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A 13-week subchronic toxicity study of vanillin propylene glycol acetal in F344 rats

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ABSTRACT

Vanillin propylene glycol acetal (VPGA) has been used as a flavoring agent. Here, we performed a 13-week subchronic toxicity study of VPGA in F344 rats with oral administration by gavage at doses of 0, 100, 300, and 1000 mg/kg body weight (BW)/day. In the 1000 mg/kg BW group, loss of vigorous activity and listlessness immediately after administration were observed for both sexes throughout the experimental period. Reduction of body weight gain was noted in both sexes at 1000 mg/kg BW. Serum biochemistry demonstrated significant increases in total protein, albumin, total cholesterol, calcium, inorganic phosphorus, and γ-glutamyl transpeptidase in both sexes at 1000 mg/kg BW and increases in the albumin/globulin ratio and urea nitrogen in the male 1000 mg/kg BW group. A significant increase in relative liver weight was detected in both sexes at 1000 mg/kg BW. Histopathologically, centrilobular hepatocellular hypertrophy in the liver was observed in both sexes at 1000 mg/kg BW. In addition, the incidence of fatty changes in hepatocytes in the male 1000 mg/kg BW group was decreased compared with that in the control. Based on these results, the no-observed-adverse-effect level for VPGA was evaluated to be 300 mg/kg BW/day for both sexes in the current study.

1. Introduction

Vanillin propylene glycol acetal (VPGA; CAS No. 68527-74-2) is a clear and colorless liquid with a sweet, chocolate, and creamy taste (Human Metabolome Project, 2012). VPGA has been used as a flavoring agent in various food such as baked goods, beverages, cereals, and confectioner frostings (Flavor and Extract Manufacturers' Association [FEMA], 1998). The dietary exposures of VPGA are estimated as 88 μg/person/day in Europe, 0.5 μg/person/day in the United States of America (USA), and 54 μg/person/day in Japan (Joint FAO/WHO Expert Committee on Additives [JECFA], 2009). In the 69th meeting of JECFA, VPGA was evaluated as a flavoring agent categorized as a hydroxy- and alkoxy-substituted benzyl derivative and was subsequently assigned to class I based on the structural classes of flavoring agents (JECFA, 2009). Structural class I substances are flavoring agents that have simple chemical structures and efficient modes of metabolism which would suggest a low order of toxicity by the oral route (JECFA, 1997). The threshold of toxicological concern for structural class I flavoring agents is 1800 μg/person/day. The Committee concluded that the use of VPGA as a flavoring agent would not present safety concerns at the estimated current levels of intake because the estimated human intake of VPGA was below the threshold for class I compounds. With regard to genotoxicity, VPGA has proved negative in the Ames tests using *Salmonella typhimurium* (TA100, TA98, TA1535, and TA1537) and *Escherichia coli* (WP *uvrA*) (unpublished data obtained in the National Institute of Health Sciences with the support of the Ministry of Health, Labour and Welfare, Japan). Although VPGA induced structural abnormality in the chromosomal aberration assay with Chinese hamster lung (CHL/IU) cells, the *in vivo* micronucleus test using mouse bone marrow showed negative results (unpublished data obtained in the National Institute of Health Sciences with the support of the Ministry of Health, Labour and Welfare, Japan).

Our laboratory has investigated the toxicological profiles of a number of food additives, including representative flavoring agents from each group of flavoring agents using rodent models (Akagi et al., 2018a, 2018b; Matsushita et al., 2018; Toyoda et al., 2014a, 2014b). Although VPGA has been used in various food products, there is little information regarding the toxicological profile of VPGA. Thus, further detailed data on the toxicological profile of VPGA is needed to provide scientific evidence supporting JECFA's evaluation. In the current study, we performed a 13-week subchronic toxicity study of VPGA with oral administration by gavage in F344 rats in order to clarify the toxicological profile of VPGA and establish the no-observed-adverse-effect level (NOAEL) for this substance.

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Abbreviations

A/G	albumin/globulin ratio
Alb	albumin
ALP	alkaline phosphatase
ALT	alanine aminotransferase
AST	aspartate aminotransferase
Bil	total bilirubin
BUN	urea nitrogen
Ca	calcium
Cl	chlorine
Cre	creatinine
Ebl	erythroblasts
γ-GTP	γ-glutamyl transpeptidase
HCT	hematocrit
HGB	hemoglobin concentration
IP	inorganic phosphorus
K	potassium
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
Na	sodium
NOAEL	no-observed-adverse-effect level
PLT	platelet count
RBC	red blood cell count
T-Chol	total cholesterol
TG	triglyceride
TP	total protein
VPGA	vanillin propylene glycol acetal
WBC	white blood cell count

2. Materials and methods

2.1. Test chemical

VPGA (Fig. 1; lot no. 115012001, purity 99.3%), produced by Inoue Perfumery MFG. Co., Ltd. (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). Gavage with corn oil (Wako Pure Chemical Industries, Osaka, Japan) as the vehicle was chosen as the route of administration because of the volatility and low water solubility of VPGA. VPGA solutions were prepared daily immediately before administration.

