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Original Article

A 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol in F344 rats

Takeshi Toyoda[1]*, Kohei Matsushita[1], Hirotoshi Akane[1], Tomomi Morikawa[1], and Kumiko Ogawa[1]

[1] Division of Pathology, National Institute of Health Sciences, 3-25-26 Tonomachi, Kawasaki-ku, Kawasaki 210-9501, Japan

Abstract: 2-(*l*-Menthoxy)ethanol has been frequently employed as a flavoring agent; however, data regarding 2-(*l*-menthoxy)ethanol toxicity remain limited. We performed a 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol in male and female F344 rats, with doses of 0, 15, 60, or 250 mg/kg body weight (BW)/day orally administered by gavage using corn oil as the vehicle. No significant toxicological changes in general condition, body weight, or food intake were observed in any groups. The hematological assessment showed decreased hemoglobin, hematocrit, mean corpuscular volume, and mean corpuscular hemoglobin and increased platelet count in the male 250 mg/kg group. Serum biochemistry revealed elevated total cholesterol in the 250 mg/kg group of male and female rats, reduced triglyceride in the female 250 mg/kg group, and increased total protein in the male 250 mg/kg group, indicating effects on lipid metabolism and protein synthesis. For organ weights, absolute and relative weights of the liver and adrenal glands were increased in the 250 mg/kg group of both sexes and the male 250 mg/kg group, respectively. Histopathological analysis showed chronic nephropathy in the male 15 mg/kg or higher groups, with increased absolute and relative kidney weights, as well as elevated serum creatinine, in the male 60 and 250 mg/kg groups. However, eosinophilic granules containing α_{2u}-globulin were identified in proximal tubules, suggesting α_{2u}-globulin nephropathy specific to male rats and without toxicological significance. These results indicated that the no-observed-adverse-effect level of 2-(*l*-menthoxy)ethanol was 60 mg/kg BW/day for both sexes. (DOI: 10.1293/tox.2020-0091; J Toxicol Pathol 2021; 34: 309–317)

Key words : 2-(*l*-menthoxy)ethanol, 2-*l*-menthoxyethanol, food additive, flavoring agent, Fischer rat, menthol

Introduction

2-(*l*-Menthoxy)ethanol (CAS No. 38618-23-4) is a clear and colorless liquid with a characteristic odor and is widely used as a flavoring agent. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) has evaluated 2-(*l*-menthoxy)ethanol as a flavoring agent, categorized as substances structurally related to menthol[1]. Based on the structural class of flavoring agents, 2-(*l*-menthoxy)ethanol was assigned to Class I. In the United States and Europe, the intake levels of 2-(*l*-menthoxy)ethanol by humans were estimated to be 12 μg/person/day and 0.01 μg/person/day, respectively, both below the Class I threshold of 1,800 μg/person/day. Based on these estimates, it is considered that 2-(*l*-menthoxy)ethanol may not present safety concerns during routine use as a flavoring agent.

As part of the risk assessment, it has been revealed that 2-(*l*-menthoxy)ethanol was not mutagenic in the Ames test, and the only *in vivo* study performed to date was an oral acute toxicity study in male and female rats (LD_{50}: >2,000 mg/kg body weight [BW])[1]. Despite the use of 2-(*l*-menthoxy)ethanol as a flavoring agent, limited data are available regarding the repeated-dose toxicity of this compound. Our laboratory has investigated the toxicity of several food additives, including representative flavoring agents from each category, in rodent models[2–7]. To clarify the toxicological profile and establish a no-observed-adverse-effect level (NOAEL), we conducted a 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol, which was orally administered to F344 rats by gavage.

Materials and Methods

Test chemical

2-(*l*-Menthoxy)ethanol (Lot No. 5I0002, purity 99.9%) produced by Takasago Int. Corp. (Tokyo, Japan) was provided by the Division of Standards and Evaluation, Department of Food Safety, Ministry of Health, Labour and Welfare, Japan, with support of the Japan Flavor & Fragrance Materials Association (Tokyo, Japan). Although the test substance was provided as a mixture of optical isomers (CAS No. 38618-23-4), it was confirmed as the *l*-enantiomer (CAS No. 75443-64-0) by the manufacturer. Oral gavage with corn oil

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*Corresponding author: T Toyoda (e-mail: t-toyoda@nihs.go.jp)

(Wako Pure Chemical Industries, Osaka, Japan) as the vehicle was selected as the route of administration owing to the aqueous insolubility and volatility of 2-(*l*-menthoxy)ethanol. Solutions of 2-(*l*-menthoxy)ethanol were prepared daily, immediately before administration. After storage for 2 h at room temperature, the residual ratios of 2-(*l*-menthoxy) ethanol in 4, 40, and 400 mg/mL solution were confirmed as 96.8%, 99.8%, and 96.5%, respectively, by gas chromatography (Japan Inspection Association of Food and Food Industry Environment, Tokyo, Japan).

Experimental animals

In total, 40 male and 40 female specific pathogen-free rats (F344/DuCrlCrlj, 5-week-old) were purchased from Charles River Laboratories Japan (Yokohama, Japan) and used after acclimation for one week. During the study, animals were housed in polycarbonate cages with soft chip bedding, maintained in a room with a barrier system to control the light/dark cycle (12 h), ventilation (air-exchange rate of 18 times/h), temperature (24 ± 1°C), and relative humidity (55 ± 5%). The cages and chip bedding were replaced twice a week. Each animal had free access to tap water and a basal diet (CRF-1; Oriental Yeast, Tokyo, Japan). At the beginning of the experiment, the animals were randomly allocated to four groups of 10 male and female rats each, based on body weights measured immediately before starting treatment with the test chemical.

