Original Article

Lack of micronucleus induction activity of ethyl *tertiary*-butyl ether in the bone marrow of F344 rats by sub-chronic drinking-water treatment, inhalation exposure, or acute intraperitoneal injection

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ABSTRACT — Ethyl *tertiary*-butyl ether (ETBE) is an oxygenated gasoline additive synthesized from ethanol and isobutene that is used to reduce CO₂ emissions. To support the Kyoto Protocol, the production of ETBE has undergone a marked increase. Previous reports have indicated that exposure to ETBE or methyl *tertiary*-butyl ether resulted in liver and kidney tumors in rats and/or mice. These reports raise concern about the effects of human exposure being brought about by the increased use of ETBE. The present study was conducted to evaluate the genotoxicity of ETBE using micronucleus induction of polychromatic erythrocytes in the bone marrow of male and female rats treated with ETBE in the drinking-water at concentrations of 0, 1,600, 4,000 or 10,000 ppm or exposed to ETBE vapor at 0, 500, 1,500 or 5,000 ppm for 13 weeks. There were no significant increases in micronucleus induction in either the drinking water-administered or inhalation-administered groups at any concentration of ETBE; although, in both groups red blood cells and hemoglobin concentration were slightly reduced in the peripheral blood in rats administered the highest concentration of ETBE. In addition, two consecutive daily intraperitoneal injections of ETBE at doses of 0, 250, 500 or 1,000 mg/kg did not increase the frequency of micronucleated bone marrow cells in either sex; all rats receiving intraperitoneal injections of ETBE at a dose of 2,000 mg/kg died after treatment day 1. These data suggest that ETBE is not genotoxic *in vivo*.

Key words: Ethyl *tertiary*-butyl ether, ETBE, Inhalation, Drinking-water, Micronucleus, Rat, Bone marrow

INTRODUCTION

Ethyl *tertiary*-butyl ether (ETBE) is chemically synthesized from bioethanol and isobutene and when blended with gasoline ETBE acts as an oxygenate: Oxygenates are added to unleaded gasoline to enhance the octane rating and reduce exhaust emissions. To support the requirement of the Kyoto Protocol for reducing CO₂ emissions, the Petroleum Association of Japan decided to use ETBE as a gasoline additive. While the use of oxygenated motor fuels can have beneficial environmental consequences, humans can be exposed to oxygenates both

orally and by inhalation (Ahmed, 2001; McGregor, 2006, 2007), and the increased use of ETBE as a gasoline additive will increase human exposure. Exposure of the public to ETBE can be by inhalation of vapor by released from gas-stations and by drinking water contaminated with ETBE by leakage of gasoline from underground storage tanks (McGregor, 2007).

The toxicity data for ETBE is limited and inconsistent; therefore, the health hazard presented by ETBE is uncertain. Medinsky *et al.* (1999) reported that inhalation exposure to ETBE for 13 weeks caused centrilobular hepatocyte hypertrophy in male and female mice, testicular

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toxicity in rats but not in mice, male rat specific α_{2u} -globin nephropathy, and an increased incidence of bone marrow congestion in female rats. Bird *et al.* (1997) exposed F344 rats and CD-1 mice to methyl *tertiary*-butyl ether (MTBE); a widely used oxygenate developed during the Second World War that is chemically similar to ETBE) for 24 months and 18 months via inhalation, respectively, and found an increased incidence of renal tubular cell tumors in male rats, increases in the incidence and severity of chronic progressive nephropathy in male and female rats, and an increased incidence of hepatocellular adenomas in female mice. However, in a review of the health hazards of MTBE, McGregor (2006) notes that the results of the carcinogenicity studies of MTBE are generally not consistent and replication of results was observed in only three cases, none of which had human relevance. In a more recent study, Saito *et al.* (2013) reported that inhalation exposure to ETBE for 104 weeks caused a significant increase in hepatocellular adenomas in male, but not in female, rats. When administered by gavage, ETBE is reported to have carcinogenesis activity. Maltoni *et al.* (1999) administered ETBE to male and female SD rats by oral gavage for 104 weeks and found a significant dose-related increase of mouth and forestomach tumors, uterus malignant tumors and hemolymphoreticular neoplasms, and Hagiwara *et al.* (2011) using a multi-organ carcinogenesis bioassay found that ETBE promoted tumorigenesis of the thyroid, forestomach, colon, liver, kidney and urinary bladder in male rats by oral gavage for 28 weeks. In contrast, Suzuki *et al.* (2012) administered ETBE to male and female rats in the drinking water for 104 weeks and found that ETBE did not have any carcinogenic effect. Thus, the results of the carcinogenicity studies of ETBE are mixed.

Studies investigating the genotoxicity of ETBE and MTBE *in vitro* have also been reported (Table 1). The results of several *in vitro* genotoxicity studies testing the mutagenic activity of ETBE and MTBE using bacterial mutation tests and chromosomal aberration tests in cultured mammalian cells were negative (Zeiger *et al.*, 1992; Kado *et al.*, 1998; Williams-Hill *et al.*, 1999; McGregor *et al.*, 2005; Zhou *et al.*, 2000; Mackerer *et al.*, 1996; McKee *et al.*, 1997; McGregor *et al.*, 2006, 2007). However, other studies report that MTBE induced mutations in *Salmonella typhimurium* TA102 and in the mouse lymphoma L5178Y cell *tk* locus assay and induced DNA-strand breaks in human HL-60 leukemia cells (Williams-Hill *et al.*, 1999; Mackerer *et al.*, 1996; McGregor, 2006, 2007).