2.2. Experimental animals

Forty male and 40 female specific pathogen-free rats (F344/DuCrlCrlj, 5 weeks old) were purchased from Charles River Japan (Yokohama, Japan) and used after acclimation for 1 week. The animals were housed in polycarbonate cages with soft chip bedding (Sankyo Labo Service, Tokyo, Japan) in a room with a barrier system and a controlled light/dark cycle (12 h), ventilation (air-exchange rate 18 times/h), temperature (23 ± 2 °C), and relative humidity (55% ± 5%) during the study. The cages and chip bedding were exchanged twice a week. Each animal had free access to tap water and a basal diet (CRF-1; Oriental Yeast Co., Ltd., Tokyo, Japan). At the beginning of the experiment, the animals were randomly allocated into four groups of 10 male and 10 female rats each based on body weights measured just before starting the test chemical administration.

2.3. Study design

Doses were selected based on recommendations in Organisation for Economic Co-operation and Development (OECD) test guideline 408 (OECD, 2018). In a preliminary 4-week study of VPGA with administration at doses of 0, 250, 500, and 1000 mg/kg body weight [BW]/day, loss of vigorous activity and listlessness immediately after administration were observed in both sexes in the 1000 mg/kg BW group, without reduction of BW or food consumption (data not shown). From these results, we selected 0, 100, 300, and 1000 mg/kg BW/day as doses of VPGA for both sexes in our 13-week toxicity study.

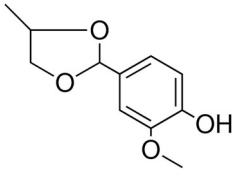

Fig. 1. Chemical structure of vanillin propylene glycol acetal.

General condition and mortality were checked daily. BWs and the amount of supplied and residual diet were measured twice a week for the first 2 weeks and once a week thereafter. All rats were fasted overnight following completion of the administration period, and blood samples for hematology and serum biochemistry were collected from the abdominal aorta under deep anesthesia induced by inhalation of isoflurane. The study design was performed in accordance with the Guidelines for Designation for Food Additives and Revision of Standards for Use of Food Additives of Japan (Ministry of Health, Labour and Welfare, Japan, 1996), without urinalysis and ophthalmological examination, and was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan.

2.4. Hematology and serum biochemistry

The following hematological parameters were analyzed using a K-4500 automatic hematology analyzer (Sysmex, Kobe, Japan): 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 white blood cell count (WBC). Blood smears were processed for Wright staining, and the numbers of erythroblasts and differential leukocytes were counted by microscopic examination. Serum biochemical analysis of the following parameters was performed by SRL (Tokyo, Japan): total protein (TP), albumin/globulin ratio (A/G), albumin (Alb), total bilirubin (Bil), glucose, triglyceride (TG), total cholesterol (T-Chol), urea nitrogen (BUN), creatinine (Cre), sodium (Na), chlorine (Cl), potassium (K), calcium (Ca), inorganic phosphorus (IP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and γ-glutamyl transpeptidase (γ-GTP).

2.5. Organ weights and histopathological assessment

Complete necropsies were performed for all animals, and the brain, thymus, heart, lungs, spleen, liver, kidneys, adrenal glands, and gonads were weighed. Paired organs were weighed separately, and statistical analysis was performed using the total value of both sides. These organs and other tissues (skin, mammary gland, sternum with marrow, femur with marrow, mandibular and mesenteric lymph nodes, salivary glands, aorta, trachea, tongue, esophagus, stomach, small and large intestines, pancreas, urinary bladder, epididymides, seminal vesicles, prostate gland, uterus, vagina, pituitary gland, thyroid glands, parathyroid glands, spinal cord with vertebrae, trigeminal nerve, sciatic nerve, harderian glands, femoral skeletal muscle, and nasal cavity) were fixed in 10% neutral-buffered formalin, and paraffin embedded sections were prepared and stained with hematoxylin and eosin (H&E) for

Table 1
General condition of F344 rats administered with vanillin propylene glycol acetal for 13 weeks.

Findings		Week												
		1	2	3	4	5	6	7	8	9	10	11	12	13
Male	Cumulated total No. of animals per week	70	70	70	70	70	70	70	70	70	70	70	70	70
0 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
300 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1000 mg/kg BW	Loss of vigorous activity	7	0	0	4	1	0	1	1	1	1	1	0	1
	Listlessness	16	8	0	2	1	6	2	3	6	13	11	6	2
Female	Cumulated total No. of animals per week	70	70	70	70	70	70	70	70	70	70	70	70	70
0 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
100 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
300 mg/kg BW	–	–	–	–	–	–	–	–	–	–	–	–	–	–
1000 mg/kg BW	Loss of vigorous activity	3	2	0	1	0	2	0	1	4	2	0	1	3
	Listlessness	10	4	3	4	0	4	7	7	9	9	9	5	6

Values are total number of occurrences in each week.
-: No abnormalities detected.

histopathological examination. The testes and eyes were fixed in Bouin's fixative and Davidson's solution, respectively. Bony tissues, including the nasal cavity, vertebrae, sternum, and femur, were decalcified with a mixture of 10% formic acid and 10% buffered formalin for up to 2 weeks. Histopathological assessment was performed on all tissues in the control and high-dose groups and in the liver, kidney, stomach, eyes, harderian gland, lung, and thymus of the low- and mid-dose groups.