Study design

In a preliminary 28-day study assessing 2-(*l*-menthoxy) ethanol administered at doses of 0, 200, 400, and 800 mg/kg BW/day, absolute and relative liver weights were significantly increased in all treated groups of both sexes (data not shown). Based on these results, we selected 2-(*l*-menthoxy) ethanol doses of 0, 15, 60, and 250 mg/kg BW/day for administration to both male and female rats in the present 13-week toxicity study.

General conditions and mortality were assessed daily. Body weights, as well as the amounts of supplied and residual diet, were measured once weekly during the experimental period. All rats were overnight fasted on completing treatment, and blood samples for hematology and serum biochemistry were collected from the abdominal aorta under deep inhalation anesthesia using isoflurane. The study design was in accordance with the Guidelines for Designation for Food Additives and Revision of Standards for Use of Food Additives of Japan (1996) and approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan.

Hematology and serum biochemistry

The following hematological parameters were analyzed using ProCyte Dx automatic hematology analyzers (IDEXX Laboratories, Westbrook, ME, USA): white blood cell count (WBC), red blood cell count (RBC), hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and differential leukocyte cells, including neutrophils (Neut), lymphocytes (Lymph), monocytes (Mono), eosinophils (Eosino), and basophils (Baso). Serum biochemical analysis was performed by Oriental Yeast for the following parameters: total protein (TP), albumin (Alb), albumin/globulin ratio (A/G), total bilirubin (Bil), glucose, total cholesterol (T-Chol), triglyceride (TG), urea nitrogen (BUN), creatinine (Cre), calcium (Ca), inorganic phosphorus (IP), sodium (Na), potassium (K), chlorine (Cl), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (γ-GTP).

Organ weights and histopathological assessment

A complete necropsy was performed for all animals, and the brain, thymus, lungs, heart, spleen, liver, adrenal glands, kidneys, testes, and ovaries were weighed. These organs, as well as tissues from the spinal cord, trigeminal nerve, pituitary gland, nasal cavity, salivary glands, tongue, esophagus, trachea, aorta, thyroid gland, parathyroid gland, pancreas, stomach, small and large intestines, submandibular and mesenteric lymph nodes, vagina, uterus, urinary bladder, prostate gland, seminal vesicles, epididymis, femur and sternum with bone marrow, vertebrae, skeletal muscles, sciatic nerve, skin, and mammary gland, were fixed in 10% neutral-buffered formalin; then, paraffin-embedded sections were prepared and stained with hematoxylin and eosin for histopathological examination. The testes and eyes with Harderian glands were fixed in Bouin's fixative and Davidson's solution, respectively. Bony tissues, including the nasal cavity, vertebrae, sternum, and femur, were decalcified using a mixture of 10% formic acid and 10% buffered formalin for up to 2 weeks. Histopathological assessment was performed for all tissues obtained from animals in the control group and the group administered the highest dose unless any treatment-related lesions were observed.

Immunohistochemistry for α_{2u}-globulin

In male rats, accumulation of α_{2u}-globulin in the kidney was examined by immunohistochemical analysis of paraffin-embedded sections. For antigen retrieval, the sections obtained from 5 rats in each group were incubated with proteinase K (DAKO, Glostrup, Denmark) for 1 min at room temperature. All sections were immersed in 3% H_2O_2/methanol solution for 10 min at room temperature for inactivation of endogenous peroxidase activity. After blocking nonspecific reactions with 10% normal rabbit serum, the sections were incubated with a primary antibody for α_{2u}-globulin (diluted 1:400; anti-rat α_{2u}-globulin antibody; R&D Systems, Minneapolis, MN, USA) overnight at 4°C[8]. Visualization of antibody binding was performed using a VectaStain Elite ABC Kit (Vector Laboratories, Burlingame, CA, USA) and 3,3′-diaminobenzidine. All sections were counterstained with hematoxylin.

Statistical analysis

Variances in data values for body weight during the experimental period, as well as for hematology, serum biochemistry, and organ weights, were assessed for homogeneity using Bartlett's test. One-way analysis of variance and the Kruskal-Wallis test, respectively, was employed for homogeneous and heterogenous data. On determining statistically significant differences, Dunnett's multiple comparison test was used to compare control and treatment groups. Comparisons of histopathological incidences and grades were analyzed by utilizing the Fisher's exact probability test and Mann-Whitney's *U* test, respectively. P values of <0.05 were considered statistically significant.

Results

In-life parameters, hematology, and serum biochemistry

No significant clinical signs were noted throughout the experimental period, and all animals survived until the scheduled necropsy. Furthermore, no significant differences in body weight and daily food intake were observed in male and female rats (Fig. 1A and B).

Hematology and serum biochemistry data are shown in Tables 1 and 2, respectively. Hematological analyses revealed significantly decreased HGB, HCT, MCV, and MCH and increased PLT in male rats administered 250 mg/kg BW/day 2-(*l*-menthoxy)ethanol (Table 1). In serum biochemistry, significant increases in TP, T-Chol, and Ca and decreases in Bil and ALP were noted in males of the 250 mg/kg group (Table 2). A significant increase in Cre and a decrease in A/G were noted in males of the 60 and 250 mg/kg groups. In females, a significant increase in Ca and a decrease in ALP were recorded in the 60 and 250 mg/kg groups, whereas elevated T-Chol and reduced A/G, Bil, TG, AST, and ALT were detected in the 250 mg/kg group. Although a significant increase in TG and a decrease in Cl were observed in males administered 60 mg/kg, the lack of any dose relationship suggested that these differences were not associated with exposure to the test substance.

