INTRODUCTION

Ethyl tertiary-butyl ether (ETBE) is chemically synthesized from bioethanol and isobutane. To support the Kyoto Protocol for reducing CO₂ emissions, the Petroleum Association of Japan has agreed to use ETBE as a gasoline blending component. ETBE is added to gasoline as an oxygenate. Oxygenates are used in unleaded gasoline as octane enhancers and to decrease exhaust emissions, particularly of carbon monoxide, unburned hydrocarbons, polycyclic aromatics, oxides of nitrogen and particulate carbon. Therefore, the use of oxygenated motor fuels can have beneficial environmental consequences. On the other hand, humans can be exposed to oxygenates by inhalation while fueling automobiles and also orally when drinking contaminated water (Ahmed, 2001; McGregor, 2006, 2007). Contamination of drinking water with Methyl tertiary-butyl ether (MTBE), the most widely used oxygenate, occurs due to leaks from underground and above ground petroleum storage tank systems and pipelines. In addition, air deposition through precipitation of industrial or vehicular emission may also contribute to surface and ground water contamination (U.S. EPA, 1997). There is the same concern with regard to ETBE. At present, there are no reports of contamination of surface or ground water by air deposition of ETBE.

The genotoxicity of ETBE has been examined by several test systems including gene mutation tests using Salmonella typhimurium and Chinese hamster ovary (CHO) cells, chromosomal aberration tests using CHO cells, and in vivo micronucleus tests using bone marrow cells of mice orally treated with ETBE and of mice exposed to ETBE by inhalation. None of the results from those studies suggested that ETBE is genotoxic (ACGIH, 2001; McGregor, 2007).

There is one long-term carcinogenicity study of ETBE. Maltoni et al. (1999) administered ETBE to Sprague-Dawley rats by stomach tube at doses of 0, 625, 2,500 or 10,000 ppm (w/w) 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 stomach, malignant tumors in the uterus, and haemolymphoreticular neoplasias. However, as not-

ABSTRACT — The carcinogenicity of ethyl tertiary-butyl ether (ETBE) was examined by oral administration using F344/DuCrlCrj rats. Groups of 50 male and 50 female rats were given drinking water containing ETBE at doses of 0, 625, 2,500 or 10,000 ppm (w/w) for 104 weeks. No significant increase in the incidence of tumors was detected in any organ of either sex. Rat-specific non-neoplastic lesions were observed in the kidney: An increase in the severity of chronic progressive nephropathy was observed in the male and female 10,000 ppm groups, and increased incidences of urothelial hyperplasia of the pelvis and mineral deposition in the renal papilla were observed in the male 2,500 and 10,000 ppm groups. Besides these lesions, no treatment-related histopathological changes were observed in any organ or tissue in either sex. Thus, the present study demonstrated that a two year administration ETBE in the drinking water did not exert any carcinogenic effects in either male or female rats.

Key words: Ethyl tertiary-butyl ether, Carcinogenicity, Oral administration, Drinking water, Rat

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ed by the authors of the study, 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 several other aspects of the study by Maltoni et al. (1999) which preclude drawing definite conclusions as to the carcinogenicity of ETBE: survival rates at 96 weeks were relatively poor, no information was provided on time of emergence of the tumor types, there was no mention of pre-neoplastic lesions, there was no treatment-related induction of forestomach dysplasias, and there was no evidence that ETBE induced lymphoid neoplasms.

A study of the carcinogenicity of MTBE, a chemical related to ETBE, administered by gavage to Sprague-Dawley rats showed increases in the incidence of leukaemias and lymphomas in females and increase in the testicular interstitial Leydig cell tumors in males (Belpoggi et al., 1995). However, the National Research Council (Washington D.C.) was unable to validate these findings (U.S. EPA, 1997).

Therefore, in order to examine the carcinogenicity of ETBE in rats, we performed a 2-year drinking water study of ETBE using F344 rats of both sexes in accordance with standard test guidelines and good laboratory practices (GLPs).

