of all three 3-MCPD fatty acid esters as well as 3-MCPD (40 mg/kg B.W./day). The initial segment of the epididymis is essential for male fertility (Yeung et al. 1998), and after orchidectomy, apoptosis first appears in this site (Fan and Robaire 1998). Blocking lumicrine factors from entering the epididymis results in a wave of apoptosis of epithelial cells of the initial segment, which does not occur in other epididymal regions and is not reversed by androgen replacement (Turner and Riley 1999). It is reported that this part of epididymis is uniquely regulated to prevention of apoptosis with testicular lumicrine factors through ERK, STST and NFKB pathways (Xu et al. 2011). Further studies regarding the effects of 3-MCPD and related fatty acid esters on the production of lumicrine factors and male infertility are required.

Our present experiment revealed a basically similar outcome to the study of Barocelli et al. (2011). As a general difference, the intensity of toxicological effects in rats treated with either CDP, CMP or CDO for 13 weeks were almost equivalent to those with equimolar 3-MCPD in our experiment, although the earlier authors mentioned that the effect of CDP was milder than that of equimolar 3-MCPD, particularly in the testis where it was one-thirds. As other differences, significant anemia was here observed only in ester-treated but not in 3-MCPD-treated rats, apoptosis in the initial segment of the epididymis being the major change instead of testicular damage. Possible reasons for the differences are (1) Higher dose of esters and volume of oil used for vehicle, which might effect on lipase induction and hydrolysis efficacy; (2) The strain difference; and (3) Interval period of two days per week for administration. With the fact of acute renal toxicity seen only by gavage administration, for risk assessment, we should consider the route and type of administration in toxicity studies and whether they are relevant to human exposure. Regarding exposure levels for 3-MCPD and its esters, based on national estimates from a wide range of foods including soy sauce and soy-sauce-related products provided for 10 countries (Denmark, Finland, France, Germany, Ireland, the Netherlands, Norway, Sweden, Thailand, UK), in 2007, JECFA estimated 3-MCPD of 0.7 μg/kg B.W. per day could be taken to represent the average for the general population, and an intake of 2.3 μg/kg B.W. per day could be taken to represent consumers with a high intake (WHO 2007). Recent analysis of 3-MCPD esters in food reported that 25.35 and 14.40 mg/kg of overall 3-MCPD esters were detected in grape seed oil and palm oil, respectively (Yamazaki et al. 2013). It might be more practical to estimate total 3-MCPD including its esters for safety evaluation, because they might be easily interconverted.

In conclusion, no obvious differences of toxicological profile among CDP, CMP and CDO were here observed. Compared with 3-MCPD, the toxicities of the 3-MCPD fatty acid esters were lower in the acute phase regarding tubular necrosis and equivalent in the subchronic phase, as evidenced by kidney organ weight gain in males and females and apoptosis in the initial segment of the epididymis. Based on significant change in absolute and relative kidney weights, no-observed-adverse-effect levels (NOAELs) in the present experiment in F344 rats were estimated to be 14 mg/kg B.W. for CDP, 8 mg/kg B.W./day for CMP and 15 mg/kg B.W./day for CDO, equimolar to 2.5 mg/kg B.W. 3-MCPD in both males and females.

Acknowledgments We thank Ms. A. Saikawa and Ms. Y. Komatsu for their expert technical assistance in processing histological materials and Dr. G. Matsui for useful discussion and comments on histopathological analysis. This study was funded by the Food Safety Commission of Japan.

Conflict of interest The authors declare that they have no conflicts of interest regarding this work.

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Food and Chemical Toxicology 74 (2014) 68–75

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

A 13-week subchronic toxicity study of ferric citrate in F344 rats

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ARTICLE INFO

Article history:
Received 29 July 2014
Accepted 10 September 2014
Available online 26 September 2014

Keywords:
Ferric citrate
Food additive
Subchronic toxicity
F344 rats
Eosinophilic enteritis

ABSTRACT

Ferric citrate has been used as a food additive for supplementation of iron. We performed a 13-week subchronic toxicity study of ferric citrate in F344 rats with oral administration in the diet at concentrations of 0%, 0.25%, 1.0%, and 4.0%. Reduction of body weight gain was noted in 4.0% males and females. On hematology assessment, decreases of red blood cells and lymphocytes and increases of platelets and eosinophils were noted in 4.0% males and females. Serum biochemistry demonstrated increased iron and decreased total protein and transferrin in both sexes treated with 4.0% ferric citrate. In addition, an increase of serum inorganic phosphorus levels was noted in 4.0% females. Regarding organ weights, an increase of relative spleen weights was detected in 4.0% males and females and a decrease of absolute and relative heart weights in 4.0% females. On histopathological assessment, colitis with infiltration of eosinophils and hyperplasia of mucosal epithelium, eosinophilic infiltration in mesenteric lymph nodes, and increased hemosiderosis in spleen were observed as treatment-related toxicological changes in 4.0% males and females. Based on the results, the no-observed-adverse-effect level (NOAEL) of ferric citrate was estimated to be 1.0% (596 mg/kg bw/day for males and 601 mg/kg bw/day for females).