Although some *in vivo* genotoxic studies of ETBE have been reported, these are unpublished and unknown for

details (Table 1). MTBE was reported to be negative in bone marrow chromosomal aberration and micronucleus tests (McKee *et al.*, 1997; Kado *et al.*, 1998; McGregor, 2006, 2007), but DNA strand breakage in peripheral lymphocytes from SD rats dosed with 800 mg/kg/day of MTBE by gavage for 28 days were reported to be significantly increased (Lee *et al.*, 1998; McGregor, 2006).

To investigate the *in vivo* genotoxicity of ETBE, we performed the bone marrow micronucleus assay after administration of ETBE to F344 rats of both sexes by three different routes: Rats were exposed to ETBE vapor for 13-weeks, ETBE in the drinking-water for 13-weeks, or ETBE by intraperitoneal injections for two consecutive days. Each 13-week study was carried out as a preliminary examination for a dosage setup of a carcinogenicity test (Suzuki *et al.*, 2012; Saitou *et al.*, 2013). All experiments were carried out in accordance with standard test guidelines and good laboratory practices (GLPs).

MATERIALS AND METHODS

This study was conducted from 2006 to 2007 in accordance with the Organization for Economic and Co-operation and Development (OECD) Guidelines for Testing of Chemicals TG408, TG413, TG474 (OECD, 1998, 1981 and 1997), and in accordance with the OECD principles of GLP (OECD, 1998). The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 1996) and the present studies were approved by the ethics committee of the Japan Bioassay Research Center (JBRC).

Chemicals

The ETBE (CAS No. 637-92-3) used in the present study was manufactured by Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), with the following specifications (quoted from the SDS in Tokyo Chemical Industry Co., Ltd., 2012): appearance, colorless transparent liquid; boiling point, 70°C; vapor pressure, 17 kPa (25°C); solubility, slightly soluble in water 1.2 g/100 g, 25°C; purity, > 99% (measured by Toray Research Center Co., Ltd., Tokyo, Japan); storage condition, in the dark at room temperature. The stability of the ETBE was measured using a gas chromatograph (Hewlett Packard 5890A, Agilent Technologies Inc., Santa Clara, CA, USA) before the beginning and at the end of the test period; the data from the two time points were compared to assess the stability of the ETBE in the administration vehicle: There were no differences between the results at the beginning and the end of the test period.

Micronucleus induction activity of ethyl *tertiary*-butyl ether**Table 1.** Summary of studies of MTBE and ETBE genotoxicity

	Endpoint	Test system	Dose	Result	Reference
MTBE	Mutation <i>in vitro</i>	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA1535	7,400 µg/plate	Negative	Kado <i>et al.</i> (1998)
		<i>Salmonella typhimurium</i> TA102	750 µg/plate	Positive	Williams-Hill <i>et al.</i> (1999)
		<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537, TA102	5,000 µg/plate	Negative	McGregor <i>et al.</i> (2005)
		mouse lymphoma L5178Y cells <i>tk</i> locus	740 µg/ml	Positive	Mackerer <i>et al.</i> (1996)
	Micronucleus formation <i>in vitro</i>	mouse NIH/3T3 cells	14,000 µg/ml	Negative	Zhou <i>et al.</i> (2000)
	DNA damage <i>in vivo</i>	SD rat peripheral lymphocytes (comet assay)	800 mg/kg/day (gavage injection for 28 days)	Positive	Lee and Quitana (1998)
	Micronucleus formation <i>in vivo</i>	male and female CD-1 mouse bone marrow cells	8,000 ppm (inhalation for 6 hr/day for 2 days)	Negative	McKee <i>et al.</i> (1997)
		male and female Swiss-Webster mouse bone marrow cells	1,750 mg/kg (single ip treatment)	Negative	Kado <i>et al.</i> (1998)
		male and female F344 rat bone marrow cells	8,000 ppm (inhalation for 6 hr/day for 5days)	Negative	McKee <i>et al.</i> (1997)
ETBE	Mutation <i>in vitro</i>	<i>Salmonella typhimurium</i> TA97, TA98, TA100, TA1535	10,000 µg/plate	Negative	Zeiger <i>et al.</i> (1992)
		Chinese hamster V79 cells hprt locus	5,000 µg/ml	Negative	Bushy Run Research Center (1995)*
	Chromosomal aberrations <i>in vitro</i>	Chinese hamster ovary cells	5,000 µg/ml	Negative	Bushy Run Research Center (1995)*
	Micronucleus formation <i>in vivo</i>	male and female OF-1 mouse bone marrow cells	5,000 mg/kg (single gavage treatment)	Negative	Insitut Pasteur de Lille (1992)*
		male and female CD-1 mouse bone marrow cells	5,000 ppm (inhalation for 6hr/day for 5days)	Negative	Bushy Run Research Center (1995)*

*: Unpublished report, quoted from McGregor (2006).

Animals and husbandry

For the 13-week studies of oral administration and inhalation exposure to ETBE, F344/DuCrI_{CrIj} rats of both sexes were obtained at the age of 4 weeks for the from Charles River Japan, Inc. (Kanagawa, Japan), and for the intraperitoneal injection study, F344/DuCrI_{CrIj} rats of both sexes were obtained at the age of 6 weeks from Charles River Japan, Inc. All rats were individually quarantined and acclimated for 1 week prior to the beginning of the ETBE administration period. Animals exposed to ETBE by inhalation were individually housed in cages placed in 1060 liter stainless steel inhalation exposure chambers. The environment in the exposure cham-

bers was maintained at a temperature of 20-24°C and a relative humidity of 30-70% with 13 air changes per hour. The exposure chambers were installed in a controlled system animal room. The animals administered ETBE in the drinking water or by intraperitoneal injection were housed individually in stainless steel wire-mesh cages in a barrier controlled animal room (temperature 23 ± 2°C, relative humidity 55 ± 15%, 15-17 ventilation frequencies per hour). Fluorescent lighting was controlled automatically to give a 12-hr light/dark cycle. All rats were given 30 kGy-γ ray irradiation sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) and sterilized water *ad libitum*.

