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Health assessment of gasoline and fuel oxygenate vapors: Micronucleus and sister chromatid exchange evaluations

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ABSTRACT

Micronucleus and sister chromatid exchange (SCE) tests were performed for vapor condensate of baseline gasoline (BGVC), or gasoline with oxygenates, methyl tert-butyl ether (G/MTBE), ethyl tert butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), t-butyl alcohol (TBA), or ethanol (G/EtOH). Sprague Dawley rats (the same 5/sex/group for both endpoints) were exposed to 0, 2000, 10,000, or 20,000 mg/m^3 of each condensate, 6 h/day, 5 days/week over 4 weeks. Positive controls (5/sex/test) were given cyclophosphamide IP, 24 h prior to sacrifice at 5 mg/kg (SCE test) and 40 mg/kg (micronucleus test). Blood was collected from the abdominal aorta for the SCE test and femurs removed for the micronucleus test. Blood cell cultures were treated with 5 μg/ml bromodeoxyuridine (BrdU) for SCE evaluation. No significant increases in micronucleated immature erythrocytes were observed for any test material. Statistically significant increases in SCE were observed in rats given BGVC alone or in female rats given G/MTBE. G/TAME induced increased SCE in both sexes at the highest dose only. Although DNA perturbation was observed for several samples, DNA damage was not expressed as increased micronuclei in bone marrow cells. Inclusion of oxygenates in gasoline did not increase the effects of gasoline alone or produce a cytogenetic hazard.

1. Introduction

The 1990 amendments to the Clean Air Act (CAA) mandated the use of oxygenates in motor gasoline, and also required manufacturers of fuels and fuel additives to provide data to the U.S. Environmental Protection Agency (EPA) regarding the potential health effects of their products. As described in more detail in a companion paper (Henley et al., 2014), requirements include inhalation exposures to evaporative emissions of the gasoline or 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). This paper describes the results of genotoxicity testing submitted to EPA. The animals evaluated for genotoxicity were exposed concurrently with animals involved in a subchronic inhalation toxicity study of the materials described above (Clark et al., 2014). Additional groups of animals were exposed to the test materials concurrently to evaluate the effects of exposure on immunotoxicity and neurotoxicity, the results of which are described elsewhere (White et al., 2014; O'Callaghan et al., 2014).

Genetic toxicity satellite studies of four week duration were incorporated with thirteen week rat inhalation studies (Clark et al., 2014) to assess the potential of seven vapor condensates of baseline gasoline (BGVC), or baseline gasoline with oxygenates, methyl tert-butyl ether (G/MTBE), ethyl tert butyl ether (G/ETBE), t-amyl methyl ether (G/TAME), diisopropyl ether (G/DIPE), t-butyl alcohol, a metabolite of MTBE and ETBE (G/TBA), or ethanol (G/EtOH) to induce *in vivo* genetic effects. These condensates represent the more easily vaporized fractions of the various gasolines and thus more accurately reproduce human exposure during vehicle fueling and other operations. The assays employed as specified in 211(b) alternative test rule (US EPA Docket, 1998a,b) were an *in vivo* bone marrow erythrocyte micronucleus test and an *in vivo/in vitro* peripheral blood sister chromatid exchange assay in the same animals.

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The bone marrow micronucleus test (Matter and Schmid, 1971; MacGregor et al., 1987) is a short term assay to identify chromosome damage and aneuploidy. Chromosome damage caused by a test substance or its metabolite can result in the formation of micronuclei containing lagging chromosome fragments or whole chromosomes which are not incorporated in the nucleus of dividing cells and remain as micronuclei in the cytoplasm of daughter cells. Increased incidence of these micronucleated immature erythrocytes is an indication of chromosome damage from recent exposure to a chromosome damaging agent.