Organ weights

Data for organ weights are summarized in Table 3. Absolute and relative liver weights were significantly increased in both sexes of the 250 mg/kg group (Table 3). A significant increase in relative liver weights was observed in males of the 60 mg/kg group. In males, absolute and relative kidney weights were significantly increased in the 60 and 250 mg/kg groups. In the male 250 mg/kg group, significant increases in absolute and relative weights of adrenal glands and relative weights of spleen and testes were noted.

Histopathology and immunohistochemistry

Data for histopathological and immunohistochemical findings are summarized in Tables 4 and 5, respectively. Histopathological findings revealed that the frequency of accumulation of eosinophilic granules (Fig. 2A–D), basophilic tubules, and hyaline cast, as well as focal interstitial inflammation in the kidney, were significantly increased in all male treatment groups when compared with the control group (Table 4). Based on immunohistochemical analyses, male rats treated with the test substance presented an increase in α_{2u}-globulin immunoreactivity in epithelial cells of proximal tubules, demonstrating a clear dose-dependency (Fig. 2E–H) (Table 5).

At necropsy, one male rat in the 250 mg/kg group showed severe enlargement of the right kidney owing to a histologically characteristic nephroblastoma. Although nephroblastoma is uncommon in most rat strains[9], this case was considered incidental, as no other animals presented similar lesions. In addition, in oral carcinogenicity studies, the incidence of spontaneous nephroblastoma in male

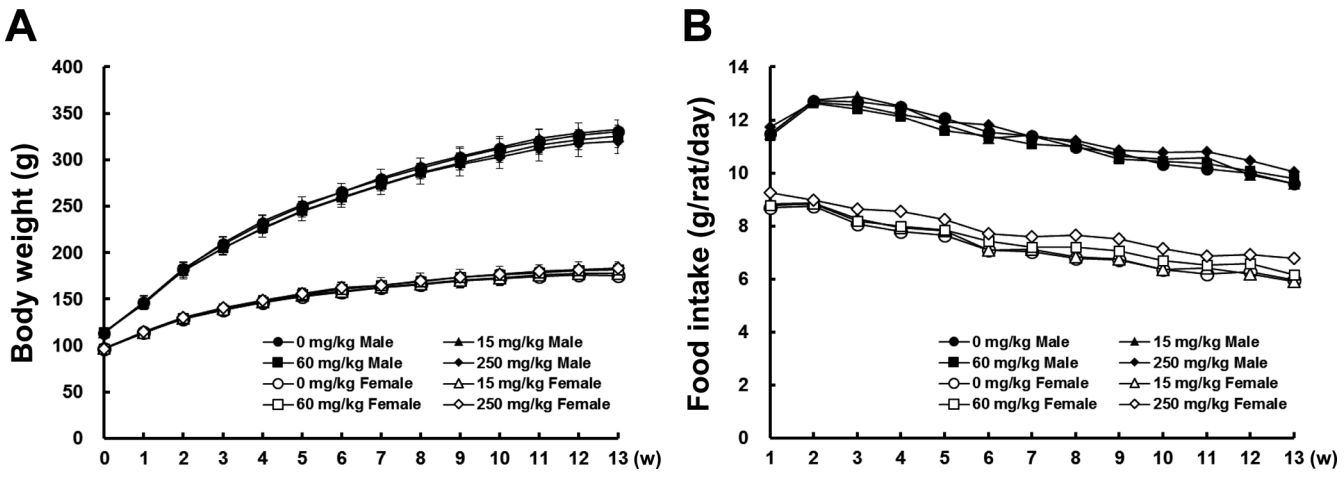

Fig. 1. Body weight (A) and daily food intake (B) data for male (closed symbols) and female (open symbols) F344 rats treated with the indicated dose of 2-(*l*-menthoxy)ethanol for 13 weeks. Each group was composed of 10 animals. The error bars represent the standard deviation for experimental groups.

Table 1. Hematology Data for F344 Rats Treated with 2-(*l*-menthoxy)ethanol for 13 weeks

