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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,