The sister chromatid exchange test (SCE) is a short term assay for the detection of reciprocal exchanges of DNA between homologous loci of two sister chromatid strands of a duplicating chromosome (Latt et al., 1981; Perry et al., 1984). In these studies peripheral blood lymphocytes are collected from inhalation exposed rats, cultured, and labeled with bromodeoxyuridine (BrdU) over two rounds of replication in culture to differentiate the sister chromatid strands [M2 chromosome consists of one chromatid unifilarly substituted with BrdU and the other bifilarly substituted]. The chromatids of such chromosomes stain differentially with Giemsa stain to detect exchanged DNA between sister strands. The exchange involves DNA breakage and reunion and is indicative of DNA perturbation but no genetic material is lost or displaced to other sites on the chromatid.

2. Materials and methods

Sprague Dawley rats (5/sex/group) were exposed by inhalation to BGVC, or G/MTBE, G/ETBE, G/TAME, G/DIPE, G/EtOH, or G/TBA at concentrations of 0, 2000, 10,000, 20,000 mg/m^3, 6 h/day, 5 days a week for a total of 20 exposures over 28 days as subgroups of 13 week rat inhalation studies performed at Huntingdon Life Sciences (East Millstone, NJ). To reduce the number of animals employed, specimens from the same animals were used for both micronucleus and SCE endpoint evaluations but a separate positive control group was used for each study. Generation and composition of the vapor concentrations as well as additional details on the exposure methodology are reported elsewhere (Clark et al., 2014). Twenty-four hours prior to sacrifice, non-exposed positive control rats (5/sex/dose) were administered a single intraperitoneal dose of cyclophosphamide (CAS No. 6055–19-2, Sigma Chemical Co., lot #108H0568, 99.2% pure) of 40 mg/kg for the micronucleus test and 5.0 mg/kg for the SCE test. On the day after the final exposure, all animals were sacrificed by CO2 asphyxiation, peripheral blood (2–4 ml in sodium heparin tubes) was collected from the abdominal aorta for SCE culture and bone marrow collected from both femurs of each rat for the micronucleus test by personnel from BioReliance Laboratories (Rockville, MD).

2.1. Micronucleus test

Studies were performed in accordance with US EPA guidelines for the micronucleus assay 79.64 CFR vol. 59, No. 122, 27 June 1994 and Health Effects Test Guidelines OPPTS 870.5395, 1998. After sacrifice and blood collection for SCE, bone marrow was collected from both femurs of each rat, aspirated into a syringe containing 0.5 ml fetal calf serum and flushed into a centrifuge tube of serum. Cells were pelleted by centrifugation at 150g for 5 min, supernatant removed and cell pellet resuspended in remaining serum. A small drop of cell suspension was spread on a clean glass slide (4 slides/rat), air dried, fixed by dipping in methanol for 3 min and aged overnight or longer until stained. Two unstained slides/animal and the refrigerated pellet were retained in storage at Huntingdon Life Sciences (East Millstone, NJ). Two unstained slides per animal were shipped via overnight delivery to Huntingdon Life Sciences' Eye Research Center (Eye Suffolk, UK) for processing and evaluation. Upon receipt slides were stained by the modified Feulgen staining method which specifically stains DNA-containing bodies a deep purple, immature erythrocytes blue and mature erythrocytes orange by acridine orange counterstaining. Slides were air dried and mounted under cover slips with DPX mountant to produce permanent preparations. Slides were coded and examined by light microscopy to determine the incidence of micronucleated cells in 2000 immature polychromatic erythrocytes per animal. One slide/animal was examined, the other held in reserve if needed. The proportion of immature erythrocytes for each animal was assessed by examination of at least 1000 total erythrocytes (mature and immature) to determine if cytotoxicity [reflected as significant decrease in the proportion of immature erythrocytes compared to control values] had occurred. The number of micronucleated mature erythrocytes in the same 1000 or more cells was also recorded.