2.6. Statistical analysis

Variances in the data for BWs during the experiment as well as hematology, serum biochemistry, and organ weights were checked for homogeneity by Bartlett's test. If the variance was homogeneous, the data were assessed by one-way analysis of variance. If not, the Kruskal-Wallis test was applied. When statistically significant differences were indicated, Dunnett's multiple tests were employed for comparisons between the control and test article groups. For comparison of histopathological changes between the control and administration groups, incidences and grades were analyzed by Fisher's exact probability test and Mann-Whitney *U* tests, respectively.

3. Results and discussion

3.1. In-life parameters

All animals survived until the scheduled necropsy. Loss of vigorous activity and listlessness immediately after administration were observed for both sexes in the 1000 mg/kg BW group throughout the experimental period, suggesting that VPGA affected the nervous system (Table 1). These findings were noted most frequently in week 1, and all animals recovered within a few hours after administration.

In the 1000 mg/kg BW groups, significant suppression of body weight gain was observed in males and females from weeks 6 and from week 7 except week 8, respectively (Fig. 2A). At the end of study, body weights of male and female rats in the 1000 mg/kg BW group were 90.4 and 92.8% of the control groups, respectively. Because no obvious changes in food intake were observed in any of the VPGA-groups compared with the control group (Fig. 2B), suppression of body weight gain was considered to be an adverse effect of VPGA.

3.2. Hematology and serum biochemistry

Hematology data are summarized in Table 2. Significant increases in RBC and HCT in male 100 and 1000 mg/kg BW groups were considered of no toxicological significance due to lack of dose dependency, findings related to dehydration, and histopathological changes in

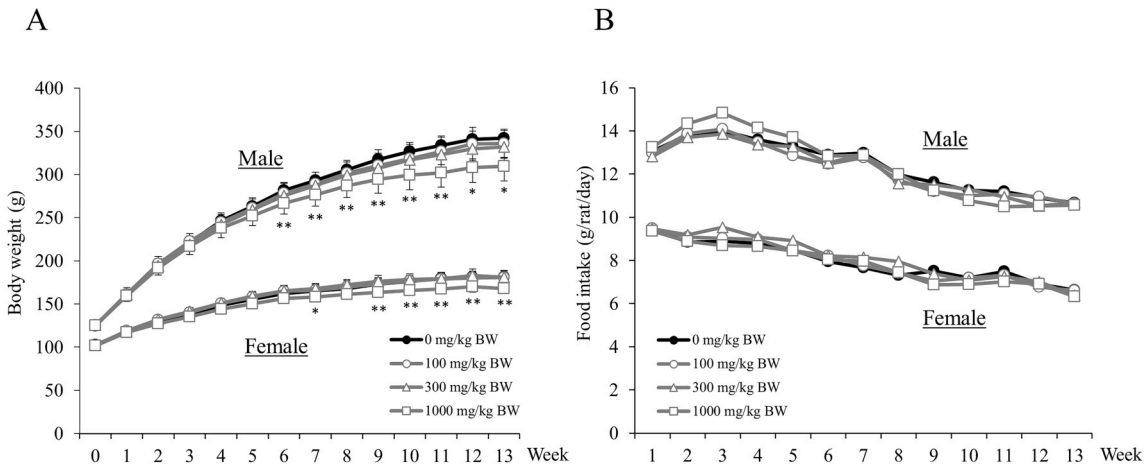

Fig. 2. Body weight (A) and daily food intake (B) data for male and female F344 rats administered with vanillin propylene glycol acetal for 13 weeks. Each group contained 10 animals. * and **: Significantly different from the 0 mg/kg BW group at $p < 0.05$ and 0.01, respectively.

Table 2
Hematology data for F344 rats administered with vanillin propylene glycol acetal (VPGA) for 13 weeks.