Experimental design 13-week drinking-water study

After quarantine and acclimation, the rats were divided by stratified randomization into 6 body weight-matched groups of each sex, each comprising 10 rats. Rats were given drinking water containing 0 (control), 250, 640, 1,600, 4,000 or 10,000 ppm (wt/wt) ETBE starting at 6 weeks of age for 13 weeks. The highest concentration of ETBE used was determined based on the maximum tolerated dose (MTD) obtained from a preliminary 28-day oral gavage toxicity study and the solubility of ETBE in water, which is 12,000 ppm. Drinking water containing ETBE was prepared by dissolving ETBE in deionized water in a stainless-steel container. After mixing for one hour in order to completely dissolve the ETBE in the water, the container was connected to the automatic watering system and pressurized to 0.05 MPa in order to prevent vaporization of ETBE from the drinking water (Kano *et al.*, 2002). ETBE-containing water or vehicle water was supplied to rats in individual groups and replaced with newly prepared drinking water once a week. The ETBE concentration in the drinking water at each dose level was analyzed by a headspace gas chromatograph (Hewlett Packard 5890A, Agilent Technologies Inc.) and found to be within 91% to 110% of the target concentrations.

13-week inhalation study

After quarantine and acclimation, the rats were divided by stratified randomization into 6 body weight-matched groups of each sex, each comprising 10 rats. The rats were individually housed in cages placed in six whole-body inhalation chambers.

Rats were exposed to airflow containing ETBE vapor at target concentrations of 0 (control), 50, 150, 500, 1,500 or 5,000 ppm for 6 hr/day, 5 days/week for 13 weeks. The highest concentration was determined by reference to Medinsky *et al.* (1999): increased incidences of bone marrow congestion were observed in female rats exposed to ETBE at concentrations of 1,750 or 5,000 ppm for 13 weeks. Concentrations of ETBE vapor in the inhalation chambers were maintained at constant levels by measuring ETBE concentration using a gas chromatograph (GC-14B, Shimadzu, Kyoto, Japan) at intervals of 15 min throughout the 13-week exposure period (Senoh *et al.*, 2003). The concentrations of ETBE in the 50, 150, 500, 1,500 and 5,000 ppm-exposed groups were 50 ± 0.2 , 150 ± 0.5 , 501 ± 2.2 , $1,505 \pm 6.1$ and $5,005 \pm 14.3$ ppm (Mean $\pm$ S.D.), respectively.

Intraperitoneal injection study

After quarantine and/or acclimation, the rats were

divided by stratified randomization into 6 body weight-matched groups of each sex, each comprising 5 rats. 8-week-old rats were given two consecutive daily intraperitoneal (ip) injections of ETBE in olive oil at doses of 0 (control), 250, 500, 1,000 or 2,000 mg/kg/day. In addition, as a positive control, 5 rats of each sex were given a single intraperitoneal injection of mitomycin C (MMC) at a dose of 1 mg/kg. Since ETBE is volatile chemical, ETBE is retained and absorbed more in the body by ip injection compared to oral administration. Intraperitoneal injection was considered suitable for assessing ETBE *in vivo* genotoxicity. The highest dose of ETBE was determined based on the upper limit dose in the OECD guideline (OECD, 1997), and the dose of MMC was the same as in our historical studies. 24 hr after the final injection, the animals were killed, and smear specimens were prepared from the bone marrows.

Micronucleus assay

The micronucleus assay was performed on cells collected from the femurs of rats exposed to 0, 1,600, 4,000, and 10,000 ppm ETBE in the drinking-water; rats exposed to 0, 500, 1,500, and 5,000 ppm ETBE vapor; rats injected with 0, 250, 500, and 1,000 mg/kg/day ETBE in olive oil (all animals injected with 2,000 mg/kg/day ETBE died after the first injection); and from rats injected with MMC. Bone marrow cells were flushed out of the left femur with fetal calf serum and smeared on glass slides. After drying, the slides were fixed with methanol and stained with acridine-orange (Hayashi *et al.*, 1983). All the slides were randomized and coded and examined at a magnification of $\times 400$ using a fluorescent microscope (BX51, Olympus, Tokyo, Japan). For each rat, 2,000 polychromatic erythrocytes (PCE) were scored and the number of PCE containing micronuclei (MNPCE) was recorded. The frequency of PCE to total erythrocytes (PCE and normochromatic erythrocytes) was determined by scoring 1,000 total erythrocytes and recording the number of PCE.

Clinical observations and pathological examinations

The animals were observed daily for their clinical signs and mortality. Body weight and food and water consumption per group were measured once a week for 13 weeks. All rats received complete necropsy. For hematology and blood biochemistry, blood was collected from the abdominal aorta under etherization at the end of the study period after overnight fasting. The blood samples were analyzed with an automatic blood cell analyzer (ADVIA 120, Bayer Co., Tarrytown, NY, USA) for hematology, and an automatic analyzer (Hitachi 7080, Hitachi,

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Fig. 1. Growth curves for male (A) and female (B) rats orally administered ETBE in the drinking-water, and male (C) and female (D) rats exposed to ETBE vapor for 13 weeks.

Ltd., Ibaraki, Japan) for blood biochemistry. Organs were removed, weighed, and examined for macroscopic lesions at necropsy. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and tissue sections 5 μ m in thickness were prepared and stained with hematoxylin and eosin (H & E).

Statistical analysis

Two-sided tests were used for all statistical analyses. Body weight, food consumption, hematological blood, biochemical parameters and organ weight were analyzed by Dunnett's test. Pathological examinations were analyzed by the chi-square test. The frequencies of micronucleated polychromatic erythrocytes (MNPCE) were analyzed by the method of Kastenbaum and Bowman (Kastenbaum and Bowman, 1970). The frequency of PCE to E was analyzed by the Wilcoxon rank-sum test ($P < 0.05$). Statistical significance at $p < 0.05$ and $p < 0.01$

are indicated in the tables.