Non-parametric statistics were employed to compare results for each treatment group with corresponding negative controls by sex and sexes combined in each study. For incidences of micronucleated immature erythrocytes, exact one-sided p-values were calculated by permutation (CYTEL, 1995). Comparison of several dose levels was made with the control using the Linear by Linear Association test for trend, in a step-down fashion if significance was detected (Agresti et al., 1990); for individual inter-group comparisons (i.e. the positive control group) this procedure simplifies to a straightforward permutation test (Gibbons, 1985). For assessment of effects on the proportion of immature erythrocytes, equivalent permutation tests based on rank scores were used, (i.e. exact versions of Wilcoxon's sum of ranks test (Wilcoxon, 1945) and Jonckheere's test for trend, (Jonckheere, 1954; Kruskal and Wallis, 1952, 1953).

A positive response was indicated by a statistically significant dose-related increase in the incidence of micronucleated immature erythrocytes (MIE) for the treatment group compared with the negative control group ($p < 0.01$); individual and/or group means should exceed the laboratory historical control range. A negative result was indicated where individual and group mean incidences of micronucleated immature erythrocytes for the treated group were not significantly greater than the negative control group and these values fall within the historical control range for the laboratory.

2.2. Sister chromatid exchange test

Studies were performed in accordance with US EPA guidelines for the sister chromatid exchange test Health Effects Guidelines OPPTS 870.5915 (US EPA, 1998b). Blood samples for the SCE study were transported to BioReliance Laboratories (Rockville, MD) immediately after sacrifice. Within 24 h after collection whole blood cultures were prepared in duplicate per animal in culture medium containing 20 μg/ml phytohemaglutinin and incubated at 37 °C. At approximately 21 h of culture 5 μg/ml BrdU (Sigma Co., St. Louis, MO) was added followed at 68 h by addition of 0.2 μg/ml colcemid. At 72 h (51 h after BrdU introduction), the cells were collected by centrifugation, the pellet resuspended in 5 ml 0.075 M KC1 and incubated at 37 °C for 20 min. followed by addition of 0.5 ml of fixative (methanol:glacial acetic acid, 3:1 v/v) to each tube. The cells were collected by centrifugation, fixed and stored in fixative overnight or longer at approximately 24 °C. Fixed cells were then centrifuged, the supernatant was aspirated, and cells resuspended in 1 ml fresh fixative twice, centrifuged and liquid decanted leaving 0.1 to 0.3 ml fixative above the cell pellet. One or 2 drops of cell suspension were dropped on glass slides and stored overnight to air dry. -Dried slides were stained by the modified Hoechst 33258 fluorescence-plus-Giemsa technique (Perry and Wolff, 1974; Wolff and Perry,

1974) Slides were prepared from all blood cultures, but only slides from the first of each duplicate culture were evaluated. Duplicate culture slides were stored unread with the study data to be available if insufficient cells were usable on the first slide or initial results required clarification. Slides were coded using random numbers and examined under oil immersion.

A minimum of 25 second division metaphases/animal were scored for SCE. At least 100 consecutive metaphase cells were evaluated for the number of cells in first, second and third division metaphase per animal as an indicator of toxicity (cell cycle delay) and 1000 cells scored for mitotic index/animal. The average generation time (AGT) was also estimated. Regression analysis (trend analysis) and a one-tailed Dunnett's *t* test for multiple comparisons were performed to compare SCE frequency of test exposure levels to negative controls, which was considered statistically significant at $p \leqslant 0.5$.

The test substance was considered positive if an exposure-level response and statistically significant increase was observed over a minimum of two exposure levels. A statistically significant increase at the high exposure level with an exposure-level response, although not a statistically significant increase at lower exposure levels was assessed as suspect. A statistically significant increase at one or more exposure levels with no evidence of an exposure level response was assessed as equivocal or as negative according to the magnitude of response and the number of exposure levels affected.

2.3. Compliance

These studies were conducted in accordance with the United States Environmental Protection Agency's (EPA) Good Laboratory Practice Standards (US EPA, 1994), and complied with all appropriate parts of the Animal Welfare Act Regulations (USDA, 1989, 1991). The study also met the requirements of EPA's guidelines for micronucleus tests (US EPA, 1998a) and sister chromatid exchanges (US EPA, 1998b).