Group (mg/kg bw/day)		0	15	60	250
No. of animals examined		10	10	10	10
Males					
WBC	$\times 10^2/\mu L$	33.6 ± 5.5	35.6 ± 8.6	36.5 ± 9.0	34.8 ± 6.4
RBC	$\times 10^4/\mu L$	880 ± 22	893 ± 24	871 ± 21	861 ± 19
HGB	g/dL	14.5 ± 0.3	14.7 ± 0.4	14.3 ± 0.3	14.0 ± 0.2**
HCT	%	43.1 ± 1.0	43.8 ± 1.3	42.4 ± 1.3	41.5 ± 0.9*
MCV	fL	49.0 ± 0.3	49.1 ± 0.3	48.7 ± 0.4	48.2 ± 0.3**
MCH	pg	16.5 ± 0.2	16.4 ± 0.1	16.4 ± 0.1	16.2 ± 0.1**
MCHC	g/dL	33.7 ± 0.1	33.5 ± 0.2	33.6 ± 0.3	33.7 ± 0.2
PLT	$\times 10^4/\mu L$	68.9 ± 3.4	70.1 ± 2.2	71.0 ± 3.1	76.2 ± 2.3**
Differential cell count					
Neutrophil	%	22.5 ± 4.3	23.9 ± 2.7	23.7 ± 3.9	24.2 ± 4.4
Lymphocyte	%	73.7 ± 5.3	71.9 ± 3.5	72.2 ± 4.5	71.1 ± 4.7
Monocyte	%	2.7 ± 0.9	3.1 ± 0.8	3.0 ± 0.8	3.4 ± 0.5
Eosinophil	%	0.7 ± 0.4	0.8 ± 0.3	0.7 ± 0.2	0.9 ± 0.3
Basophil	%	0.4 ± 0.1	0.4 ± 0.2	0.4 ± 0.2	0.5 ± 0.1
Females					
WBC	$\times 10^2/\mu L$	24.6 ± 5.6	20.8 ± 2.9	22.2 ± 6.3	25.9 ± 5.2
RBC	$\times 10^4/\mu L$	818 ± 31	822 ± 24	817 ± 13	816 ± 32
HGB	g/dL	14.3 ± 0.6	14.4 ± 0.5	14.4 ± 0.2	14.1 ± 0.7
HCT	%	42.2 ± 1.9	42.7 ± 1.5	42.4 ± 0.6	41.6 ± 2.1
MCV	fL	51.6 ± 0.4	51.9 ± 0.7	51.9 ± 0.2	51.0 ± 0.9
MCH	pg	17.5 ± 0.2	17.6 ± 0.2	17.6 ± 0.1	17.3 ± 0.3
MCHC	g/dL	34.0 ± 0.3	33.9 ± 0.2	34.0 ± 0.1	33.9 ± 0.2
PLT	$\times 10^4/\mu L$	66.5 ± 5.3	64.0 ± 7.5	68.7 ± 5.4	71.5 ± 4.6
Differential cell count					
Neutrophil	%	17.5 ± 3.1	17.4 ± 3.7	16.1 ± 1.6	20.2 ± 4.4
Lymphocyte	%	77.7 ± 3.5	77.8 ± 4.0	80.0 ± 1.8	75.6 ± 4.5
Monocyte	%	2.9 ± 0.7	3.2 ± 0.8	2.6 ± 0.4	2.8 ± 0.4
Eosinophil	%	1.5 ± 1.0	1.2 ± 0.9	0.9 ± 0.3	1.0 ± 0.4
Basophil	%	0.4 ± 0.2	0.4 ± 0.2	0.3 ± 0.3	0.5 ± 0.1

Values are mean ± SDs. * and **; Significantly different from the control at $p<0.05$ and <0.01, respectively.

F344/DuCrlCrlj rats reportedly ranged between 0–2.0%[10]. Although several lesions were sporadically detected in other organs, no significant treatment-dependent alterations in the incidence of these lesions were apparent.

Discussion

To date, 24 flavoring agents, including 2-(*l*-menthoxy) ethanol, have been evaluated by JECFA as substances structurally related to menthol, and all are listed as "no safety concern" at the current estimated levels of intake[1, 11]. 2-(*l*-Menthoxy)ethanol is classified in a subgroup of alicyclic alcohols or ethers and is considered to be primarily conjugated with glucuronic acid, followed by urinary excretion[1]. Although oral LD_{50} values of these substances in rodents are relatively high, data from repeated-dose toxicity studies remain limited. A 13-week repeated-dose toxicity study has revealed that menthyl pyrrolidone carboxylate (CAS No. 68127-22-0), a compound structurally related to menthol, can be associated with increased relative liver and kidney weights in male and female Wistar rats. Accumulation of α_{2u}-globulin in the proximal tubules of male rats was also observed at a dose of 1,111 mg/kg BW/day, and the NOAEL was determined as 109 mg/kg BW/day[1].

In the present 13-week subchronic toxicity study of 2-(*l*-menthoxy)ethanol, no toxicological changes in general condition, body weight, and food intake were observed. Following hematological assessment, we observed a decrease in HGB (96.6% to the control mean values), HCT (96.3%), MCV (98.4%), and MCH (98.2%) and an increase in PLT (111%) in the male 250 mg/kg group, which were considered to be toxicologic influences of 2-(*l*-menthoxy)ethanol treatment. Both absolute (115% in both sexes) and relative (119% in males and 111% in females) liver weights were significantly increased in both sexes in the 250 mg/kg groups. Although there were no histopathological changes in the liver, increased levels of serum T-Chol (110% in males and 121% in females) and TP (105% in males) and a reduction in TG (60.1% in females) in the 250 mg/kg groups indicate toxic changes associated with lipid metabolism and protein synthesis in the liver. Reduced HGB and MCH and elevated PLT, T-Chol, and TP in males and decreased TG and increased absolute and relative liver weights in both sexes were also detected, with statistical significance in the

Table 2. Serum Biochemistry Data for F344 Rats Treated with 2-(*l*-menthoxy)ethanol for 13 weeks