RESULTS

13-week drinking-water study

The average daily ETBE-intake throughout the treatment period in the 250, 640, 1,600, 4,000 and 10,000 ppm groups were 17 (12-27), 40 (30-62), 101 (77-149), 259 (208-354) and 626 (484-804) mg/kg/day (values in parenthesis indicate the range of drinking water consumption) for males and 27 (22-33), 58 (46-71), 120 (98-166), 267 (222-393) and 629 (497-835) mg/kg/day for females. Neither mortality nor clinical signs were found in any of the groups. There was a slight, but statistically significant, decrease in the body weight of males, but not females, administered 10,000 ppm ETBE compared with the control throughout the treatment period (Fig. 1). Average body weights at the end of the treatment period in

rats administered 250, 640, 1,600, 4,000 and 10,000 ppm ETBE were 100%, 100%, 99%, 98% and 92% of the control for males and 102%, 102%, 104%, 101% and 97% of the control for females. Food consumption by males administered 10,000 ppm ETBE was suppressed at week 1 and there was a tendency to decrease water consumption dose-dependently in both sexes (data not shown), however, there were no significant differences in food or water consumption during the administration period in either sex.

The micronucleus assay was performed on cells collected from the femurs of the rats administered 0, 1,600, 4,000, and 10,000 ppm ETBE. The percentages of PCE with micronuclei to total PCE (MNPCE (%)) and PCE to total erythrocytes (PCE (%)) in males and females are shown in Table 2. The MNPCE (%) and PCE (%) in the controls ($0.16 \pm 0.08\%$ and $24.2 \pm 3.5\%$ in males and $0.10 \pm 0.07\%$ and $23.7 \pm 3.8\%$ in females) were within the background data of F344 rats in our facility. No significant differences in the MNPCE (%) or PCE (%) were observed in any of the treated groups of either sex compared to the control groups.

There was a slight, but significant, decrease in the red blood cell count and hemoglobin concentration in females administered 640, 4,000 and 10,000 ppm ETBE (Table 3). There was also a slight, but significant, decrease in hematocrit in females administered 640 and 10,000 ppm ETBE. No other significant changes in hematological parameters were observed in any of the treatment groups of either sex.

Analysis of blood biochemistry revealed a significant and dose-dependent increase of urea nitrogen in males administered 640 ppm and higher levels of ETBE; there was no significant change in urea nitrogen in females (data not shown). No other significant changes in blood biochemistry were observed in any of the treatment groups of either sex.

Histopathological examination revealed significantly increased incidences of regeneration and granular casts in the proximal tubules in the kidney in males given 1,600 ppm or higher levels of ETBE. There was also a significant enhancement of the degree of hyaline droplet accumulation in the cytoplasm of proximal tubular epithelium of the kidney in males receiving 10,000 ppm ETBE. These renal lesions were not observed in any of the female groups. Significant increases in kidney weights were observed in males given 1,600 ppm or higher levels of ETBE and in females given 640 ppm or higher levels of ETBE (data not shown).

13-week inhalation study

Neither mortality nor clinical signs were found in any of the groups. There was a slight, but statistically significant, decrease in the body weight of males, but not females, exposed to 5,000 ppm ETBE compared with the control throughout the treatment period (Fig. 1). Average body weights at the end of the treatment in rats exposed to 50, 150, 500, 1,500 and 5,000 ppm ETBE were 94%, 95%, 95%, 97%, and 89% of the control for males and 95%, 97%, 96%, 94%, and 95%, of the control for females. Food consumption by males exposed to 5,000 ppm ETBE was suppressed early during the exposure period (data not shown).

The micronucleus assay was performed on cells collected from the femurs of the rats exposed to 0, 500, 1,500, and 5,000 ppm ETBE. The MNPCE (%) and PCE (%) in males and females is shown in Table 4. The MNPCE (%) and PCE (%) in the controls ($0.14 \pm 0.06\%$ and $23.3 \pm 2.6\%$ in males and $0.16 \pm 0.13\%$ and $24.2 \pm 2.6\%$ in females) were within the background data of F344 rats in our facility. No significant differences in the MNPCE (%) or PCE (%) were observed in any of the treated groups of either sex compared to the control groups.

There was a slight, but significant, decrease in the red blood cell count and hemoglobin concentration in males exposed to 5,000 ppm ETBE (Table 5). No other significant changes in hematological parameters were observed in any of the treatment groups of either sex.

Analysis of blood biochemistry revealed significant changes in total protein, parameters related to lipids, and urea nitrogen in the 5,000 ppm-exposed males (data not shown). No other significant changes in blood biochemistry were observed in any of the treatment groups of either sex.

Histopathological examination revealed significantly increased incidences of regeneration in the proximal tubules in the kidney in the 5,000 ppm-exposed males (data not shown). Significant increases in kidney weights were found in both sexes exposed to 1,500 ppm and above levels of ETBE, and significant increases in liver weights were found in both sexes exposed to 5,000 ppm ETBE.