**MATERIALS AND METHODS**

This study was conducted with reference to the Organisation for Economic Co-operation and Development (OECD) Guideline for Testing of Chemicals 451; “Carcinogenicity Studies” (OECD, 1981), 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).

**Test material**

The ETBE used in the present study was manufactured by Nippon Refine Co., Ltd. (Gifu, Japan) and had the following properties: appearance, colorless transparent liquid; boiling point, 70°C; vapor pressure, 17 kPa (25°C); solubility, slightly soluble in water (1.2 g/100 g, 20°C); lot no., L-506251; purity, > 99% (measured by Toray Research Center Co., Ltd., Tokyo, Japan). The ETBE was stored at room temperature in the dark.

A single batch of ETBE was used in this study, and its stability was determined by analysis using gas chromatography (GC) (Agilent Technologies 5890A, Santa Clara, CA, USA) before the beginning and at the end of the administration period and comparing the data from the two time points. There were no differences between the results obtained at these two time points, indicating that the test substance was stable throughout the 104-week period.

**Animals and husbandry**

F344/DuCrI Crj rats of both sexes were obtained at the age of 4 weeks from Charles River Laboratories Japan, Inc. (Katagawa, Japan). The animals were quarantined and acclimated for 2 weeks, and then divided by stratified randomization into 4 body weight-matched groups, each composed of 50 rats of each sex. The animals were housed individually in stainless steel wire-mesh cages (170W × 294D × 176H mm) in a barrier system animal room (temperature 23 ± 2°C, relative humidity 55 ± 15%, 15-17 room air changes/hr). Fluorescent lighting was controlled automatically to give a 12-h light/dark cycle. All rats were given 30 kGy-γ ray irradiation sterilized commercial pellet diet (CRF-1, Oriental Yeast Co., Ltd., Tokyo, Japan) ad libitum. The body weight measured immediately before the first dosing with ETBE or control water was 133 ± 5 g (mean ± S.D.) for male and 103 ± 4 g for female rats.

**A preliminary study**

In a preliminary study, both sexes of F344/DuCrI Crj rats were given drinking water containing 0 (control), 250, 640, 1,600, 4,000 or 10,000 ppm (wt/wt) ETBE for 13 weeks. No mortality was found in any of the groups. Significant inhibition of body weight gain was found in males administered 10,000 ppm ETBE. Average body weights at the end of the treatment in the male groups administered 250, 640, 1,600, 4,000 and 10,000 ppm ETBE were 100, 100, 99, 98 and 92% of the control and those in the female groups were 102, 102, 104, 101 and 97% of the control. A significant suppression of food consumption was noted during the early phase of the administration period in males administered 10,000 ppm ETBE. Average body weights at the end of the treatment in the male groups administered 250, 640, 1,600, 4,000 and 10,000 ppm ETBE were 100, 100, 99, 98 and 92% of the control and those in the female groups were 102, 102, 104, 101 and 97% of the control. A significant suppression of food consumption was noted during the early phase of the administration period in males administered 10,000 ppm ETBE and sporadically during the administration period in females administered 10,000 ppm. A significant lowering of water consumption was noted in both sexes of all treated groups. Significant, but very slight, decreases of red blood cell counts and hemoglobin concentrations were found in females administered 640, 4,000 and 10,000 ppm ETBE. Analysis of blood biochemistry revealed a significant increase of urea nitrogen in males given 640 ppm or higher levels of ETBE; no significant changes in the blood biochemistry of females were noted. 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. Histopathological examination revealed significantly increased incidences of regeneration of proximal renal tubules and granular casts in the kidney in males given 1,600 ppm or higher levels of ETBE, and significant enhancement of the degree of hyaline droplet accumulation in the proximal epithelium of the kidney was noted in males receiving 10,000 ppm ETBE. These renal lesions were not observed in any of the female groups.