3. Results

The results of the genetic assays performed with baseline gasoline vapor condensate (BGVC) or vapor condensates generated from baseline gasoline blended with MTBE, TAME, ETBE, DIPE, TBA or ETOH are summarized in Table 1a (Micronucleus test), Table 1b (Micronucleus test cytotoxicity) and Table 2 (Sister Chromatid Exchange test). In all studies the cyclophosphamide positive controls produced statistically significant increases in the frequency of micronucleated immature erythrocytes or increased sister chromatid exchange events, respectively. Results for other endpoints within each assay (incidence of micronucleated mature erythrocytes or average generation time and mitotic index for sister chromatid exchange assay) were not statistically different from negative controls, and these data are not shown.

3.1. Micronucleus tests

No statistically significant increases in the frequency of micronucleated immature erythrocytes (MIE) or positive dose response trends were observed for either sex or sexes combined for rats exposed to baseline gasoline vapor condensate (BGVC), G/MTBE, G/TAME, G/DIPE or G/TBA. Although there were slight non-significant increases in MIE in males exposed to 2000 and 10,000 mg/m^3

Table 1a
Micronucleus assay results in rat bone marrow erythrocytes.

Test vapor	Micronucleated immature erythrocytes (MIE) mean ± std. dev. (frequency/2000 IE)				Positive control
	Control	2000 mg/m^3	10,000 mg/m^3	20,000 mg/m^3	CP
BGVC					
Male	0.4 ± 0.5	0.6 ± 0.4	1.0 ± 0.2	0.6 ± 0.8	18.2 ± 2.5[b]
Female	0.6 ± 0.7	0.8 ± 0.7	0.6 ± 0.5	0.6 ± 0.8	9.8 ± 3.6[b]
M & F	0.6 ± 0.7	0.7 ± 0.7	0.8 ± 0.6	0.6 ± 0.8	14.0 ± 5.5[b]
G/MTBE					
Male	1.8 ± 1.5	2.0 ± 1.9	1.2 ± 0.4	1.2 ± 0.8	19.2 ± 6.5[b]
Female	2.4 ± 2.1	0.8 ± 0.8	0.6 ± 0.5	1.8 ± 1.3	10.6 ± 3.6[b]
M & F	2.1 ± 1.7	1.4 ± 1.5	0.9 ± 0.6	1.5 ± 1.1	14.9 ± 6.7[b]
G/EtOH					
Male	0.0 ± 0.0[c]	0.0 ± 0.0[c]	0.2 ± 0.4[c]	0.6 ± 0.5[c]	14.2 ± 5.3[b]
Female	0.2 ± 0.4	0.8 ± 1.1	0.0 ± 0.0	1.0 ± 1.4	8.0 ± 3.9[b]
M & F	0.1 ± 0.3	0.4 ± 0.8	0.1 ± 0.3	0.8 ± 1.0	11.1 ± 5.5[b]
G/TAME					
Male	1.0 ± 1.7	1.6 ± 0.9	0.4 ± 0.5	0.8 ± 0.8	13.2 ± 9.7[b]
Female	0.4 ± 0.9	0.8 ± 1.3	0.4 ± 0.5	0.8 ± 0.0	14.0 ± 10.3[b]
M & F	0.7 ± 1.3	1.2 ± 1.1	0.4 ± 0.5	0.8 ± 0.8	13.6 ± 9.5[b]
G/ETBE					
Male I	1.6 ± 0.9	0.6 ± 0.5	1.6 ± 0.8	2.8 ± 0.8	25.8 ± 4.5[b]
Male II	2.2 ± 0.8	1.2 ± 0.8	2.4 ± 1.1	2.8 ± 2.5	30 ± 10.9[b]
Female I	0.8 ± 1.1	1.0 ± 0.7	3.8 ± 0.4[a]	2.0 ± 2.0	19.6 ± 8.0[b]
Female II	1.4 ± 1.1	3.2 ± 1.8	3.2 ± 1.9	2.6 ± 0.9	17.6 ± 5.1[b]
M & F I	1.2 ± 1.0[c]	0.8 ± 0.6[c]	2.7 ± 1.1[c]	2.4 ± 1.5[c]	22.7 ± 1.9[b]
M & F II	1.8 ± 1.0	2.2 ± 1.7	2.8 ± 1.5	2.7 ± 1.8	19.6 ± 8.0[b]
G/DIPE					
Male	0.8 ± 0.4	1.0 ± 1.0	0.8 ± 0.4	0.6 ± 0.5	9.8 ± 1.0[b]
Female	0.8 ± 0.8	0.6 ± 0.5	1.0 ± 0.7	0.8 ± 0.4	20.4 ± 6.8[b]
M & F	0.8 ± 0.6	0.8 ± 0.8	0.9 ± 0.6	0.7 ± 0.5	15.1 ± 7.5[b]
G/TBA					
Male	2.2 ± 0.9	0.8 ± 0.4	2.4 ± 1.5	1.4 ± 0.8	18.0 ± 3.5[b]
Female	1.0 ± 0.5	1.0 ± 0.5	0.8 ± 1.2	2.2 ± 1.2	9.4 ± 1.8[b]
M & F	1.6 ± 1.5	0.9 ± 0.6	1.6 ± 1.6	1.8 ± 1.1	13.7 ± 5.9[b]