Intraperitoneal injection study

All animals given 2,000 mg/kg/day ETBE died after treatment day 1. The micronucleus data are shown in Table 6. No significant differences in the MNPCE (%) and PCE (%) were observed in any of the treated groups of either sex compared to the control groups. The MNPCE (%) in the positive control group, the MMC-treated group, was markedly increased in both males ($1.45 \pm 0.33\%$) and females ($1.04 \pm 0.10\%$) compared to the vehicle control

Micronucleus induction activity of ethyl *tertiary*-butyl ether**Table 2.** Results of micronucleus induction in the bone marrow of rats orally administrated ETBE in the drinking-water for 13 weeks

Dose	Number of animal	PCE/E (%)		MNPCE/PCE (%)	
Male	10	24.2 ± 3.5 ^a	(18.1 ^b - 29.4 ^c)	0.16 ± 0.08 ^d	(0.05 ^e - 0.30 ^f)
0 ppm					
1,600 ppm	10	26.3 ± 5.4	(17.3 - 35.3)	0.17 ± 0.12	(0 - 0.35)
4,000 ppm	10	24.7 ± 4.9	(17.7 - 32.3)	0.14 ± 0.06	(0.05 - 0.25)
10,000 ppm	10	23.8 ± 5.4	(16.5 - 31.4)	0.19 ± 0.15	(0 - 0.45)
Female	10	23.7 ± 3.8	(18.1 - 29.4)	0.10 ± 0.07	(0.05 - 0.25)
0 ppm					
1,600 ppm	10	24.7 ± 3.4	(21.1 - 30.3)	0.16 ± 0.09	(0.05 - 0.30)
4,000 ppm	10	24.8 ± 2.6	(20.5 - 29.8)	0.09 ± 0.06	(0 - 0.20)
10,000 ppm	10	23.6 ± 4.2	(15.1 - 29.3)	0.13 ± 0.07	(0.05 - 0.25)

^a) Average ± S.D. of PCE/E^b) Minimum value of PCE/E^c) Maximum value of PCE/E^d) Average ± S.D. of MNPCE/PCE^e) Minimum value of MNPCE/PCE^f) Maximum value of MNPCE/PCE**Table 3.** Results of hematology in rats orally administrated ETBE in the drinking-water for 13 weeks

Dose	Number of animal	RBC (10 ⁶ /μl)	Hb (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	Platelet counts (10 ³ /μl)	Reticulocyte (%)
Male	10	9.35 ± 0.15	16.3 ± 0.3	43.5 ± 0.8	46.5 ± 0.4	17.5 ± 0.3	644 ± 50	1.9 ± 0.2
0 ppm								
250 ppm	10	9.30 ± 0.14	16.4 ± 0.3	43.6 ± 0.6	46.9 ± 0.3	17.6 ± 0.2	673 ± 47	2.0 ± 0.2
640 ppm	10	9.29 ± 0.12	16.3 ± 0.2	43.3 ± 0.5	46.6 ± 0.4	17.6 ± 0.1	672 ± 40	1.9 ± 0.2
1,600 ppm	10	9.28 ± 0.16	16.4 ± 0.2	43.4 ± 0.5	46.8 ± 0.5	17.6 ± 0.2	677 ± 67	1.9 ± 0.2
4,000 ppm	10	9.24 ± 0.19	16.3 ± 0.2	43.2 ± 0.8	46.8 ± 0.5	17.6 ± 0.3	637 ± 45	1.8 ± 0.2
10,000 ppm	10	9.28 ± 0.15	16.4 ± 0.3	43.3 ± 0.9	46.7 ± 1.1	17.6 ± 0.4	627 ± 79	1.6 ± 0.4
Female	10	8.77 ± 0.15	16.5 ± 0.3	42.8 ± 0.8	48.8 ± 0.6	18.8 ± 0.2	721 ± 59	1.7 ± 0.2
0 ppm								
250 ppm	10	8.64 ± 0.14	16.3 ± 0.3	42.4 ± 0.6	49.1 ± 0.4	18.8 ± 0.1	702 ± 61	1.8 ± 0.2
640 ppm	10	8.46 ± 0.28**	16.0 ± 0.5**	41.5 ± 1.1**	49.1 ± 0.5	18.9 ± 0.2	725 ± 31	1.8 ± 0.3
1,600 ppm	10	8.58 ± 0.15	16.2 ± 0.2	42.2 ± 0.8	49.2 ± 0.4	18.9 ± 0.2	702 ± 41	1.8 ± 0.3
4,000 ppm	10	8.54 ± 0.15*	16.1 ± 0.2*	42.0 ± 0.8	49.2 ± 0.4	18.9 ± 0.1	698 ± 34	1.8 ± 0.2
10,000 ppm	10	8.48 ± 0.24**	15.9 ± 0.4**	41.5 ± 1.1**	49.0 ± 0.3	18.8 ± 0.2	674 ± 54	1.8 ± 0.2

Values are average ± S.D.

RBC: Red blood cell counts, Hb: Hemoglobin concentration, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin.

* and **: Statistical difference from control group at $p < 0.05$ and $p < 0.01$ (Dunnnett's test).

group. The MNPCE (%) and PCE (%) in the vehicle controls ($0.13 \pm 0.08\%$ and $26.4 \pm 3.0\%$ in males and $0.11 \pm 0.07\%$ and $23.4 \pm 5.4\%$ in females) were within the background data of F344 rats in our facility.

DISCUSSION

In the present study, administration of ETBE in the drinking water for 13 weeks resulted in significantly decreased red blood cell counts and hemoglobin concentration in female rats administered 640, 4,000 and 10,000 ppm ETBE. Similarly, exposure to ETBE vapor for

Table 4. Results of micronucleus induction in the bone marrow of rats exposed to ETBE vapor for 13 weeks