Overall, 10,000 ppm ETBE caused (i) a significant inhibition of body weight gain in males, (ii) a significant increase in kidney weight in both sexes, and (iii) histopathological lesions in males. Given these results plus the fact that 10,000 ppm ETBE is close to the maximum technically feasible concentration in a drinking water study (the solubility of ETBE in water is 1.2 g/100 g [12,000 ppm]), 10,000 ppm was determined to be the maximum tolerated dose (MTD) for ETBE and was selected as the highest dose for the present 2-year study. Based on the fact that while slight changes in hematology, blood biochemistry, urinalyses, and kidney weight were noted at 640 ppm ETBE, treatment-related changes were not found in body weight or histopathological examination of rats given this dose of ETBE, the lowest dose for the 2-year study was set at 625 ppm. 2,500 ppm was chosen as the middle dose with a proportional factor of 4.

**Experimental groups and preparation of drinking water containing ETBE**

Groups of 50 rats of both sexes were given drinking water containing 0 (control), 625, 2,500 or 10,000 ppm (wt/wt) ETBE for 104 weeks. Drinking water containing ETBE was prepared by dissolving ETBE in UV-irradiated, deionized and then filtered tap water in a stainless-steel container. After mixing vigorously for one hour in order to completely dissolve 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 replaced with newly prepared drinking water once a week. The ETBE concentrations in the drinking water at each dose level at preparation were analyzed by GC with a headspace sampler (HS) (Agilent Technologies 7694, Santa Clara, CA, USA) and were found to be 90.1% to 110% of the target concentrations.

The stability of ETBE in the drinking water was determined during the preliminary 13-week study. Drinking water containing ETBE (250 and 10,000 ppm) was stored at room temperature for 8 days and then analyzed by a HS/GC. This analysis confirmed that ETBE in the formulated water was stable.

**Water consumption and ETBE intake**

Water consumption was measured for each group once a week for the first 14 weeks and every 4 weeks thereafter. ETBE intake (as mg/kg body weight/day) was calculated from the target ETBE concentration, water consumption, and body weights.

**Clinical observations and pathological examinations**

Animals were observed daily for clinical signs and mortality. Body weight and food consumption were measured once a week for the first 14 weeks and every 4 weeks thereafter. Urinary parameters were measured in the last week of the 2-year study period with Multisticks (Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA). All rats received a complete necropsy. Surviving animals were weighed after overnight fasting and prior to the terminal necropsy. For hematology and blood biochemistry, blood was collected from the abdominal aorta of animals under deep ether anesthesia at the terminal necropsy. The blood samples were analyzed with an automatic blood cell analyzer (ADVIA 120, Siemens Healthcare Diagnostics, Inc., Tarrytown, NY, USA) for hematology and an automatic analyzer (Hitachi 7080, Hitachi, Ltd., Ibaraki, Japan) for blood biochemistry. Organs were removed, weighed, and examined for macroscopic lesions at necropsy. Organs and tissues designated in the OECD test guidelines (OECD, 1981) were examined for histopathology in all rats. The tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and tissue sections of 5 μm in thickness were prepared and stained with hematoxylin and eosin (H & E).

**A peer review by the Pathology Working Group**

The kidneys were histopathologically reviewed by the Pathology Working Group (PWG) in the USA. The PWG consisted of 5 members: All PWG members were senior pathologists with extensive experience in chemically-induced nephrotoxicity and neoplasia in laboratory animals. The PWG microscopically examined kidney sections in the male control and 10,000 ppm groups, and in all female groups. Histopathological findings by the PWG were in close agreement with our observations.

**Statistical analysis**

Incidence of neoplastic lesions were analyzed for a dose-response relationship by Peto's test (Peto, 1980) and for a significant difference from the control group.
by Fisher’s exact test. Incidences of pre- and non-neo-
plastic lesions and urinary parameters were analyzed by
the chi-square test as a parameter with the severity. The
average severity grade was calculated using the follow-
ing equation. Σ (grade × number of animals with grade) /
number of affected animals. Body weight, food consump-
tion, hematological and blood biochemical parameters,
and organ weight were analyzed by Dunnett’s test. A two-
tailed test was used for all statistics except for Peto’s test.
In all cases, statistical significance at $p < 0.05$ and $p <
0.01$ are indicated in the tables.