Group (mg/kg bw/day)		0	15	60	250
No. of animals examined		10	10	10	10
Males					
TP	(g/dL)	6.2 ± 0.2	6.3 ± 0.2	6.3 ± 0.2	6.5 ± 0.1**
Alb	(g/dL)	4.3 ± 0.2	4.3 ± 0.1	4.3 ± 0.1	4.4 ± 0.1
A/G		2.3 ± 0.1	2.2 ± 0.1	2.2 ± 0.1*	2.1 ± 0.1**
Bil	(mg/dL)	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01	0.03 ± 0.01*
Glucose	(mg/dL)	159 ± 19	163 ± 22	175 ± 26	171 ± 23
T-Chol	(mg/dL)	53.5 ± 5.7	55.1 ± 2.0	58.0 ± 3.2	60.0 ± 2.7**
TG	(mg/dL)	46.6 ± 8.1	56.4 ± 15.3	62.6 ± 12.5*	45.8 ± 16.4
BUN	(mg/dL)	18.8 ± 1.1	18.3 ± 1.9	19.9 ± 1.6	19.3 ± 1.3
Cre	(mg/dL)	0.36 ± 0.04	0.39 ± 0.04	0.41 ± 0.04*	0.43 ± 0.04**
Ca	(mg/dL)	10.1 ± 0.3	10.1 ± 0.2	10.3 ± 0.2	10.4 ± 0.2*
IP	(mg/dL)	5.4 ± 0.5	5.3 ± 0.5	5.5 ± 0.4	5.8 ± 0.5
Na	(mEq/L)	145 ± 1.0	145 ± 0.8	145 ± 0.9	145 ± 0.7
K	(mEq/L)	4.7 ± 0.3	4.7 ± 0.1	4.6 ± 0.2	4.7 ± 0.1
Cl	(mEq/L)	106 ± 0.8	106 ± 1.6	104 ± 2.5*	106 ± 1.6
AST	(IU/L)	84.1 ± 23.4	75.1 ± 11.3	78.4 ± 8.2	70.6 ± 6.8
ALT	(IU/L)	43.1 ± 8.0	42.0 ± 3.9	42.2 ± 5.6	39.1 ± 5.5
ALP	(IU/L)	433 ± 25	427 ± 35	429 ± 23	397 ± 35*
γ-GTP	(IU/L)	< 3	< 3	< 3	< 3
Females					
TP	(g/dL)	5.7 ± 0.2	5.8 ± 0.2	5.9 ± 0.3	5.9 ± 0.1
Alb	(g/dL)	4.1 ± 0.2	4.2 ± 0.2	4.3 ± 0.2	4.2 ± 0.1
A/G		2.7 ± 0.1	2.6 ± 0.2	2.7 ± 0.1	2.5 ± 0.2*
Bil	(mg/dL)	0.06 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01*
Glucose	(mg/dL)	96 ± 18	95 ± 17	89 ± 13	97 ± 10
T-Chol	(mg/dL)	66.3 ± 4.6	65.8 ± 6.1	71.4 ± 7.2	80.4 ± 11.6*
TG	(mg/dL)	40.1 ± 17.1	32.2 ± 9.4	32.3 ± 9.9	24.1 ± 6.2*
BUN	(mg/dL)	17.5 ± 2.5	16.3 ± 1.8	16.5 ± 1.6	15.0 ± 1.6
Cre	(mg/dL)	0.37 ± 0.03	0.36 ± 0.02	0.36 ± 0.04	0.34 ± 0.03
Ca	(mg/dL)	9.9 ± 0.2	9.9 ± 0.2	10.1 ± 0.2*	10.1 ± 0.2*
IP	(mg/dL)	4.9 ± 0.6	4.9 ± 0.7	5.1 ± 0.4	5.4 ± 0.4
Na	(mEq/L)	145 ± 1.0	146 ± 1.2	145 ± 1.5	145 ± 0.4
K	(mEq/L)	4.5 ± 0.2	4.3 ± 0.3	4.4 ± 0.2	4.5 ± 0.2
Cl	(mEq/L)	106 ± 1.9	107 ± 2.1	105 ± 3.3	107 ± 2.1
AST	(IU/L)	77.4 ± 6.5	76.3 ± 6.1	75.2 ± 9.6	68.9 ± 5.2*
ALT	(IU/L)	41.0 ± 4.6	38.0 ± 5.1	39.6 ± 8.1	33.1 ± 3.0**
ALP	(IU/L)	352 ± 72	302 ± 45	283 ± 27*	270 ± 25**
γ-GTP	(IU/L)	< 3	< 3	< 3	< 3

Values are mean ± SDs. * and **; Significantly different from the control at $p<0.05$ and <0.01, respectively.

28-day preliminary study (data not shown), corroborating that these altered hematological and serum biochemical parameters, as well as liver weights, were not incidental but test substance-related effects. In contrast, the significant increase in relative liver weights detected in the male 60 mg/kg group in the present study was not accompanied by an increase in absolute weights or changes in related biochemical parameters, suggesting that this increase was an adaptive change.

Significantly increased absolute (120%) and relative (118%) weights of adrenal glands in the male 250 mg/kg group were considered to be associated with the test chemical. Furthermore, increased absolute and relative weights of adrenal glands in both sexes showed a clear dose-dependency in the 28-day preliminary study (data not shown), indicating the association with test chemical treatment. Although adrenal hypertrophy could result from stress, there were no findings suggestive of stress, such as reduced body weight gain or atrophy of the thymus and other lymphoid organs, in both the present and preliminary studies. Since there may be no histopathological changes in the adrenal gland even with impaired glucocorticoid production in cases of inhibition of steroidogenesis-related enzymes[12], the possibility that the increased adrenal gland weight is a toxic finding cannot be excluded. In contrast, increased relative weights of the spleen and testes in the male 250 mg/kg group, not observed in the preliminary study, were considered toxicologically insignificant due to the absence of histopathological lesions or significantly increased absolute weights. Similarly, serum biochemistry revealed a decrease in A/G and an increase in