	VPGA (mg/kg BW)			
	0	100	300	1000
Males				
No. of animals examined	10	10	10	10
RBC ($\times 10^4$/μL)	859.9 ± 14.4	884.1 ± 26.7*	880.3 ± 20.2	897.3 ± 20.6**
HGB (g/dL)	14.2 ± 0.3	14.4 ± 0.4	14.5 ± 0.3	14.4 ± 0.3
HCT (%)	45.9 ± 0.9	47.5 ± 1.3**	47.0 ± 1.1	47.2 ± 1.0*
MCV (fL)	53.3 ± 0.5	53.7 ± 0.4	53.4 ± 0.5	52.6 ± 0.8*
MCH (pg)	16.6 ± 0.3	16.3 ± 0.4	16.4 ± 0.3	16.0 ± 0.4**
MCHC (g/dL)	31.0 ± 0.4	30.4 ± 0.6*	30.8 ± 0.6	30.5 ± 0.5
PLT ($\times 10^4$/μL)	66.6 ± 2.9	66.3 ± 4.0	67.1 ± 2.9	66.2 ± 12.5
WBC ($\times 10^2$/μL)	37.7 ± 5.3	34.5 ± 5.9	31.8 ± 3.3*	38.9 ± 2.6
Differential leukocyte counts				
Band form neutrophils (%)	0.7 ± 0.6	0.6 ± 0.5	0.7 ± 0.8	0.7 ± 0.6
Segmented neutrophils (%)	24.0 ± 3.1	24.4 ± 2.7	24.6 ± 2.1	21.3 ± 1.6*
Eosinophils (%)	0.9 ± 0.5	0.7 ± 0.5	0.7 ± 0.6	0.6 ± 0.6
Basophils (%)	0.1 ± 0.2	0 ± 0	0 ± 0	0 ± 0
Lymphocytes (%)	73.5 ± 2.9	72.8 ± 3.3	72.7 ± 2.3	75.7 ± 2.6
Monocytes (%)	1.2 ± 0.6	1.7 ± 0.8	1.4 ± 0.5	1.7 ± 0.8
Ebl (/100 cells)	0.61 ± 0.47	0.76 ± 0.27	0.81 ± 0.64	0.91 ± 0.47
Females				
No. of animals examined	10	10	10	10
RBC ($\times 10^4$/μL)	851.2 ± 36.2	852.3 ± 47.1	837.8 ± 54.2	823.3 ± 23.7
HGB (g/dL)	14.9 ± 0.5	14.5 ± 1.2	14.9 ± 0.8	14.5 ± 0.4
HCT (%)	48.1 ± 2.0	48.1 ± 2.6	47.4 ± 3.1	46.2 ± 1.4
MCV (fL)	56.6 ± 0.2	56.5 ± 0.2	56.5 ± 0.3	56.2 ± 0.3**
MCH (pg)	17.6 ± 0.5	17.1 ± 1.4	17.8 ± 0.6	17.7 ± 0.2
MCHC (g/dL)	31.0 ± 0.8	30.2 ± 2.5	31.5 ± 1.0	31.4 ± 0.4
PLT ($\times 10^4$/μL)	70.0 ± 4.3	69.4 ± 3.2	68.2 ± 4.7	68.8 ± 4.8
WBC ($\times 10^2$/μL)	31.9 ± 8.7	29.2 ± 12.6	29.8 ± 7.7	28.5 ± 6.9
Differential leukocyte counts				
Band form neutrophils (%)	0.5 ± 0.4	0.1 ± 0.2*	0.4 ± 0.4	0.5 ± 0.5
Segmented neutrophils (%)	19.4 ± 4.4	21.0 ± 3.9	20.2 ± 4.3	19.1 ± 2.4
Eosinophils (%)	0.9 ± 0.6	1.2 ± 0.5	1.2 ± 0.7	1.1 ± 0.6
Basophils (%)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Lymphocytes (%)	77.6 ± 4.5	76.5 ± 4.0	76.2 ± 4.6	77.7 ± 2.2
Monocytes (%)	1.6 ± 0.5	1.3 ± 0.7	1.7 ± 0.6	1.7 ± 1.0
Ebl (/100 cells)	0.50 ± 0.48	0.81 ± 0.55	0.66 ± 0.64	0.86 ± 0.42

Values are mean ± SDs.
*, **: Significantly different from the 0 mg/kg BW group at $p < 0.05$ and 0.01, respectively.

hematopoietic organs. Although significant decreases in MCV were observed for both sexes in the 1000 mg/kg BW group and a significant decrease in MCH was observed in males in the 1000 mg/kg BW group, these findings were considered to have no toxicological significance due to lack of toxicological effects on RBC and HCT and histopathological findings related to hemorrhage or anemia. The significant decrease in segmented neutrophils in males in the 1000 mg/kg BW group was considered of no toxicological significance due to lack of fluctuations in WBC, histopathological findings related to inflammation, and changes in the hematopoietic system. Although several other parameters were altered with statistical significance, the lack of any dose relation suggested that these changes were not associated with VPGA administration.

Data for serum biochemistry are summarized in Table 3. There were significant increases in TP, Alb, and T-Chol for both sexes in the 1000 mg/kg BW group, and a significant increase in the A/G ratio was observed for males in the 1000 mg/kg BW group. These changes indicated abnormalities in protein and lipid metabolism and were considered to be associated with changes in the liver detected upon histopathological examination. Although a significant increase in the A/G ratio was also observed for males in the 300 mg/kg BW group, the fluctuation was considered to be of no toxicological significance due to lack of increases in TP and Alb and histopathological changes in the liver. In addition, the values of γ-GTP in one male and four female rats in the 1000 mg/kg BW group were higher than the detection limit, suggesting adverse effects of VPGA on the liver. The significant decrease in AST for females in the 1000 mg/kg BW group was contradictory to typical toxicological changes. Significant increases in Ca and IP in both sexes of the 1000 mg/kg BW group and an increase in BUN in the male 1000 mg/kg BW group were also observed. Because parameters in serum biochemistry are generally altered prior to histopathological changes and clinical signs, these changes were considered to be adverse effects possibly due to renal dysfunction, although, no obvious histopathological lesions were detected in the kidneys.