RESULTS

Survival, general condition, body weight and
food consumption

There was no significant difference in the survival rate
between any ETBE-treated group of either sex and their
respective controls. At the end of the treatment period,
the survival rates of the control, 625, 2,500 and 10,000
ppm groups were 76, 74, 68 and 68% for males and 72,
74, 76 and 76% for females. There were no treatment
related changes in clinical signs in any of the groups.
Body weights of males administered 2,500 and 10,000
ppm ETBE and females administered 10,000 ppm ETBE
were significantly lower than their controls throughout
the treatment period. Body weights of males administered
625 ppm ETBE were significantly lower than the control
from 26 to 98 weeks, and females administered 625 and
2,500 ppm ETBE were significantly lower than their con-
trols from 38 and 14 weeks to the end of the treatment
period. Final body weights of the 625, 2,500 and 10,000
ppm groups were 96, 93 and 91% of the control for males
and 90, 89 and 83% of the control for females (Fig. 1,
Table 1). There was decreased food consumption by all
treated groups of both sexes in most weeks during the
study period. Average food consumption throughout the
treatment period in the 625, 2,500 and 10,000 ppm groups
was 97, 97 and 96% of the control for males and 95, 97
and 95% of the control for females.

Water consumption and ETBE intake

Water consumption by males administered 625 and
2,500 ppm ETBE and by all female groups was lower
than their respective control groups throughout the treat-
ment period. Water consumption by males administered
10,000 ppm ETBE was lower during the early phase of
the treatment period (before week 22). Average water
consumption throughout the treatment period in the 625,
2,500 and 10,000 ppm groups was 81, 85 and 94% of the
control for males and 77, 73 and 58% of the control for
females.

Average daily ETBE-intake throughout the treatment
period in the 625, 2,500 and 10,000 ppm groups was 28,
121 and 542 mg/kg body weight for males and 46, 171
and 560 mg/kg body weight for females (Table 1).

![Fig. 1. Growth curves for male and female rats treated with ETBE in the drinking water for 2 years.](image-url)
There was a decrease in mean cell volume (MCV) of the female 625 and 10,000 ppm groups (data not shown). No other significant changes in hematological parameters were observed in any of the treatment groups of either sex.

Significant elevation of urea nitrogen was observed in the male 2,500 and 10,000 ppm groups. Significantly increased values for total cholesterol, triglyceride, phospholipid and inorganic phosphorus and decreased values for total protein and albumin were found in the male 10,000 ppm group (data not shown). No treatment-related changes in blood biochemistry were observed in any of the female groups.

A lowering of urine pH was observed in the male 10,000 ppm group, and an increase in animals positive for occult blood was observed in the female 10,000 ppm group (data not shown). No other significant changes in urinalysis were observed in any of the treatment groups of either sex.

Organ weight
In males, absolute kidney weight in the 10,000 ppm group was significantly increased, and the relative kidney weights were significantly increased in the 2,500 and 10,000 ppm groups. In females, absolute kidney weights were significantly increased in the 2,500 and 10,000 ppm groups, and the relative kidney weights were significantly increased in all of the treatment groups (Table 1).

**Table 1.** Chemical intake, terminal body weight and organ weight of male and female rats treated with ETBE in the drinking water for 2 years