1. Introduction

Ferric citrate has been used as a food additive for nutritional supplementation of iron in various types of foodstuffs, such as flour or formula milk. It is generally synthesized from ferric hydroxide and citric acid and is officially registered on the list of designated additives by the Ministry of Health, Labour and Welfare of Japan. In the US, monohydrate of ferric citrate has also been used as a food ingredient and listed in the Code of Federal Regulations (CFR) as one of the Direct Food Substances Affirmed as Generally Recognized as Safe (21 CFR 184.1298). In recent years, ferric citrate has demonstrated potential as a novel adsorbent of phosphate for the prevention and treatment of hyperphosphatemia in hemodialysis patients (Dwyer et al., 2013; Iida et al., 2013; Nastou et al., 2014; Sinsakul et al., 2012; Yang et al., 2002; Yokoyama et al., 2012).

While it is well known that iron exerts strong toxicity on intravenous injection or subcutaneous administration, iron absorption from the diet in the intestinal mucosa is functionally regulated to prevent an iron overload by excessive intake. However, a recent study has suggested that oral administration of ferric citrate in the diet at a concentration of 2% induced fatal weight loss and intestinal disorders in mice (Shirase et al., 2010). In addition, it has been reported that an iron-rich diet with ferric citrate supplementation promoted estrogen-induced renal tumorigenesis in Syrian hamsters (Wyllie and Liehr, 1998). Although these experiments were not appropriate for toxicological evaluation, the results suggest a possibility of toxicity of ferric citrate.

As part of safety evaluation of ferric citrate, a two-year chronic toxicity and carcinogenicity study was earlier performed in B6C3F$_1$ mice (Inai et al., 1994), in which treatment with 0.12% ferric citrate in drinking water for 96 weeks caused no significant toxicity or increase of tumor incidence. With regard to genotoxicity, ferric citrate has proved negative in the Ames test and chromosomal aberration assay with mammalian cells (Ishidate et al., 1984). In spite of the history of usage as a food additive and possible application as a medical product, there have been few reports of *in vivo* animal experiments investigating toxicological effects of ferric citrate with dietary administration. Our laboratory has provided new knowledge concerning the toxicology of a number of food additives and contaminants such as sodium iron chlorophyllin, garden balsam extract and 3-monochloropropane-1,2-diol in rodent models (Onami

Abbreviations: ACE, angiotensin converting enzyme; A/G, albumin/globulin ratio; Alb, albumin; ALP, alkaline phosphatase; ALT, alanine aminotransferase; ANOVA, analysis of variance; AST, aspartate aminotransferase; Bil, total bilirubin; BUN, urea nitrogen; Ca, calcium; CFR, Code of Federal Regulations; Cl, chlorine; Cre, creatinine; Ebl, erythroblasts; EGIDs, eosinophilic gastrointestinal disorders; Fe, iron; 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; WBC, white blood cell count.

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http://dx.doi.org/10.1016/j.fct.2014.09.005
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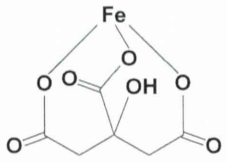

Fig. 1. Chemical structure of ferric citrate.

et al., 2014; Toyoda et al., 2013, 2014). In the present study, a 13-week subchronic toxicity study with oral administration of ferric citrate in the diet to F344 rats was performed to establish a no-observed-adverse-effect level (NOAEL).

2. Materials and methods

2.1. Test chemical

The ferric citrate (Fig. 1) sample provided by the Division of Standards and Evaluation, Department of Food Safety, Ministry of Health, Labour and Welfare of Japan with the cooperation of Nichia Corp. (Lot No. 5265-01-1108; Tokushima, Japan) was a powder with reddish brown color. We confirmed that an iron composition of the test chemical was 17.6% which meets the criteria (16.5–18.5%) and other contaminants were below the reference value, according to the Japanese Standards of Food Additives (Ministry of Health, Labour and Welfare of Japan, 2009). For the present study, the compound was mixed into powdered basal diet (CRF-1; Oriental Yeast, Tokyo, Japan) every 2 weeks and kept in a refrigerator until use. The stability of ferric citrate in the mixed diet was evaluated by Japan Frozen Foods Inspection Corporation (Tokyo, Japan). After storage for 4 weeks at 4°C and 8 days at room temperature, the residual amount of ferric citrate in the experimental diet was confirmed to be 90.2% and 95.1% (as contents of citric acid), respectively.