Dose	Number of animal	PCE/E(%)		MNPCE/PCE(%)	
Male	10	23.3 ± 2.6 ^a	(18.4 ^b - 26.9 ^c)	0.14 ± 0.06 ^d	(0.05 ^e - 0.20 ^f)
0 ppm					
500 ppm	10	24.4 ± 3.2	(19.4 - 28.9)	0.16 ± 0.07	(0.05 - 0.25)
1,500 ppm	10	23.5 ± 4.3	(17.5 - 30.2)	0.17 ± 0.07	(0.05 - 0.30)
5,000 ppm	10	23.5 ± 5.4	(16.4 - 33.3)	0.18 ± 0.10	(0.05 - 0.40)
Female	10	24.2 ± 2.6	(19.1 - 27.7)	0.16 ± 0.13	(0.05 - 0.40)
0 ppm					
500 ppm	10	23.4 ± 2.4	(19.7 - 27.1)	0.18 ± 0.08	(0.10 - 0.35)
1,500 ppm	10	25.1 ± 5.4	(14.5 - 34.4)	0.16 ± 0.09	(0 - 0.30)
5,000 ppm	10	25.0 ± 2.4	(22.4 - 29.7)	0.16 ± 0.08	(0 - 0.25)

^a) Average ± S.D. of PCE/E^b) Minimum value of PCE/E^c) Maximum value of PCE/E^d) Average ± S.D. of MNPCE/PCE^e) Minimum value of MNPCE/PCE^f) Maximum value of MNPCE/PCE**Table 5.** Results of hematology in rats exposed to ETBE vapor for 13 weeks

Dose	Number of animal	RBC (10 ⁶ /μl)	Hb (g/dl)	Hematocrit (%)	MCV (fl)	MCH (pg)	Platelet counts (10 ³ /μl)	Reticulocyte (%)
Male	10	9.34 ± 0.22	16.2 ± 0.4	42.7 ± 1.0	45.7 ± 0.4	17.3 ± 0.1	663 ± 36	1.8 ± 0.2
0 ppm								
50 ppm	10	9.31 ± 0.19	16.2 ± 0.3	42.6 ± 0.9	45.8 ± 0.5	17.4 ± 0.2	676 ± 32	1.7 ± 0.2
150 ppm	10	9.40 ± 0.24	16.3 ± 0.4	43.0 ± 1.1	45.8 ± 0.4	17.4 ± 0.3	671 ± 33	1.5 ± 0.1
500 ppm	10	9.41 ± 0.32	16.3 ± 0.3	43.2 ± 1.3	45.9 ± 0.5	17.3 ± 0.4	664 ± 25	1.6 ± 0.1
1,500 ppm	10	9.31 ± 0.20	16.1 ± 0.2	42.7 ± 0.7	45.9 ± 0.5	17.3 ± 0.3	681 ± 34	1.6 ± 0.2
5,000 ppm	10	9.06 ± 0.15*	15.8 ± 0.2*	41.8 ± 0.5	46.2 ± 0.5	17.4 ± 0.3	719 ± 32	1.6 ± 0.2
Female	10	8.76 ± 0.12	16.3 ± 0.2	42.3 ± 0.5	48.4 ± 0.4	18.6 ± 0.2	696 ± 60	1.5 ± 0.2
0 ppm								
50 ppm	10	8.74 ± 0.19	16.3 ± 0.4	42.3 ± 0.9	48.4 ± 0.4	18.7 ± 0.2	694 ± 30	1.6 ± 0.2
150 ppm	10	8.72 ± 0.23	16.3 ± 0.4	42.2 ± 1.1	48.4 ± 0.3	18.6 ± 0.2	705 ± 40	1.7 ± 0.2
500 ppm	10	8.67 ± 0.20	16.2 ± 0.3	42.1 ± 0.9	48.6 ± 0.4	18.7 ± 0.2	700 ± 31	1.6 ± 0.2
1,500 ppm	10	8.65 ± 0.20	16.1 ± 0.3	41.8 ± 1.1	48.3 ± 0.3	18.6 ± 0.1	703 ± 42	1.6 ± 0.2
5,000 ppm	10	8.72 ± 0.21	16.1 ± 0.3	42.0 ± 0.8	48.2 ± 0.4	18.5 ± 0.2	739 ± 51	1.6 ± 0.3

Values are average ± S.D.

RBC: Red blood cell counts, Hb: Hemoglobin concentration, MCV: Mean corpuscular volume, MCH: Mean corpuscular hemoglobin.

* and **: Statistical difference from control group at $p < 0.05$ and $p < 0.01$ (Dunnett's test).

13 weeks significantly decreased red blood cell counts and hemoglobin concentration in male rats exposed to 5,000 ppm ETBE. These results indicate that effective levels of ETBE and/or its metabolites were delivered to the bone marrow, and therefore test animals were exposed

to levels of ETBE sufficient to evaluate its genotoxicity in bone marrow cells. ETBE did not increase the frequency of micronucleated polychromatic erythrocytes in the bone marrow in any of the ETBE-exposed groups of either sex.

Micronucleus induction activity of ethyl *tertiary*-butyl ether**Table 6.** Results of micronucleus induction in the bone marrow of rats intraperitoneally injected with ETBE on two consecutive days

Dose	Number of animal	PCE/E (%)		MNPCE/PCE (%)	
Male	5	26.4 ± 3.0a	(22.8 ^b - 30.6 ^c)	0.13 ± 0.08d	(0.05 ^e - 0.20 ^f)
0 mg/kg/day					
250 mg/kg/day	5	26.5 ± 4.0	(22.1 - 32.8)	0.12 ± 0.06	(0.05 - 0.20)
500 mg/kg/day	5	27.7 ± 3.4	(22.7 - 31.5)	0.12 ± 0.04	(0.05 - 0.15)
1,000 mg/kg/day	5	24.5 ± 5.4	(18.6 - 33.9)	0.18 ± 0.04	(0.15 - 0.25)
2,000 mg/kg/day	5 ^g				
MMC 1 mg/kg	5	24.3 ± 3.2	(18.8 - 26.7)	1.45 ± 0.33**	(0.95 - 1.80)
Female	5	23.4 ± 5.4	(14.4 - 27.1)	0.11 ± 0.07	(0.05 - 0.20)
0 mg/kg/day					
250 mg/kg/day	5	26.2 ± 4.7	(21.1 - 32.1)	0.18 ± 0.06	(0.10 - 0.25)
500 mg/kg/day	5	26.6 ± 4.2	(21.5 - 33.2)	0.12 ± 0.08	(0.05 - 0.20)
1,000 mg/kg/day	5	29.5 ± 5.2	(25.3 - 38.1)	0.12 ± 0.04	(0.10 - 0.20)
2,000 mg/kg/day	5 ^g				
MMC 1 mg/kg	5	23.6 ± 6.6	(16.6 - 34.4)	1.04 ± 0.10**	(0.95 - 1.15)