Table 3. Organ Weight Data for F344 Rats Treated with 2-(*l*-menthoxy)ethanol for 13 weeks

Group (mg/kg bw/day)	0	15	60	250
No. of animals examined	10	10	10	10
Males				
Body weight (g)	321.5 ± 12.2	323.5 ± 8.9	315.5 ± 11.4	310.4 ± 13.6
Absolute (g)				
Brain	1.99 ± 0.05	1.98 ± 0.03	1.98 ± 0.02	1.99 ± 0.03
Thymus	0.178 ± 0.012	0.178 ± 0.013	0.169 ± 0.016	0.174 ± 0.009
Lungs	0.98 ± 0.05	0.99 ± 0.05	0.95 ± 0.06	1.02 ± 0.09
Heart	0.898 ± 0.049	0.868 ± 0.039	0.870 ± 0.029	0.885 ± 0.048
Spleen	0.579 ± 0.043	0.597 ± 0.022	0.603 ± 0.018	0.623 ± 0.035
Liver	7.35 ± 0.51	7.37 ± 0.29	7.64 ± 0.36	8.43 ± 0.49**
Adrenals	0.035 ± 0.005	0.036 ± 0.005	0.035 ± 0.006	0.042 ± 0.003*
Kidneys	1.81 ± 0.15	1.89 ± 0.08	2.07 ± 0.08*	2.50 ± 0.27**
Testes	3.01 ± 0.32	3.11 ± 0.12	3.11 ± 0.10	3.25 ± 0.29
Relative (%)				
Brain	0.62 ± 0.02	0.61 ± 0.02	0.63 ± 0.02	0.64 ± 0.03
Thymus	0.055 ± 0.004	0.055 ± 0.004	0.054 ± 0.005	0.056 ± 0.003
Lungs	0.305 ± 0.019	0.306 ± 0.019	0.300 ± 0.024	0.327 ± 0.025
Heart	0.280 ± 0.019	0.268 ± 0.009	0.276 ± 0.006	0.285 ± 0.008
Spleen	0.180 ± 0.010	0.185 ± 0.007	0.191 ± 0.004	0.201 ± 0.004**
Liver	2.28 ± 0.09	2.28 ± 0.08	2.42 ± 0.08**	2.71 ± 0.07**
Adrenals	0.011 ± 0.001	0.011 ± 0.001	0.011 ± 0.002	0.013 ± 0.001**
Kidneys	0.562 ± 0.034	0.585 ± 0.024	0.658 ± 0.018**	0.803 ± 0.071**
Testes	0.94 ± 0.09	0.96 ± 0.02	0.99 ± 0.05	1.05 ± 0.08**
Females				
Body weight (g)	170.8 ± 4.93	172.0 ± 7.9	175.8 ± 8.1	176.7 ± 4.3
Absolute (g)				
Brain	1.81 ± 0.03	1.82 ± 0.04	1.82 ± 0.03	1.83 ± 0.03
Thymus	0.152 ± 0.008	0.143 ± 0.014	0.157 ± 0.010	0.149 ± 0.010[a]
Lungs	0.70 ± 0.05	0.69 ± 0.05	0.68 ± 0.05	0.71 ± 0.04
Heart	0.558 ± 0.030	0.550 ± 0.026	0.549 ± 0.028	0.566 ± 0.033
Spleen	0.372 ± 0.032	0.365 ± 0.024	0.388 ± 0.026	0.363 ± 0.027
Liver	3.65 ± 0.09	3.66 ± 0.23	3.80 ± 0.21	4.21 ± 0.09**
Adrenals	0.037 ± 0.004	0.039 ± 0.003	0.039 ± 0.003	0.040 ± 0.004
Kidneys	1.05 ± 0.04	1.02 ± 0.05	1.04 ± 0.08	1.06 ± 0.08
Ovaries	0.048 ± 0.005	0.044 ± 0.007	0.047 ± 0.005	0.048 ± 0.005
Relative (%)				
Brain	1.06 ± 0.03	1.06 ± 0.04	1.04 ± 0.05	1.04 ± 0.03
Thymus	0.089 ± 0.006	0.083 ± 0.007	0.089 ± 0.004	0.084 ± 0.005[a]
Lungs	0.412 ± 0.027	0.401 ± 0.033	0.388 ± 0.037	0.402 ± 0.030
Heart	0.327 ± 0.019	0.320 ± 0.015	0.313 ± 0.015	0.320 ± 0.015
Spleen	0.218 ± 0.019	0.212 ± 0.013	0.221 ± 0.012	0.205 ± 0.015
Liver	2.14 ± 0.06	2.13 ± 0.08	2.16 ± 0.08	2.38 ± 0.08**
Adrenals	0.022 ± 0.002	0.023 ± 0.002	0.022 ± 0.001	0.023 ± 0.002
Kidneys	0.614 ± 0.025	0.593 ± 0.023	0.591 ± 0.033	0.602 ± 0.046
Ovaries	0.028 ± 0.003	0.026 ± 0.004	0.027 ± 0.003	0.027 ± 0.003

Values are mean ± SDs. * and **; Significantly different from the control at $p<0.05$ and <0.01, respectively. a; The number of effective animals was reduced to nine due to failure in tissue sampling.