3.3. Organ weights

The data for final BW and organ weights are shown in Table 4. The significant increase in relative liver weights for both sexes in the 1000 mg/kg BW group was considered to be related to centrilobular hepatocellular hypertrophy observed histopathologically. Moreover, the increase in relative liver weights for males in the 300 mg/kg BW group was considered to be of no toxicological significance due to the lack of related changes in parameters of serum biochemistry and histopathological examinations. The significant decrease in absolute kidney weights for males in the 100 and 1000 mg/kg BW groups and increase in relative kidney weights for males in the 1000 mg/kg BW group were considered to have no toxicological significance because the changes were very slight and because related histopathological changes were not detected. The significant decrease in absolute thymus weights for males in the 1000 mg/kg BW group and increase in relative spleen weights for males in the 300 and 1000 mg/kg BW groups were also

Table 3
Serum biochemical data for F344 rats administered with vanillin propylene glycol acetal (VPGA) for 13 weeks.

	VPGA (mg/kg BW)			
	0	100	300	1000
Male				
No. of animals examined	10	10	10	10
TP (g/dL)	6.3 ± 0.2	6.3 ± 0.2	6.4 ± 0.2	6.7 ± 0.1**
A/G ratio	2.3 ± 0.1	2.4 ± 0.1	2.4 ± 0.1*	2.4 ± 0.1*
Alb (g/dL)	4.4 ± 0.2	4.5 ± 0.1	4.5 ± 0.2	4.7 ± 0.1**
Bil (mg/dL)	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
Glucose (mg/dL)	146.8 ± 23.4	148.1 ± 11.1	145.1 ± 12.0	142.4 ± 26.5
TG (mg/dL)	58.8 ± 16.2	58.8 ± 19.2	51.6 ± 15.3	71.6 ± 25.2
T-Chol (mg/dL)	57.0 ± 7.8	58.5 ± 6.4	60.2 ± 5.9	74.7 ± 9.7**
BUN (mg/dL)	16.3 ± 1.1	16.1 ± 1.0	18.2 ± 2.4	20.7 ± 2.8**
Cre (mg/dL)	0.33 ± 0.03	0.34 ± 0.02	0.34 ± 0.02	0.35 ± 0.02
Na (mEQ/L)	142.4 ± 1.6	143.7 ± 1.7	143.2 ± 2.5	143.6 ± 1.4
Cl (mEQ/L)	103.6 ± 1.7	105.5 ± 2.1	103.8 ± 2.4	105.4 ± 1.8
K (mEQ/L)	4.3 ± 0.1	4.4 ± 0.1	4.4 ± 0.2	4.5 ± 0.3
Ca (mg/dL)	10.4 ± 0.2	10.4 ± 0.1	10.5 ± 0.2	10.7 ± 0.3**
IP (mg/dL)	5.4 ± 0.3	5.3 ± 0.6	5.5 ± 0.5	6.0 ± 0.5*
AST (IU/L)	90.5 ± 14.6	81.7 ± 12.5	89.4 ± 19.9	83.1 ± 28.0
ALT (IU/L)	51.7 ± 4.4	49.9 ± 5.0	61.8 ± 9.6	67.4 ± 30.0
ALP (IU/L)	423.0 ± 45.8	422.8 ± 30.3	418.0 ± 30.7	405.5 ± 26.0
γ−GTP (IU/L)	< 3	< 3	< 3	3 ± 0[a]
Females				
No. of animals examined	10	10	10	10
TP (g/dL)	5.8 ± 0.2	5.7 ± 0.2	5.8 ± 0.2	6.2 ± 0.2**
A/G ratio	2.7 ± 0.2	2.7 ± 0.2	2.7 ± 0.1	2.8 ± 0.2
Alb (g/dL)	4.2 ± 0.1	4.2 ± 0.1	4.2 ± 0.2	4.6 ± 0.2**
Bil (mg/dL)	0.05 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.04 ± 0.01
Glucose (mg/dL)	99.5 ± 16.7	99.8 ± 15.1	109.2 ± 19.6	114.8 ± 8.0
TG (mg/dL)	38.1 ± 14.4	30.5 ± 10.4	35.7 ± 14.3	39.1 ± 11.5
T-Chol (mg/dL)	66.7 ± 4.6	68.2 ± 7.5	70.4 ± 9.4	78.6 ± 6.0**
BUN (mg/dL)	17.0 ± 2.5	16.7 ± 1.2	16.2 ± 1.3	18.0 ± 2.5
Cre (mg/dL)	0.35 ± 0.03	0.35 ± 0.02	0.36 ± 0.02	0.34 ± 0.01
Na (mEQ/L)	144.5 ± 1.1	144.2 ± 1.4	144.7 ± 1.7	144.5 ± 1.4
Cl (mEQ/L)	106.8 ± 1.8	106.7 ± 1.9	108.6 ± 3.1	107.7 ± 3.7
K (mEQ/L)	4.2 ± 0.1	4.2 ± 0.2	4.2 ± 0.1	4.4 ± 0.3
Ca (mg/dL)	9.8 ± 0.1	9.8 ± 0.2	9.9 ± 0.2	10.2 ± 0.2**
IP (mg/dL)	4.3 ± 0.4	4.5 ± 0.4	4.6 ± 0.5	5.0 ± 0.5**
AST (IU/L)	79.3 ± 4.6	75.6 ± 4.8	74.0 ± 5.4	72.8 ± 5.1*
ALT (IU/L)	48.2 ± 10.3	46.4 ± 4.6	45.3 ± 7.0	46.7 ± 4.2
ALP (IU/L)	301.3 ± 35.5	288.2 ± 34.0	272.7 ± 25.0	290.3 ± 49.3
γ-GTP (IU/L)	< 3	< 3	< 3	3.3 ± 0.5[b]