^a) Average ± S.D. of PCE/E^b) Minimum value of PCE/E^c) Maximum value of PCE/E^d) Average ± S.D. of MNPCE/PCE^e) Minimum value of MNPCE/PCE^f) Maximum value of MNPCE/PCE^g) All animals given 2,000 mg/kg/day ETBE died after treatment day 1.**: Statistical difference from control group $p < 0.01$ (Kastenbaum and Bowman, 1970).

Administration of 10,000 ppm ETBE in the drinking water resulted in an average daily ETBE-intake of approximately 626 mg/kg/day in males and 629 mg/kg/day in females. Exposure to 5,000 ppm ETBE vapor resulted in an average ETBE-intake of approximately 640 mg/kg/day, assuming a respiratory level of 254 ml/min/kg (Mauderly, 1982) with a respiratory uptake of 32-34% for ETBE (Nihlen *et al.*, 1998). In addition to these exposure levels, rats were acutely exposed to ETBE levels of 250, 500, 1,000, and 2,000 mg/kg by intraperitoneal injection of ETBE once a day for two consecutive days. Injection of ETBE at doses of 250, 500 and 1,000 mg/kg for two consecutive days did not increase the frequency of micronucleated polychromatic erythrocytes in the bone marrow of rats of either sex: injection of 2,000 mg/kg resulted in the death of all injected animals after treatment day 1. Therefore, exposure to ETBE at a level of approximately 600 mg/kg administered in the drinking water for 13 weeks or exposure to ETBE at a level of approximately 600 mg/kg administered via inhalation for 13 weeks or acute exposure to ETBE at a level of 1,000 mg/kg administered via intraperitoneal injection for 2 days had no genotoxic activity in the micronucleus assay. Although the positive control group was not set up

in the drinking water and inhalation studies for 13 weeks, it is considered that the appropriate evaluation of the micronucleus studies was performed because the positive control group was set to acute ip injection study and the MNPCE (%) and PCE (%) in the controls were within the background data of F344 rats in our facility.

The genotoxicity of ETBE has been reviewed by McGregor (2007): ETBE is negative in the *Salmonella typhimurium* mutation assay, the CHO cell chromosome aberration assay, and the V79 cell *hprt* locus assay. ETBE is also negative when assessed by micronucleus induction in the bone marrow of mice treated with a single gavage administration of ETBE at doses up to 5,000 mg/kg or mice administered ETBE via inhalation for 6 hr/day for 5-days at concentrations up to 5,000 ppm. Likewise, MTBE, a structural analog of ETBE, is negative in the *Salmonella typhimurium* mutation assay, the CHO cell chromosome aberration assay, the V79 cell *hprt* locus assay, induction of unscheduled DNA synthesis in primary cultured rat hepatocytes, and micronucleus induction in NIH/3T3 cells (McGregor, 2006). MTBE is also negative in inducing sex-linked recessive lethal mutations in *Drosophila melanogaster*, induction of unscheduled DNA synthesis induction in hepatocytes and micro-

nucleus induction in bone marrow cells of mice treated with a single gavage administration of MTBE at a dose of 1,750 mg/kg or mice administered 8,000 ppm MTBE via inhalation for 6 hr/day for 2 days, and chromosomal aberration in the bone marrow cells of rats administered 8,000 ppm MTBE via inhalation for 6 hr/day for 5 days (McGregor, 2006). In contrast, Mackerer *et al.* (1996) reported that MTBE was genotoxic in mouse lymphoma L5178Y *tk* assay with a rat liver-induced metabolic activation system. In this system, the results were negative in the presence of formaldehyde dehydrogenase. Therefore, the genotoxic activity was concluded to be from the formaldehyde generated by metabolism of MTBE (Mackerer *et al.*, 1996; McGregor, 2006). There are also a few reports that MTBE was positive in the reverse mutation assay using *Salmonella typhimurium* TA102 and induction of DNA strand breakage in human HL-60 leukemia cells (Williams-Hill *et al.*, 1999; McGregor, 2006). Although McGregor re-assessed the ETBE and MTBE literature and concluded that these two compounds were not genotoxic, the genotoxicity of ETBE was evaluated with unpublished data and is unknown for details (McGregor, 2006, 2007).

Metabolism of ETBE is similar in rats and humans (Miller *et al.*, 1997; Amberg *et al.*, 2000; McGregor, 2006, 2007). After inhalation or ingestion, ETBE is promptly absorbed into the blood and rapidly distributed throughout the body. ETBE is metabolized to *tertiary*-butyl alcohol (TBA) and acetaldehyde in the liver and TBA is further oxidized into 2-methyl-1,2-propanol and 2-hydroxyisobutyric acid and these metabolites are excreted into urine (Bernauer *et al.*, 1998; Amberg *et al.*, 1999, 2000). Of these ETBE metabolites, acetaldehyde is a genotoxic in rodents, inducing gene mutation, sister chromatid exchanges, chromosomal aberrations and micronuclei in cultured mammalian cells and sister chromatid exchange in bone marrow cells of mice and hamsters administered acetaldehyde by intraperitoneal injection (IARC, 1999, Obe *et al.*, 1979, Korte *et al.*, 1981); acetaldehyde is classified as a Group 2B carcinogen by IARC (IARC, 1999). Aldehydes are ordinarily detoxified by acetaldehyde dehydrogenase (ALDH) in the liver and converted into acetate (Gemma *et al.*, 2006). The other metabolites — TBA, 2-methyl-1,2-propandiol, and 2-hydroxyisobutyric acid — are not genotoxic (McGregor, 2006). Recently, Weng *et al.* (2011, 2012) found ETBE induced DNA strand breaks in leukocytes of *Aldh2* knockout C57BL/6 mice exposed to 500, 1,750 and 5,000 ppm ETBE vapor for 13 weeks, and DNA strand brakes, modified oxidative DNA strand breaks with hOGG1, and 8-hydroxydeoxyguanosin in hepatocytes of