Positive control CP–cyclophosphamide 40 mg/kg.
[a] $p < 0.01$.
[b] $p < 0.001$ permutation test.
[c] $p < 0.01$ linear by linear trend test for dose response.

Table 1b
Cytotoxicity results in rat bone marrow erythrocytes in the micronucleus assay.

Test vapor	Proportion of immature erythrocytes (% IE) in ⩾1000 total erythrocytes/rat (mean ± S.D.)				Positive control
	Control	2000 mg/m^3	10,000 mg/m^3	20,000 mg/m^3	CP 40 mg/kg
BGVC					
Male	47 ± 3.9	37 ± 9.9	42 ± 5.1	42 ± 7.7	30 ± 5.2 [b]
Female	56 ± 7.3[c]	48 ± 3.4[a]	48 ± 3.0 [a]	46 ± 8.5 [a]	23 ± 4.3 [b]
M & F	51 ± 7.7	43 ± 9.8	45 ± 5.6	44 ± 8.0	27 ± 6.2 [b]
G/MTBE					
Male	44 ± 3.2	40 ± 6.0	38 ± 5.0	38 ± 2.6	25 ± 7.2 [a]
Female	47 ± 6.0	44 ± 5.3	48 ± 2.3	49 ± 4.2	26 ± 0.8 [a]
M & F	45 ± 4.8	42 ± 5.8	43 ± 6.4	43 ± 6.8	26 ± 4.8 [b]
G/EtOH					
Male	46 ± 2.7	41 ± 5.2	43 ± 2.3	46 ± 3.1	33 ± 3.5 [a]
Female	44 ± 5.4	39 ± 6.5	44 ± 3.9	40 ± 7.3	34 ± 2.9 [a]
M & F	45 ± 4.2	40 ± 5.6	43 ± 3.1	43 ± 6.3	33 ± 3.1 [b]
G/TAME					
Male	49 ± 2.1	51 ± 2.0	49 ± 0.9	50 ± 2.4	44 ± 3.5 [b]
Female	49 ± 2.0	50 ± 4.3	51 ± 1.7	50 ± 3.7	45 ± 2.8 [a]
M & F	49 ± 1.9	51 ± 3.2	50 ± 1.7	50 ± 2.9	44 ± 3.1 [b]
G/ETBE					
Male I	45 ± 2.4	46 ± 2.3	45 ± 3.8	46 ± 1.8	37 ± 1.4 [a]
Male II	50 ± 2.7	51 ± 2.6	51 ± 4.4	48 ± 7.5	32 ± 3.8 [a]
Female I	45 ± 4.7	45 ± 2.9	45 ± 2.8	44 ± 3.2	38 ± 4.8 [a]
Female II	49 ± 4.6	44 ± 4.1	48 ± 4.2	47 ± 6.8	30 ± 6.3 [a]
M & F I	45 ± 3.6	46 ± 2.5	45 ± 3.2	45 ± 2.5	37 ± 3.4 [b]
M & F II	49 ± 3.6	48 ± 5.0	50 ± 4.4	48 ± 6.8	31 ± 5.1 [b]
G/DIPE					
Male	51 ± 3.8	53 ± 1.9	49 ± 2.0	53 ± 3.6	44 ± 2.8 [b]
Female	51 ± 2.4	51 ± 1.6	49 ± 4.2	53 ± 6.4	41 ± 2.6 [b]
M & F	51 ± 3.0	52 ± 1.9	49 ± 3.1	53 ± 4.9	43 ± 3.0 [b]
G/TBA					
Male	44 ± 3.6	45 ± 2.7	44 ± 2.2	44 ± 6.0	37 ± 3.2 [b]
Female	43 ± 4.8	47 ± 2.2	45 ± 3.3	47 ± 0.7	44 ± 2.9
M & F	43 ± 4.5	46 ± 2.9	44 ± 3.1	46 ± 4.9	41 ± 4.9