Ca in both sexes, which was considered to possess no toxicological significance, considering no abnormalities were detected in related parameters. Furthermore, decreased Bil and ALP levels in both sexes and decreased AST and ALT in females were in contrast to expected toxic effects and were considered not meaningful.

Chronic nephropathy characterized by basophilic tubules, hyaline cast, and focal interstitial inflammation was observed with statistical significance in males of all treated groups. In addition, increased serum Cre and absolute and relative kidney weights were detected in the male 60 and 250 mg/kg groups; these changes were not observed in female treatment groups. In these cases, the degree of accumulation of eosinophilic granules in proximal tubular epithelial cells was increased in an apparent dose-dependent manner. These granules were confirmed as α_{2u}-globulin by immu-

Table 4. Histopathological Findings for F344 Rats Treated with 2-(*l*-menthoxy)ethanol for 13 weeks

	Group (mg/kg bw/day)	0	15	60	250
Organs and findings	No. of animals examined	10	10	10	10
Males					
Liver	Microgranuloma (±)	3	-	-	2
	Necrosis, focal (±)	1	-	-	0
Kidney	Accumulation of eosinophilic granule (±, +, ++)	0	10** (10, 0, 0)‡	10** (0, 9, 1)‡	10** (0, 1, 9)‡
	Basophilic tubule (±, +, ++)	1 (1, 0, 0)	10** (0, 10, 0)‡	10** (0, 7, 3)‡	10** (0, 3, 7)‡
	Hyaline cast (±, +, ++)	0	9** (4, 5, 0)‡	10** (0, 0, 10)‡	10** (0, 0, 10)‡
	Inflammation, interstitial, focal (±, +, ++)	4 (4, 0, 0)	10* (9, 1, 0)‡	10* (1, 6, 3)‡	10* (0, 5, 5)‡
	Mineralization (±)	0	0	0	4
	Nephroblastoma, unilateral	0	0	0	1
Heart	Mononuclear cell infiltration, focal (±)	6	-	-	3
Lung	Mineralization (±)	5	-	-	5
Forestomach	Inflammation, focal (±)	1	-	-	0
Pancreas	Mononuclear cell infiltration, focal (±)	0	-	-	2
Parotid gland	Mononuclear cell infiltration, focal (±)	2	-	-	0
	Basophilic cell foci	4	-	-	3
Testis	Atrophy, tubular, unilateral	1	-	-	0
	Dilation, tubular, unilateral	0	-	-	1
Epididymis	Atrophy, ductal, unilateral	1	-	-	0
	Epithelial degeneration with inflammation (±)	2	-	-	0
	Sperm stasis, unilateral	0	-	-	1
Prostate	Mononuclear cell infiltration, focal (±)	1	-	-	3
Urinary bladder	Mononuclear cell infiltration, focal (±)	0	-	-	1
Thyroid gland	Ultimobranchial cyst	1	-	-	2
Nasal cavity	Chronic inflammation, focal (±, +)	6 (5, 1)	-	-	4 (4, 0)
	Mucous cell metaplasia, respiratory epithelium (±)	0	-	-	4
Harderian gland	Mononuclear cell infiltration, focal (±)	0	-	-	1
Females					
Liver	Microgranuloma (±)	3	-	-	3
Kidney	Basophilic tubule (±)	0	-	-	1
	Inflammation, interstitial, focal (±)	1	-	-	0
	Mineralization (±)	3	-	-	1
	Hydronephrosis, bilateral	0	-	-	1
Heart	Mononuclear cell infiltration, focal (±)	1	-	-	3
Lung	Mineralization (±)	5	-	-	5
Tongue	Inflammation, focal (+)	1	-	-	0
Pancreas	Mononuclear cell infiltration, focal (±)	1	-	-	1
Parotid gland	Mononuclear cell infiltration, focal (±)	1	-	-	0
	Basophilic cell foci	0	-	-	1
Pituitary gland	Cyst, pars distalis	2	-	-	0
	Cyst, pars intermedia	0	-	-	1
Thyroid gland	Ultimobranchial cyst	1	-	-	2
Bone marrow	Granuloma	1	-	-	1
Nasal cavity	Chronic inflammation, focal (±, +)	7 (6, 1)	-	-	8 (6, 2)
	Mucous cell metaplasia, respiratory epithelium (±)	2	-	-	2
Eye	Retinal atrophy	1	-	-	0
Harderian gland	Mononuclear cell infiltration, focal (±, +, ++)	3 (2, 0, 1)	-	-	1 (0, 1, 0)

-; Not evaluated. ± , +, and ++; Slight, mild, and moderate, repectively. * and **; Significantly different from the control at $p<0.05$ and <0.01, respectively (Fisher's exact test). ‡; Significantly different from the control at $p<0.01$ (Mann-Whitney *U* test).