Values are means ± SDs.
[a,b]: The mean value was calculated from data above the detection limit (3.0). The number of animals which showed the value below detection limit was 9([a]) and 6([b]).
*, **: Significantly different from the 0 mg/kg BW group at $p < 0.05$ and 0.01, respectively.

considered to not be toxicologically significant because there were no associated histopathological changes. Significant increases in relative brain weights for both sexes in the 1000 mg/kg BW group and increases in relative heart and testes weights for males in the 1000 mg/kg BW group were considered to be associated with the observed decrease in the final BW.

3.4. Macroscopic and histopathological observations

At necropsy, there were no macroscopic findings related to VPGA administration. Histopathological findings are summarized in Table 5. VPGA administration-related findings were detected in the liver. Centrilobular hepatocellular hypertrophy was observed for both sexes in the 1000 mg/kg BW group (Fig. 3). Although the detailed mechanisms remain unclear, the hepatocellular changes could be attributable to an increase in cytosolic protein or in the number of some organelles (Thoolen et al., 2010). Additionally, decreased fatty changes in hepatocytes were observed for males in the 1000 mg/kg BW group. Hepatocellular hypertrophy with a decrease in fatty changes could reflect imbalances in protein and lipid metabolism (Hafner et al., 2011; Maronpot et al., 2010), which could be related to the increases in T-Chol, TP, and A/G ratio observed for both sexes in the 1000 mg/kg BW group. Focal necrosis of hepatocytes was sporadically observed with no significant differences between the control group and VPGA administration group.

Histopathological changes observed in other organs were detected only in the control groups or were well-known spontaneous lesions in F344 rats (Boorman et al., 1990; Frazier et al., 2012; Renne et al., 2009), indicating that these lesions were not adverse effects of VPGA. Although rats of both sexes in the 1000 mg/kg BW group showed clinical signs of loss of vigorous activity and listlessness, there were no histopathological changes in the nervous systems.

3.5. Comparison with toxicological profiles of other vanilloid compounds

The toxicological profiles of hydroxy- and alkoxy-substituted benzyl derivatives used as flavor ingredients, including vanillin and ethyl vanillin, were reviewed by FEMA GRAS assessment (Adams et al., 2005). A 13-week subchronic toxicity study for ethyl vanillin at estimated daily doses of 0, 500, 1000, and 2000 mg/kg BW in the diet using Sprague-Dawley rats demonstrated that no compound-related changes were detected in observations of the general condition of rats and in

Table 4
Organ weights data for F344 rats administered with vanillin propylene glycol acetal (VPGA) for 13 weeks.