% IE = Proportion of immature erythrocytes (IE) divided by [IE + mature erythrocytes] in 1000 or more total erythrocytes. $N = 5$/sex/group. Combined $N = 10$/group.
[a] $p < 0.01$.
[b] <0.001 Permutation/Wilcoxin.
[c] BGVC female control value high for these data sets.

Table 2
Sister chromatid exchange results in rat peripheral lymphocytes.

Test vapor	SCE/cell mean ± std. dev.				Positive control
	Control	2000 mg/m^3	10,000 mg/m^3	20,000 mg/m^3	CP
BGVC					
Male	5.9 ± 1.6	6.7 ± 1.9[b]	8.1 ± 1.7[a,b]	9.2 ± 2.2[a,b]	18.3 ± 6.4[a]
Female	5.0 ± 1.4	6.7 ± 2.2[a,b]	9.0 ± 2.1[a,b]	11.4 ± 2.4[a,b]	19.7 ± 2.7[a]
G/MTBE					
Male	7.5 ± 2.3	7.7 ± 1.8[b]	8.6 ± 2.4[ab]	8.2 ± 1.9[b]	22.9 ± 4.0[a]
Female	7.1 ± 1.9	8.6 ± 1.8[a,b]	8.5 ± 2.4[a,b]	8.7 ± 1.5[b]	22.3 ± 3.6[a]
G/EtOH					
Male	6.1 ± 1.2	5.5 ± 1.1[a]	5.7 ± 1.3	5.8 ± 1.6	25.1 ± 4.2[a]
Female	6.2 ± 1.2	5.7 ± 1.5	6.1 ± 1.3	5.8 ± 1.7	22.4 ± 3.5[a]
G/TAME					
Male	6.8 ± 2.0	6.0 ± 1.7[b]	7.0 ± 1.9[b]	8.2 ± 2.1[a,b]	19.7 ± 3.1[a]
Female	6.7 ± 1.8	6.2 ± 1.4[b]	6.9 ± 1.8[b]	7.7 ± 2.3[a,b]	19.7 ± 3.0[a]
G/ETBE					
Male	5.8 ± 1.3	5.4 ± 1.4	5.7 ± 1.3	6.1 ± 1.4	21.0 ± 2.8[a]
Female	5.4 ± 1.4	5.8 ± 1.3	6.0 ± 1.5[a]	5.7 ± 1.2	19.6 ± 2.1[a]
G/DIPE					
Male	7.5 ± 1.8	7.4 ± 1.7	6.7 ± 1.4	7.2 ± 1.7	22.7 ± 4.9[a]
Female	7.1 ± 1.8	7.0 ± 1.5	6.9 ± 1.4	6.9 ± 1.5	24.3 ± 4.3[a]
G/TBA					
Male	6.6 ± 1.8	6.4 ± 1.6	6.5 ± 1.7	6.2 ± 1.8	21.9 ± 3.5[a]
Female	6.2 ± 1.6	6.4 ± 1.8	6.5 ± 1.6	6.3 ± 1.6	22.1 ± 3.4[a]

Positive control CP–cyclophosphamide 5 mg/kg.
[a] $p \leqslant 0.05$ Dunnett's t test (one tailed).
[b] $p \leqslant 0.01$ regression (trend) analysis for dose response.