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>0 (Control)</th>
<th>625</th>
<th>2500</th>
<th>10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>No. of surviving animals (rate %)</td>
<td>38 (76)</td>
<td>37 (74)</td>
<td>34 (68)</td>
<td>34 (68)</td>
</tr>
<tr>
<td>ETBE intake (mg/kg bw/day)</td>
<td>-</td>
<td>28</td>
<td>121</td>
<td>542</td>
</tr>
<tr>
<td>Terminal body weight (g)</td>
<td>400 ± 44</td>
<td>382 ± 30</td>
<td>372 ± 26**</td>
<td>363 ± 47**</td>
</tr>
<tr>
<td>Organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>2.82 ± 0.26</td>
<td>2.72 ± 0.21</td>
<td>2.96 ± 0.35</td>
<td>3.33 ± 0.93**</td>
</tr>
<tr>
<td>Kidneys (%) b</td>
<td>0.77 ± 0.15</td>
<td>0.77 ± 0.10</td>
<td>0.86 ± 0.15**</td>
<td>1.01 ± 0.34**</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of animals examined</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>No. of surviving animals (rate %)</td>
<td>36 (72)</td>
<td>37 (74)</td>
<td>38 (76)</td>
<td>38 (76)</td>
</tr>
<tr>
<td>ETBE intake (mg/kg bw/day)</td>
<td>-</td>
<td>46</td>
<td>171</td>
<td>560</td>
</tr>
<tr>
<td>Terminal body weight (g)</td>
<td>290 ± 31</td>
<td>261 ± 19**</td>
<td>259 ± 28**</td>
<td>241 ± 28**</td>
</tr>
<tr>
<td>Organ weight</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidneys (g)</td>
<td>1.81 ± 0.12</td>
<td>1.86 ± 0.14</td>
<td>1.99 ± 0.19**</td>
<td>2.06 ± 0.26**</td>
</tr>
<tr>
<td>Kidneys (%) b</td>
<td>0.68 ± 0.08</td>
<td>0.77 ± 0.06**</td>
<td>0.83 ± 0.10**</td>
<td>0.93 ± 0.20**</td>
</tr>
</tbody>
</table>

* Mean ± S.D. of the body weight of the surviving animals to the end of the 2-year administration period.

b Relative organ weight was calculated using the following equation: absolute organ weight / fasted body weight × 100.

**: Significantly different from control at $p < 0.01$ by Dunnett’s test.

**Hematology, blood biochemistry and urinalysis**
There was a decrease in mean cell volume (MCV) of the female 625 and 10,000 ppm groups (data not shown). No other significant changes in hematological parameters were observed in any of the treatment groups of either sex.

Significant elevation of urea nitrogen was observed in the male 2,500 and 10,000 ppm groups. Significantly increased values for total cholesterol, triglyceride, phospholipid and inorganic phosphorus and decreased values for total protein and albumin were found in the male 10,000 ppm group (data not shown). No treatment-related changes in blood biochemistry were observed in any of the female groups.

A lowering of urine pH was observed in the male 10,000 ppm group, and an increase in animals positive for occult blood was observed in the female 10,000 ppm group (data not shown). No other significant changes in urinalysis were observed in any of the treatment groups of either sex.

**Organ weight**
In males, absolute kidney weight in the 10,000 ppm group was significantly increased, and the relative kidney weights were significantly increased in the 2,500 and 10,000 ppm groups. In females, absolute kidney weights were significantly increased in the 2,500 and 10,000 ppm groups, and the relative kidney weights were significantly increased in all of the treatment groups (Table 1).

**Histopathology**

**Neoplastic lesions**
No significant increases in the incidences of neoplastic lesions were found in any of the treatment groups of either sex (Table 2). Significantly decreased incidences of adenomas of the pituitary gland in the male 625 and 2,500 ppm groups and C-cell adenomas of thyroid gland in the male 625 ppm group were detected by Fisher’s exact test. However, decreased incidences of these lesions cannot be attributed to ETBE-treatment because the changes were not dose-related.
Significant increases in treatment-related non-neoplastic lesions were found in the kidney (Table 2). A significant increase in the degree of chronic progressive nephropathy (CPN) was observed in both the male and female 10,000 ppm groups; CPN was evaluated for severity using the report of Kawai (1980).

