotated hindpaw					
BGVC – fetuses	3/313	4/338	1/320	2/242	N/A
Litters	3/23	4/25	1/24	2/21	N/A
G/MTBE – fetuses	5/297	2/252	6/303	5/255	N/A
Litters	4/24	2/20	6/25	5/20	N/A
G/MTBE-2 fetuses	0/276	0/336	0/225	0/252	0/407
Litters	0/23	0/21	0/19	0/20	0/33

N/A – not applicable – 30,000 mg/m^3 concentration not included in study.

The repeat of the G/MTBE study (G/MTBE-2) was conducted to confirm or refute the finding of ectopia cordis observed in the first study. In that study the exposure concentrations used were extended to include a group at 30,000 mg/m^3, approximately 75% of the lower explosive limit. The 30,000 mg/m^3 dose group was exposed on GD 5–10 to encompass the time of embryonic ventral wall closure, the failure of which is likely responsible for both ectopia cordis and gastroschisis. The group size was increased to 38 plug-positive females to improve the possibility of detection of the malformation at 30,000 mg/m^3. The CD-1 mice used in the first study were from the Charles River facility in Portage, MI; those used in the repeat study were from the Charles River, Raleigh, NC facility to preclude the possibility that the fetal findings in the first study were due to a different spontaneous rate of these two fetal malformations in the mice at the Portage colony (due to founder effects, genetic drift, etc.).

The repeat study did not confirm the presence of ectopia cordis in any mouse fetus at any exposure concentration out of a total of 122 litters and 1396 fetuses. In the absence of a clear dose–response pattern to the finding in the first study, and the total absence of the finding in the repeat study, it is appropriate to conclude that the finding is unlikely to be related to maternal exposure to the test material. In support of this conclusion, ectopia cordis was not seen in a companion developmental toxicity study of BGVC in rats conducted at similar concentrations (Roberts et al., 2014) nor in a two-generation reproductive toxicity study in rats (Gray et al., 2014).

In the original G/MTBE study a single fetus was observed with gastroschisis in the 10,000 mg/m^3 dose group. In the repeat study, gastroschisis was also observed in a single fetus in the 30,000 mg/mg^3 dose group, and only in the presence of profound developmental toxicity for that fetus (very low body weight and cleft palate). Historical control data from governmental studies with the Charles River CD-1® (Swiss) mouse at RTI, with 288 litters and 3641 fetuses, show no fetuses with gastroschisis or ectopia cordis. The historical control data reports 18 fetuses in 11 control litters (in 6 studies) with cleft palate (1 to 4 litters affected/affected study) and two fetuses in two control litters (1/litter) with exencephaly in two studies, 1 litter affected/affected study. The absence of gastroschisis in any of the 3,641 control CD-1 mouse fetuses in 288 control litters lends support to the possibility that gastroschisis in this study may be treatment related, occurring in a compromised fetus at 30,000 mg/m^3 with likely concomitant maternal toxicity. No other historical control data on maternal and fetal findings in the Charles River CD-1® mouse could be found in the open literature. However, gastroschisis was also reported by the authors who conducted studies with ionic liquids and observed ectopia cordis in this mouse strain (Bailey et al., 2010) with 1-decyl-3-methylimidazaolium chloride.

Neither gastroschisis nor ectopia cordis was observed in CD-1 mouse fetuses from mothers exposed to 0, 3600, 14,390, or 28,780 mg/m^3 MTBE by whole-body inhalation (in the presence of maternal and embryofetal toxicity at 14,390 and 28,780 mg/m^3 MTBE; (Bevan et al., 1997), nor was either of these malformations observed in the BGVC study. It does not appear that exposure to either MTBE or gasoline vapor, at atmospheric concentrations <28,780 mg/m^3, causes ectopia cordis or gastroschisis in mice, and thus it is questionable and certainly not definitive whether G/MTBE induced these malformations in these studies. Maternal ataxia, hypoactivity, prostration, labored breathing, and lacrimation were observed at 14,390 and 28,780 mg/m^3 MTBE, and the resultant stress was considered most likely responsible for (or at least exacerbated) the increased incidence of fetal cleft palate at 28,780 mg/m^3.

No tenable explanation or specific causes have been identified for gastroschisis, a ventral wall defect. One hypothesis is that gastroschisis results from an intrauterine interruption of the omphalomesenteric artery (Hoyme et al., 1981). Feldkamp et al. (2007)

proposed that abnormal folding of the body wall results in a ventral body wall defect through which the gut herniates, leading to the clinical presentation of gastroschisis. She further speculated that such a mechanism could explain the origin of other developmental defects of the ventral wall. Another nonvascular explanation for the origin of gastroschisis includes failure of incorporation of the vitelline duct into the umbilical cord (Stevenson et al., 2009).

While there are numerous reports associating various drugs and over-the-counter medications with an increased risk of gastroschisis, none are definitive. Increased rates of gastroschisis among infants born to mothers who had used vasoactive medications (including salicylates, pseudoephedrine and phenylpropanolamine) have also been reported (Husain et al., 2008; Werler et al., 2009), but other evidence is contradictory (Mac Bird et al., 2009). One of the challenges for interpretation of ectopia cordis and gastroschisis is that causes for these defects in humans are not well understood.