BGVC which dropped back in the 20,000 mg/m^3 group, no statistically significant trend in dose response was identified.

For rats exposed to G/EtOH no statistically significant increases in frequency of MIEs or positive dose response trends were observed in female rats or sexes combined. In male rats, a statistically significant dose response was seen with the trend test but was not considered biologically relevant because individual male and male group mean values were within the historical negative control values for this laboratory [0.0–0.2 MIE (20% of test population) and 0.6–0.8 MIE (16% of test population)] and the statistical significance was enhanced by low MIE frequency in negative control males.

In the G/ETBE test, two sets of slides were evaluated for frequency of MIE in bone marrow cells of treated rats. In the first set of slides, no statistically significant increases in the frequency of MIE were observed for male rats or sexes combined. Female rats treated with 10,000 mg/m^3 G/ETBE demonstrated a statistically significant increase in MIE. Linear by linear trend analysis of combined sex data showed a statistically significant dose response trend but analysis for individual sexes did not show a significant dose response trend. To clarify the first set of results, the second set of slides that had been held in reserve was evaluated. Although some increases in group mean MIE were seen, none of the values from the second set of slides were statistically significant compared to negative controls for individual sexes or sexes combined and the trend test for dose response was not significant for individual sexes or sexes combined. The second set of data did not confirm the results of the first set. No cytotoxicity was seen; % IE in each slide set was comparable to controls and similar to each other (Table 1b).

Table 1b presents the proportion of immature erythrocytes as measure of cytotoxicity in the micronucleus test. Proportions of IE in exposed groups were generally comparable to control values demonstrating a ratio of 45–50% immature erythrocytes in relation to 1000–1200 total erythrocytes counted. The control % IE value for female animals in the BGVC study was high compared to control values for BGVC males and in the other studies resulting in a statistically significant decrease in % IE at all dose groups which was not expressed when sexes were combined. This effect was not considered relevant to treatment. The cyclophosphamide positive control groups showed cellular toxicity expressed as statistically significant lower % IE in most studies.

3.2. Sister chromatid exchange tests

Statistically significant increases in frequency of SCE were observed in groups exposed to BGVC (females at all doses, 10,000 and 20,000 mg/m^3 males), G/MTBE (females all doses, 10,000 mg/m^3 males) and G/TAME (20,000 mg/m^3 both sexes). Regression (trend) analyses for dose response were statistically significant for both sexes in these studies.

No statistically significant increases in SCE were observed for male or female rats at any dose level and regression (trend) analyses were negative for G/EtOH, G/DIPE and G/TBA exposed rats. In the G/ETBE test, a statistically significant increase in SCE frequency was observed for females in the 10,000 mg/m^3 group only and no statistically significant increase in SCE was observed for males. The regression (trend) analysis was negative for dose responses in either sex.

4. Discussion

None of the vapor condensates induced biologically significant micronucleus increases or cytogenetic damage in rat bone marrow cells. Exposure to G/ETBE produced a statistically significant dose response trend in one set of slides which was not reproducible when a second set of slides was analyzed. Further, the SCE test for DNA perturbation in the same animals was considered negative as only 10,000 mg/m^3 females showed a statistically significant increase and no dose response trend was evident. On this basis G/ETBE was not demonstrated conclusively to induce genetic damage in these test systems.