		VPGA (mg/kg BW)			
		0	100	300	1000
Males					
No. of animals examined		10	10	10	10
Final body weight	(g)	334.3 ± 9.4	328.2 ± 16.6	323.4 ± 11.4	300.6 ± 16.6**
Brain	(g)	1.96 ± 0.03	1.98 ± 0.04	1.95 ± 0.03	1.94 ± 0.03
	(g/100 g BW)	0.59 ± 0.02	0.60 ± 0.03	0.60 ± 0.02	0.65 ± 0.03**
Thymus	(g)	0.17 ± 0.02	0.16 ± 0.02	0.17 ± 0.01	0.15 ± 0.02*
	(g/100 g BW)	0.05 ± 0.01	0.05 ± 0.01	0.05 ± 0.00	0.05 ± 0.01
Heart	(g)	0.92 ± 0.04	0.89 ± 0.06	0.90 ± 0.06	0.88 ± 0.05
	(g/100 g BW)	0.28 ± 0.01	0.27 ± 0.01	0.28 ± 0.01	0.29 ± 0.02*
Lungs	(g)	1.02 ± 0.17	0.95 ± 0.08	0.91 ± 0.07	0.90 ± 0.08[a]
	(g/100 g BW)	0.31 ± 0.05	0.29 ± 0.03	0.28 ± 0.02	0.30 ± 0.03[a]
Spleen	(g)	0.58 ± 0.03	0.56 ± 0.03	0.59 ± 0.02	0.56 ± 0.03
	(g/100 g BW)	0.17 ± 0.01	0.17 ± 0.01	0.18 ± 0.01*	0.19 ± 0.01**
Liver	(g)	7.84 ± 0.34	7.61 ± 0.5	7.78 ± 0.36	8.09 ± 0.65
	(g/100 g BW)	2.34 ± 0.06	2.32 ± 0.08	2.40 ± 0.06*	2.69 ± 0.10**
Kidneys	(g)	1.98 ± 0.10	1.86 ± 0.09*	1.90 ± 0.08	1.85 ± 0.11*
	(g/100 g BW)	0.59 ± 0.03	0.57 ± 0.02	0.59 ± 0.02	0.62 ± 0.02*
Adrenals	(mg)	37.9 ± 5.5	35.0 ± 3.9	33.3 ± 2.8[a]	35.5 ± 4.5
	(mg/100 g BW)	11.3 ± 1.6	10.7 ± 1.2	9.3 ± 3.4[a]	11.8 ± 1.6
Testes	(g)	3.06 ± 0.12	3.02 ± 0.14	3.09 ± 0.10	3.09 ± 0.09
	(g/100 g BW)	0.92 ± 0.04	0.92 ± 0.05	0.96 ± 0.04	1.03 ± 0.06**
Females					
No. of animals examined		10	10	10	10
Final body weight	(g)	175.4 ± 7.5	175.0 ± 6.1	176.4 ± 7.5	163.0 ± 6.1**
Brain	(g)	1.80 ± 0.04	1.81 ± 0.03	1.82 ± 0.03	1.79 ± 0.03
	(g/100 g BW)	1.03 ± 0.04	1.03 ± 0.03	1.03 ± 0.04	1.10 ± 0.04**
Thymus	(g)	0.14 ± 0.01	0.15 ± 0.01	0.15 ± 0.01	0.13 ± 0.01[a]
	(g/100 g BW)	0.08 ± 0.01	0.09 ± 0.01	0.08 ± 0.01	0.07 ± 0.03[a]
Heart	(g)	0.54 ± 0.03	0.54 ± 0.02	0.56 ± 0.03	0.53 ± 0.02
	(g/100 g BW)	0.31 ± 0.01	0.31 ± 0.01	0.32 ± 0.02	0.32 ± 0.02
Lungs	(g)	0.62 ± 0.06	0.60 ± 0.03	0.61 ± 0.05	0.60 ± 0.06
	(g/100 g BW)	0.35 ± 0.03	0.34 ± 0.01	0.35 ± 0.03	0.37 ± 0.04
Spleen	(g)	0.38 ± 0.02	0.38 ± 0.02	0.39 ± 0.02	0.36 ± 0.02
	(g/100 g BW)	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01	0.22 ± 0.01
Liver	(g)	3.80 ± 0.14	3.84 ± 0.18	3.95 ± 0.21	4.00 ± 0.17
	(g/100 g BW)	2.17 ± 0.07	2.20 ± 0.04	2.24 ± 0.09	2.45 ± 0.10**
Kidneys	(g)	1.10 ± 0.06	1.08 ± 0.06	1.10 ± 0.05	1.06 ± 0.06
	(g/100 g BW)	0.63 ± 0.03	0.61 ± 0.02	0.63 ± 0.03	0.65 ± 0.04
Adrenals	(mg)	39.6 ± 5.5	40.5 ± 4.0	36.8 ± 2.0	38.5 ± 3.8
	(mg/100 g BW)	22.6 ± 3.6	23.2 ± 2.0	21.0 ± 1.5	23.7 ± 2.7
Ovaries	(mg)	45.7 ± 2.7	49.4 ± 6.7	47.2 ± 7.0	44.4 ± 6.3
	(mg/100 g BW)	26.1 ± 2.2	28.2 ± 4.1	26.7 ± 3.5	27.2 ± 3.4

Values are mean ± SDs.
[a]: Number of effective animals was reduced to nine due to the failure of measurement.
*, **: Significantly different from the 0 mg/kg BW group at $p < 0.05$ and 0.01, respectively.

hematological examinations (Adams et al., 2005). In this study, histopathological analysis revealed peribiliary inflammatory changes in the livers of rats in the 2000 mg/kg BW group without parenchymal changes. A 2-year combined study for chronic toxicity and carcinogenicity of vanillin and ethyl vanillin in rats at estimated daily doses of 250, 500, and 1000 mg/kg BW in the diet showed no toxic or carcinogenic effects of both compounds with regard to general condition, hematological examinations, and histopathological examinations (Hagan et al., 1967). Based on these reports, we concluded that the toxic effects of VPGA are clearly different from those of vanillin and ethyl vanillin, indicating that even compounds with similar structure could have different toxicological profiles.

4. Conclusion

In the current 13-week subchronic toxicity study of VPGA, loss of vigorous activity and listlessness was observed immediately after administration in both sexes at 1000 mg/kg BW. Reduction of BW gain was noted in both sexes at 1000 mg/kg BW. Serum biochemistry analyses demonstrated increases in TP, Alb, T-Chol, Ca, IP, and γ-GTP in both sexes at 1000 mg/kg BW and increases in the A/G ratio and BUN for males in the 1000 mg/kg BW group. Increased relative liver weights were detected for both sexes at 1000 mg/kg BW. Following histopathological examination, centrilobular hepatocellular hypertrophy in the liver was observed for both sexes at 1000 mg/kg BW. In addition, the incidence of fatty changes in hepatocytes in males in the 1000 mg/kg BW group was decreased compared with that in control rats. Taken together, we concluded that the NOAEL for VPGA was 300 mg/kg BW/day for both sexes based on our current findings. The NOAEL of 300 mg/kg BW/day is about 200,000-fold greater than the estimated dietary intake of 88 μg/person/day in Europe based on an average human body weight of 60 kg. Thus, these results provide scientific evidence to support the JECFA's evaluation that there are no safety concerns using VPGA as a flavoring agent.