The treatment-related increases in absolute (at 10,000 mg/m^3) and relative (at 2000, 10,000, and 20,000 mg/m^3) maternal liver weights in the G/MTBE-2 study are most likely due to the induction of hepatic metabolizing enzymes, with the concomitant increase in liver weight (Conney, 1967). This is not considered maternal toxicity, per se, but an adaptive metabolic response to exposure to a xenobiotic. The absence of an increased liver weight at 30,000 mg/m^3 might reflect the 7-day interval between the end of treatment and necropsy of animals in this group.

The incidence of fetal cleft palate in the G/MTBE study was observed in only one fetus at 20,000 mg/m^3 (that study's highest concentration). In the repeat study, cleft palate was observed in all five groups, including the air control group. An increased incidence of cleft palate observed at 30,000 mg/m^3, although not statistically significantly different from the control value, is considered to be biologically relevant. Cleft palate in fetal mice is inducible by increased corticosterone levels in the dam (and presumably transported to the fetuses) (Carmichael et al., 2007; Senda et al., 2005; Pradat et al., 2003; Hemm et al., 1977).

The presence of fetal cleft palate in all groups, (including the control group), was not unexpected. Maternal increased corticosterone levels have been attributed to the increased maternal stress from inhalation study activities such as being moved into and out of the inhalation chambers, exposure to dynamic air flows, the absence of feed and water during exposure periods, and no solid flooring in exposure cages, as well as from test materials at anesthetic concentrations. In fact, maternal inhalation of MTBE has been shown to produce cleft palates in fetuses from CD-1 mouse dams which exhibited lethargy and apparent unconsciousness (Bevan et al., 1997). Maternal lethargy during exposures was also observed by laboratory staff during the daily exposure periods of G/MTBE at 30,000 mg/m^3 in the range-finding study at HLS but was not reported in the definitive study. Therefore, the presence of fetal cleft palate in all groups (including the control group) was not unexpected, and the increased incidence at 30,000 mg/m^3 (from both inhalation procedures, per se, and the anesthetic qualities of the MTBE in the G/MTBE at this atmospheric concentration) was also anticipated.

The increased cleft palate incidence at 30,000 mg/m^3 is interpreted as exposure-related and most likely secondary to maternal stress effects. Maternal stress may have also played a role in the single case of gastroschisis in a compromised (low weight, cleft palate) fetus developing in a dam exposed to very high levels of G/MTBE. Since the maternal exposures to 30,000 mg/m^3 ended on GD 10, seven days before necropsy, it is likely that indications of maternal stress such as increased adrenal weights would have resolved during the post exposure period (GD 10–17) and were therefore not present at scheduled necropsy on GD 17.

Table 10
No observed adverse effect levels.

	No observed adverse effect levels (mg/m^3)		
	BGVC	G/MTBE	G/MTBE-2
Maternal	10,000	20,000	10,000
	Reduced body weight and body weight gain	No adverse effects	Labored breathing
Developmental	2000	20,000	20,000
	Reduced fetal weight	No adverse effects	Cleft palate

5. Conclusions

Administration of "baseline" gasoline vapor condensates to mice by whole-body inhalation exposure during the period of organogenesis and fetal growth produced maternal toxicity that was evident at the target exposure level of 20,000 mg/m^3 as reduced body weights and body weight change. The target exposure levels of 10,000 mg/m^3 and 20,000 mg/m^3 produced reduced fetal body weights. Therefore, the no observable adverse effect levels (NOAELs) for developmental and maternal toxicity in the BGVC study were considered to be the 2000 and 10,000 mg/m^3 target concentrations, respectively (Table 10). In the G/MTBE study administration of the test material produced no evidence of maternal toxicity or adverse fetal effects, so the NOAELs are considered to be 20,000 mg/m^3, the highest exposure level, for both developmental and maternal toxicity (Table 10).

In the G/MTBE-2 study, maternal effects were observed at 20,000 and 30,000 mg/m^3 and developmental effects at 30,000 mg/m^3, resulting in NOAEL values for maternal and developmental toxicity of 10,000 mg/m^3 and 20,000 mg/m^3, respectively.

In the G/MTBE-2 study, one fetus with gastroschisis occurred in the group exposed to the test chemical at 30,000 mg/m^3, an exposure level that also produced maternal toxicity during the embryonic period of ventral body wall closure. Although there were no statistically significant changes to any of the developmental data for this study, the developmental NOAEL was selected as the highest exposure group without equivocal findings.

Conflict of interest

Dr. Murray reports receiving personal fees from the American Petroleum Institute as a consultant during the conduct of the study. Drs. Clark and Schreiner report receiving personal fees from the American Petroleum Institute as consultants to assist in preparing manuscripts from the original laboratory study reports. All other authors have declared that there is no conflict of interest.

Acknowledgments

Funding for these studies was provided by the 211(b) Research Group ("Research Group"), an unincorporated group of over two hundred fuel, oxygenate, and fuel additive manufacturers affiliated by contractual obligation to meet the Tier 1 and Tier 2 testing requirements of Section 211(b)(2) and 211(e) of the Clean Air Act.

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