For all studies, micronucleated immature erythrocyte values were compared with the laboratory historical control data. Although at the lower end of the historical control ranges, all control and treated groups in the studies described here were considered acceptable. In reviewing the available literature, cytogenetic studies of unadditized gasoline and its blending streams did not report increases in chromosome aberrations or positive micronucleus findings in laboratory animals (US EPA, 2008). Negative results in standard cytogenetic tests for some individual oxygenates have also been reported: MTBE (Bushy Run Research Center, 1989; Kado et al., 1998; McKee et al., 1997; Ward et al., 1995; Zhou et al., 2000); TAME (Daughtrey and Bird, 1995), and ETBE (McGregor, 2007).

Three of the vapor condensates [G/EtOH, G/DIPE, G/TBA] were negative in both micronucleus and SCE tests indicating an absence of DNA perturbation or cytogenetic hazard from inhalation of these materials. The negative results for gasoline/ethanol blend [G/EtOH] is consistent with the generally negative results in chromosome and micronucleus assays for EtOH alone summarized by Phillips and Jenkinson (2001), although some drinking water studies in rodents showed small increases in SCE induction but no increased SCEs in cultured cells were reported. TBA did not cause either cytogenetic damage *in vivo* in bone marrow cells or sister chromatid exchanges in cultured Chinese hamster ovary cells (NTP, 1994).

Sister chromatid exchange test results were positive for baseline gasoline vapor condensate in both sexes, positive for G/MTBE females and equivocal for males due to a statistically significant response at 10,000 mg/m^3 and a positive dose trend, and suspect for G/TAME due to statistically significant increases in SCE at the highest dose tested and a positive trend in dose response. Positive results in the SCE assay showed that these vapor condensates were capable of inducing DNA perturbation in peripheral blood lymphocytes of treated rats. However this DNA effect was not expressed as cytogenetic damage in bone marrow erythrocytes of the same animals raising the question of whether a mutation had in fact occurred. The exact mechanism of SCE formation and its significance as a genotoxic endpoint is still unclear (Phillips and Jenkinson, 2001).

Although gasoline is the base fuel for all the vapor condensates tested, it is difficult to speculate on the reasons for the variation in SCE results. The vapor fractions resulting from some of the oxygenate blends may contain lower ratios of the chemical constituents responsible for the induction of sister chromatid exchanges, or the presence of biologically inactive constituents in these vapor fractions may block the genetic action of other constituents resulting in lower or no increases in DNA perturbation events.

Positive results in the SCE DNA perturbation assay are considered a marker of chemical exposure but in the absence of positive results in an assay measuring actual chromosome damage does not represent mutation. It is not sufficient proof to consider a vapor condensate to be clastogenic and induction of increased SCE alone does not constitute a health concern.

5. Conclusions

Overall, exposure to vapor condensates of baseline gasoline or gasoline/oxygenate blends demonstrated none or minimal cytogenetic activity. Exposures of rats to concentrations of up to 50% of

the lower explosive limits of evaporative emissions (20,000 mg/m^3) from these condensates indicate minimal genetic impact at concentrations well in excess of potential human exposure at refueling, which typically measure less than 1.0 mg/m^3 but excursions could reach 7.0 mg/m^3 for 5 min (Clayton, 1993; NATLSCO, 1995). The results from both the micronucleus and SCE tests demonstrate that the presence of oxygenates blended with gasoline do not increase genetic expression induced by gasoline evaporative emissions alone and present minimal likelihood of significant cytogenetic hazard.

Conflict of interest

Dr. Clark reports that while employed at Phillips 66 Company, he was involved in the API technical work group that designed and oversaw the conduct of the studies. The American Petroleum Institute employed him after retirement as a consultant to assist in preparing manuscripts from the original laboratory study reports. Dr. Gudi has nothing to disclose. Dr. Hoffman has nothing to disclose. Dr. Schreiner reports that while employed at Mobil Oil Corp., she was involved in the API technical workgroup that oversaw the design and conduct of the studies. The American Petroleum Institute employed her after retirement as a consultant to assist in preparing manuscripts from the original laboratory study reports. There was no influence exerted on evaluation of the scientific data and manuscript content.

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