Table 5
Histopathological findings for F344 rats administered with vanillin propylene glycol acetal (VPGA) for 13 weeks.

Organs	Findings	VPGA (mg/kg BW)			
		0	100	300	1000
Male	No. of animals examined	10	10	10	10
Liver	Hypertrophy, hepatocytes, centrilobular (±)	0	0	0	3
	Fatty change, hepatocytes (± /+)	10 (6/4)	10 (6/4)	10 (9/1)	0**
	Focal necrosis, hepatocytes (±)	1	0	1	2
Kidney	Regenerative tubules (±)	4	–	–	4
Lung	Osseous metaplasia (±)	1	–	–	0
	Foamy cells, alveoli (±)	1	–	–	0
Testis	Atrophy, seminiferous tubules (+)	1	–	–	0
Epididymis	Exfoliated germinal cells, epididymal ducts (±)	1	–	–	0
Forestomach	Degeneration, keratin layer, focal (±)	1	–	–	0
Heart	Myocardial necrosis, focal (± /+)	4 (3/1)	–	–	2 (1/1)
Pituitary	Cyst, anterior lobe (±)	0	–	–	1
Thyroid	Ultimobranchial rest (±)	0	–	–	2
Nasal cavity	Intracytoplasmic eosinophilic body (±)	2	–	–	1
Female	No. of animals examined	10	10	10	10
Liver	Hypertrophy, hepatocytes, centrilobular (±)	0	0	0	5*
	Focal necrosis, hepatocytes (±)	2	1	1	0
Kidney	Regenerative tubules (±)	1	–	–	1
Lung	Osseous metaplasia (±)	1	–	–	0
Forestomach	Cyst, squamous (±)	1	–	–	0
Heart	Myocardial necrosis, focal (±)	2	–	–	0
Adrenal	Focal inflammation, cortex, unilateral (±)	1	–	–	0
Thyroid	Ultimobranchial rest (±)	0	–	–	2
Harderian gland	Focal inflammation, unilateral (±)	1	–	–	0
Nasal cavity	Intracytoplasmic eosinophilic body (± /+)	3 (2/1)	–	–	0
Bone marrow	Microgranuloma (±)	0	–	–	1

-: Not examined, ± : Very slight, +: Slight.
*, **: Significantly different from the 0 mg/kg BW group at $p < 0.05$ and 0.01, respectively.

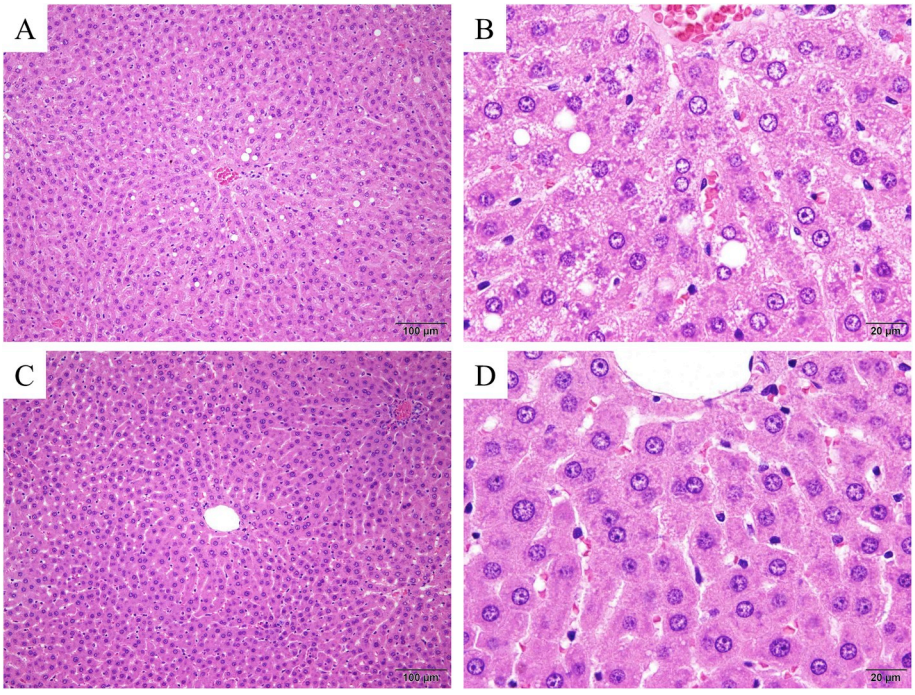

Fig. 3. Representative findings in the livers of male F344 rats administered with corn oil (control) or 1000 mg/kg BW/day vanillin propylene glycol acetal for 13 weeks. (A and B) Normal livers in the control group. (C and D) Centrilobular hepatocellular hypertrophy with decreased fatty changes in hepatocytes in the 1000 mg/kg BW group. Hematoxylin & eosin (H&E) stain.

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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