knockout mice exposed to 5,000 ppm ETBE vapor. Therefore, in the absence of ALDH, exposure to ETBE results in a genotoxic response. These results are especially relevant for people with low levels of ALDH activity.

ETBE is toxic, but the results of the carcinogenicity studies on ETBE are not consistent. Medinsky *et al.* (1999) exposed F344 rats and CD-1 mice to 0, 500, 1,750 or 5,000 ppm ETBE vapor for 6 hr/day, 5 days/week for 13 weeks. They found treatment-dependent bone marrow congestion, but without changes in the hematopoietic cell population, in female rats exposed to 1,750 and 5,000 ppm ETBE vapor; testicular seminiferous tubules with spermatocyte degeneration in male rats exposed to 1,750 and 5,000 ppm ETBE vapor; male rat specific increases in the incidence of regenerative foci, rates of renal cell proliferation, and α 2u-globulin containing protein droplets in the kidneys of all treated male rats; and increases in the incidence of centrilobular hepatocyte hypertrophy and rates of hepatocyte cell proliferation in the liver of male and female mice exposed to 5,000 ppm ETBE vapor. Bird *et al.* (1997) exposed F344 rats and CD-1 mice to inhalation of MTBE, which is chemically related to ETBE, for 24 months (rats) and 18 months (mice) and found that MTBE, like ETBE, targeted the male rat kidney and the mouse liver: They found an increase in the incidence and severity of chronic progressive nephropathy and increased incidence of renal tubular cell tumors in male rats and an increased incidence of hepatocellular adenomas in female mice. Recently, Saito *et al.* (2013) reported significant increases in the incidence of hepatocellular adenomas in male rats exposed to 5,000 ppm ETBE vapor for 2 years. When administered by gavage ETBE also has carcinogenesis activity. Maltoni *et al.* (1999) administered ETBE to Sprague-Dawley rats by gavage at doses of 0, 250, or 1,000 mg/kg/day, 4 days/week for 104 weeks and observed the rats until natural death. They reported that ETBE caused an increase in total malignant tumors, oncological lesions of the mouth epithelium and forestomach, malignant tumors in the uterus, and hemolymphoreticular neoplasias. However, they also noted that only two doses of ETBE were used and a dose-response relationship between the ETBE concentration and tumor formation was not found. In addition, McGregor (2007) pointed out other limitations of the study: survival rates at 96 weeks were relatively poor, there was insufficient histopathological analysis (no information was provided on pre-neoplastic lesions or time of emergence of the tumor types), there was no treatment-related induction of forestomach dysplasias, and there was no evidence that ETBE induced lymphoid neoplasms. Hagiwara *et al.* (2011) investigated the promoting potential of ETBE on

tumor development in a medium-term multi-organ carcinogenesis bioassay using rats. They administered 5 carcinogens with different target sites to male F344 rats for 4 weeks to initiate multi-organ carcinogenesis. ETBE was then administered by gavage at daily dose levels of 0, 300 or 1,000 mg/kg until experimental week 28. They found that ETBE had tumor promoting potential for the thyroid, forestomach, colon, liver, kidney and urinary bladder. In contrast with these reports, Suzuki *et al.* (2012) reported that administration of ETBE in the drinking-water at doses up to 10,000 ppm for 104 weeks did not have any carcinogenic effect in any organ.

It is notable that the results of the carcinogenicity studies on ETBE noted above appear to correlate with detoxification in the liver. The detoxification of MTBE in hepatocytes prepared from F344 rats reaches saturation at high concentration (Casanova and Heck, 1997), thus detoxification of ETBE is also presumed to reach saturation similarly. A far larger bolus of ETBE is delivered to the liver in the animals treated by gavage compared to animals administered ETBE in the drinking water. Consequently, it is likely that less ETBE is detoxified in gavage treated animals than in drinking water treated animals, and ETBE administered by gavage appears to have carcinogenesis activity (Maltoni *et al.*, 1999; Hagiwara *et al.*, 2011; Suzuki *et al.*, 2012). In inhalation exposed animals, ETBE enters the systemic circulation and is not delivered to the liver and is not detoxified, and ETBE administered via inhalation is carcinogenic (Saito *et al.*, 2013).

In conclusion, the results of present study indicate that ETBE administered in the drinking water or via inhalation caused changes relevant to anemia such as a decrease in red blood cell count and hemoglobin concentration, however, ETBE did not increase the frequency of micronucleated polychromatic erythrocytes in the bone marrow of exposed rats. Intraperitoneal injection of sub-lethal doses of ETBE also did not increase the frequencies of micronucleated polychromatic erythrocytes in the bone marrow of injected rats. Our results are in agreement with the negative results of *in vitro* mutation tests. Consequently, our results indicate that ETBE has no genotoxic effects on somatic cells *in vivo*. Therefore, the tumorigenic activities of ETBE administered via inhalation are suggested due to non-genotoxic mechanisms.

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