Table 5. Accumulation Levles of α_{2u}-globulin in the Kidney of Male F344 Rats

Group (mg/kg bw/day)	0	15	60	250
No. of animals examined	5	5	5	5
Normal	5	0	0	0
Slight	0	5**	0	0
Mild	0	0	5**	0
Moderate	0	0	0	5**

**; Significantly different from the control at $p<0.01$ (Fisher's exact test).

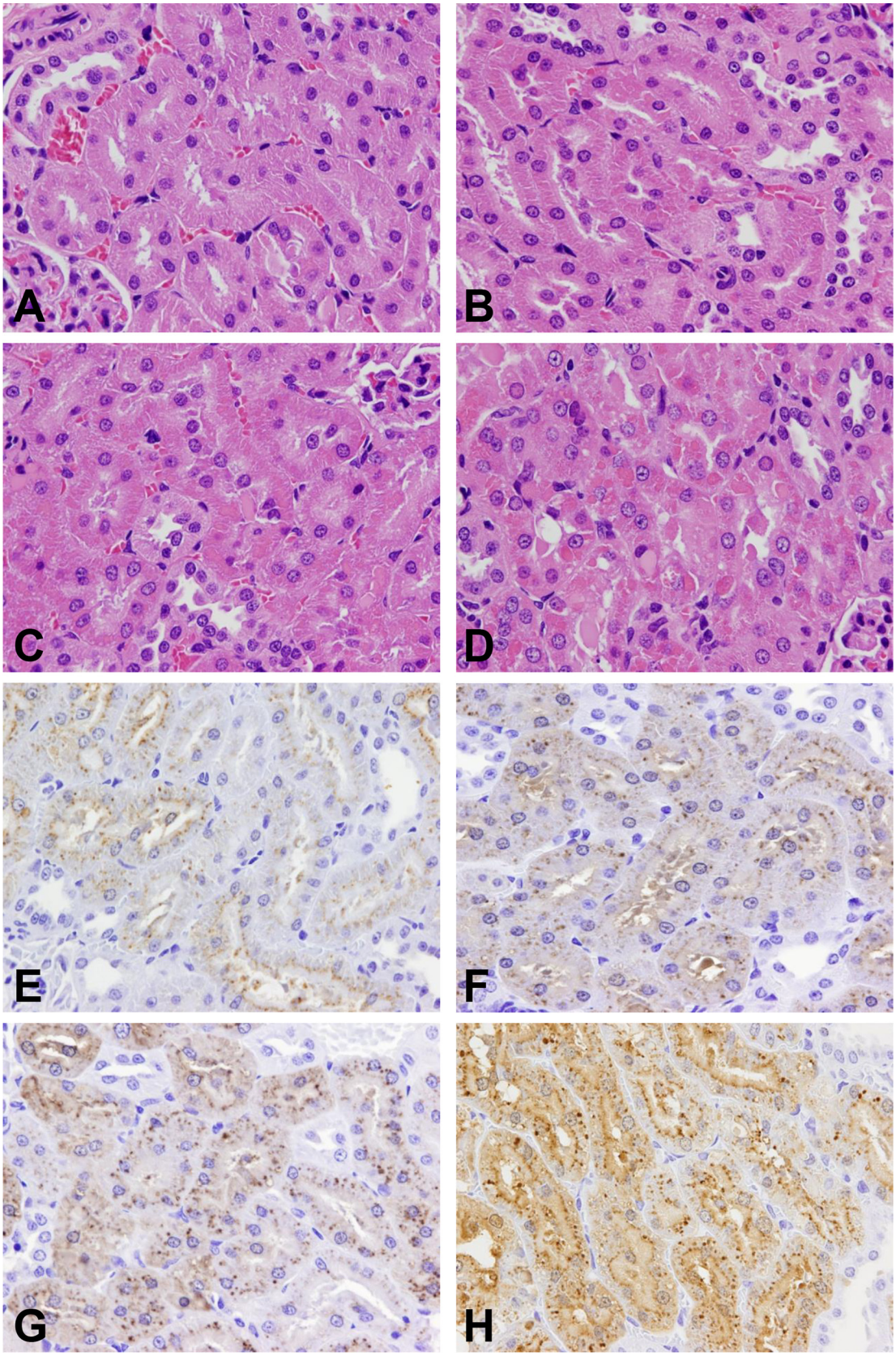

Fig. 2. Representative histopathological and immunohistochemical findings in the kidney of male F344 rats treated with 2-(*l*-menthoxy)ethanol for 13 weeks. Original magnifications: 400×. (A–D) Accumulation of eosinophilic granules in epithelial cells of proximal tubules in control (A), 15 (B), 60 (C), and 250 mg/kg (D) groups. (E–H) A dose-dependent increase in immunoreactivity of α_{2u}-globulin is apparent in control (E), 15 (F), 60 (G), and 250 mg/kg (H) groups.

nohistochemistry, indicating the occurrence of α_{2u}-globulin nephropathy. α_{2u}-Globulins bound to certain chemicals, such as *d*-limonene, accumulate in lysosomes and are observed in the proximal tubules as eosinophilic or hyaline granules[9]. This accumulation can damage epithelial cells, leading to chronic progressive nephropathy and consequent tumor formation[9]. Thus, although the NOAEL for 2-(*l*-menthoxy)ethanol associated with α_{2u}-globulin nephropathy in male rat kidneys was not identified, this type of nephropathy is reportedly specific to male rats and thus not relevant to human risk assessment[13]. An increased risk of renal failure mediated via mechanisms related to α_{2u}-globulin accumulation is highly improbable, as this protein is absent in humans[14]. Therefore, it was considered reasonable to exclude the changes associated with chronic nephropathy observed in males from the determination of the NOAEL.

In conclusion, the present 13-week subchronic toxicity study demonstrated that 250 mg/kg BW/day 2-(*l*-menthoxy) ethanol treatment caused toxic changes in hematology, serum biochemistry, and organ weights in male and female F344 rats. Based on the observed results, the NOAEL of 2-(*l*-menthoxy)ethanol was evaluated as 60 mg/kg BW/day for both sexes.

Disclosure of Potential Conflicts of Interest: The authors declare that there is no conflict of interest.

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