In addition, significantly increased incidences of urothelial hyperplasia of the pelvis and mineral deposition in the renal papilla were found in the kidney of the

Table 2. Incidences of neoplastic and non-neoplastic lesions in male and female rats treated with ETBE in the drinking water for 2 years

<table>
<thead>
<tr>
<th>Dose (ppm)</th>
<th>Male 0</th>
<th>Male 625</th>
<th>Male 2500</th>
<th>Male 10000</th>
<th>Female 0</th>
<th>Female 625</th>
<th>Female 2500</th>
<th>Female 10000</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**<Neoplastic lesions>**

Subcutis
- Fibroma
  - Male: 6, 2, 2, 2, 0, 0, 0, 0
  - Female: 6, 7, 4, 2, 1, 0, 1, 0

Pituitary
- Adenoma
  - Male: 25, 13*, 15*, 16
  - Female: 15, 14, 13, 15

Thyroid
- C-cell adenoma
  - Male: 13, 5*, 11, 9
  - Female: 8, 8, 6, 13

Adrenal
- Pheochromocytoma
  - Male: 11, 6, 11, 10
  - Female: 1, 0, 3, 2

Testis
- Interstitial cell tumor
  - Male: 29, 32, 37, 34
  - Female: - , - , - , -

Uterus
- Endometrial stromal polyp
  - Male: - , - , - , -
  - Female: 6, 9, 3, 7

Mammary gland
- Fibroadenoma
  - Male: 1, 1, 0, 0
  - Female: 6, 4, 12, 7

Spleen
- Mononuclear cell leukemia
  - Male: 3, 4, 7, 7
  - Female: 6, 7, 9, 2

**<Non-neoplastic lesions>**

Kidney
- Chronic progressive nephropathy
  - Male: 49, 43, 45, 48*, 41, 37, 37, 39*
    - Grade: (2.1), (2.0), (2.0), (2.4), (1.2), (1.2), (1.5), (1.5)
  - Female: 0, 0, 16**, 42**, 0, 0, 1, 3
    - Grade: (0), (0), (1.0), (1.4), (0), (0), (1.0), (1.0)

- Mineralization: papilla
  - Male: 0, 0, 16**, 42**, 0, 0, 1, 3
    - Grade: (0), (0), (1.0), (1.4), (0), (0), (1.0), (1.0)
  - Female: 0, 0, 10**, 25**, 0, 0, 0, 0
    - Grade: (0), (0), (1.0), (1.0), (0), (0), (0), (0)

- Urothelial hyperplasia: pelvis
  - Male: 0, 0, 10**, 25**, 0, 0, 0, 0
    - Grade: (0), (0), (1.0), (1.0), (0), (0), (0), (0)
  - Female: 0, 0, 10**, 25**, 0, 0, 0, 0
    - Grade: (0), (0), (1.0), (1.0), (0), (0), (0), (0)

The values in parentheses indicate the average severity grade index of the lesion. The average severity grade was calculated using the following equation: Σ (grade × number of animals with grade) / number of affected animals.

Grade: 1 = slight, 2 = moderate, 3 = marked, 4 = severe.

* : Significantly different from control at \( p < 0.05 \) by Fisher’s exact test.
** : Significantly different at \( p < 0.05 \) and \( p < 0.01 \) by Chi-square test.

Non-neoplastic lesions

Significant increases in treatment-related non-neoplastic lesions were found in the kidney (Table 2). A significant increase in the degree of chronic progressive nephropathy (CPN) was observed in both the male and female 10,000 ppm groups; CPN was evaluated for severity using the report of Kawai (1980).
male 2,500 and 10,000 ppm groups. Urothelial hyperplasia of the renal pelvis was characterized by multilayer thickening of the papillary epithelium with protrusion into the pelvis lumen; it was of low grade and limited to small areas of the renal papilla (Table 2). Mineral deposition featured linear basophilic material in Henle’s loop in the papilla.

No other renal lesions and no treatment-related histopathological changes in other organs or tissues were observed in any of the treatment groups of either sex.

**DISCUSSION**

The results of the present study showed no increases in either neoplastic or pre-neoplastic lesions of any organs or tissues in the rats administered up to 10,000 ppm ETBE in the drinking water for 104 weeks. The highest dose of ETBE used in this study, 10,000 ppm, was selected based on the results of a preliminary 13-week drinking water study. 10,000 ppm ETBE in the drinking water resulted in a significant inhibition of body weight, 91% and 83% of the control values for males and females, respectively, at the end of the treatment period. Histopathological changes in the kidney were also observed in both males and females administered 10,000 ppm ETBE. However, survival rates of the male and female 10,000 ppm groups were comparable to the control groups, and no significant increases in the incidences of neoplastic lesions were found in any of the rats administered ETBE. These results confirm that the MTD of ETBE administered in the drinking water of rats is approximately 10,000 ppm.

Hagiwara et al. (2011) investigated the modifying 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 (control), 300 or 1,000 mg/kg body weight until experimental week 28. They found that ETBE had tumor promoting potential for the thyroid and forestomach at dose levels of 300 and 1,000 mg/kg/day and for the colon, liver, kidney and urinary bladder at 1,000 mg/kg/day. In the present study, the average daily ETBE-intake at 10,000 ppm was 542 mg/kg body weight for males, but there was no increase in neoplastic lesions. Further studies are required to determine the specific mechanisms by which ETBE promotes, but does not initiate, carcinogenesis.

In contrast to the lack of induction of neoplastic lesions, administration of ETBE in the drinking water did result in an increase of rat-specific non-neoplastic lesions. An increase in the severity of CPN was observed in both male and female rats administered 10,000 ppm ETBE. ETBE is metabolized to tertiary-butyralcohol (TBA) and acetaldehyde by human liver microsomal enzymes, and the metabolism of ETBE in rats pathways is similar to that in man (McGregor, 2007). TBA in the drinking water of F344 rats enhances the severity of CPN in both male and female rats (NTP, 1995). However, CPN is a rodent specific disease commonly encountered in aging laboratory rats and has no apparent similar human kidney disease condition; therefore, CPN is regarded as having no relevance for human risk assessment (Barthold, 1979; Hard and Khan, 2004; Seely and Hard, 2008).

The increased incidences of urothelial hyperplasia of the pelvis and mineral deposition in the renal papilla observed in the present study have been reported to be associated with hyaline droplet nephropathy resulting from excessive accumulation of α2u-globulin in the cytoplasm of renal tubular epithelium (Alden and Frith, 1991). In the 13-week preliminary study preceding the current 2-year study, a significant enhancement of the degree of hyaline droplet accumulation in the renal tubular epithelium was noted in the kidneys of male rats administered 10,000 ppm ETBE in their drinking water. In another study, an increased incidence of α2u-globulin containing protein droplets was reported in male F344 rats exposed to ETBE vapor by inhalation for 13 weeks (Medinsky et al., 1999). These results suggest that the urothelial hyperplasia of the pelvis and the mineral deposition in the renal papilla observed in male rats administered ETBE in the present study resulted from accumulation of α2u-globulin in the renal tubular epithelium. Induction of α2u-globulin nephropathy in male rats has been reported for many chemicals including d-Limonene and unleaded gasoline (Alden and Frith, 1991). Because there is no evidence that the nephropathy induced by α2u-globulin accumulation occurs in humans, the U.S. EPA proposes that nephropathy in male rats associated with the induction of α2u-globulin accumulation in hyaline droplets is not an appropriate endpoint to determine renal toxic effects potentially occurring in humans (U.S. EPA, 1991).

No other treatment-related histopathological changes were observed in any other organs or tissues of the ETBE-treated rats of either sex. Finally, although some statistically significant changes were found in hematology, blood biochemistry and urinalysis, these effects were minor, did not result from histological changes or appear to negatively impact on the health of the rats administered ETBE, and were likely due to rat-specific kidney lesions. Therefore, the major finding of the present study is that exposure to ETBE resulted in rat-specific non-neoplastic
lesions in the kidney.

In conclusion, the two year administration ETBE by drinking water did not exert any carcinogenic effects in either male or female rats.

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