2.2. Experimental animals

A total of 40 male and 40 female specific pathogen-free rats (F344/DuCrj, 5-week-old) were purchased from Charles River Japan (Yokohama, Japan) and used after one-week acclimatization. The animals were housed 3–4 rats per polycarbonate cage with soft chip bedding (Sankyo Labo Service, Tokyo, Japan) in a room with a barrier system controlled for the light/dark cycle (12-hr), ventilation (air-exchange rate 18 times per hr), 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 basal diet with or without the test chemical. At the beginning of the experiment, the animals were randomly allocated to four groups of 10 male and 10 female rats each, based on their body weights measured just before starting the test chemical treatment.

2.3. Study design

In a preliminary 4-week palatability study of ferric citrate with administration at the highest dose of 5.0% (w/w), significant decrease of body weight gain (>20% compared to the controls) was observed in males and females (data not shown). From these results, doses of ferric citrate were decided as 0%, 0.25%, 1.0%, and 4.0% (w/w) in both sexes for the present 13-week toxicity study, with diet replacement every week.

General conditions and mortality were checked daily and body weights were measured once a week during the experimental period. The amounts of supplied and residual diet were weighed weekly in order to calculate the average daily food intake and test chemical intake through the entire treatment period. All rats were fasted overnight at the completion of the treatment, and blood samples were collected from the abdominal aorta under deep anesthesia, caused by inhalation of isoflurane, for hematology and serum biochemistry. The present study design was basically in accordance with Guidelines for Designation for Food Additives and for Revision of Standards for Use of Food Additives of Japan (Ministry of Health, Labour and Welfare of Japan, 1996), with minor changes, and 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): 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), and platelet count (PLT). Blood smears were processed for May–Giemsa staining and counting of erythroblasts (Ebl) and differential leukocytes using a Microx HEG-50S (Sysmex). 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), γ-glutamyl transpeptidase (γ-GTP), transferrin, and iron (Fe).

2.5. Organ weights and histopathological assessment

Complete necropsy was performed for all animals, and the brain, thymus, lungs, heart, spleen, liver, adrenal glands, kidneys, and gonads were weighed. These organs and the following tissues were fixed in 10% neutral-buffered formalin, and paraffin-embedded sections were routinely prepared and stained with hematoxylin and eosin for histopathological examination: 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, bulbourethral glands, uterus, vagina, pituitary gland, thyroid glands, parathyroid glands, spinal cord with vertebrae, trigeminal nerve, sciatic nerve, harderian glands, femoral skeletal muscle, and nasal cavity. 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 first performed on all tissues of the control and highest dose groups. If a chemical treatment-related change appeared at the highest dose, the relevant tissues from the lower dose groups then also underwent examination.

2.6. Statistical analysis

Variance in data for body and organ weights, as well as the results of hematology and serum biochemistry, was checked by Bartlett's test for homogeneity. When the data were homogeneous, one-way analysis of variance (ANOVA) was conducted. In heterogeneous cases, the Kruskal–Wallis test was applied. When statistically significant differences were indicated, the Dunnett's or non-parametric Dunnett's multiple test were employed for comparisons between control and other groups. For incidences of histopathological findings, the Fisher's exact probability test was applied. P values of < 0.05 were considered to be statistically significant.

3. Results

3.1. In-life parameters

No clinical signs were noted throughout the experimental period, and all animals survived until the scheduled necropsy. Body weight gain of both sexes was significantly reduced with 4.0% ferric citrate-treatment compared to the controls from the 1st week until the end of the experiment except in the 9th and 12th weeks in females (Fig. 2a). Comparing with the control groups, the average body weight gain during the experiment in 4.0% males and females were reduced by 13.4% and 5.9%, respectively. Daily food intake of 4.0% males and females was slightly higher than in the other groups (Fig. 2b). Since there was no avoidance of the experimental diet, average intakes of ferric citrate per body weight was dose-dependent (Table 1).

Table 1
Body weight and food intake data for F344 rats treated with ferric citrate for 13 weeks.

Group (%)	Body weight (g)		Food intake	Chemical intake
	Initial	Final	(g/rat/day)	(mg/kg bw/day)
Males				
0	106.0 ± 5.2	305.0 ± 10.7	13.9	0
0.25	105.8 ± 4.1	301.5 ± 12.8	13.4	143.9
1.0	105.9 ± 4.4	304.5 ± 18.7	13.9	595.9
4.0	105.7 ± 4.1	266.6 ± 10.1**	14.5	2834.7
Females				
0	94.1 ± 3.0	174.8 ± 5.8	9.2	0
0.25	93.3 ± 3.5	173.2 ± 9.5	8.8	147.7
1.0	93.7 ± 3.4	174.7 ± 6.1	9.0	601.4
4.0	93.4 ± 2.8	166.1 ± 6.0*	10.0	2845.6

Values are mean ± S.D. * and ** Significantly different from the controls at $p < 0.05$ and < 0.01, respectively.

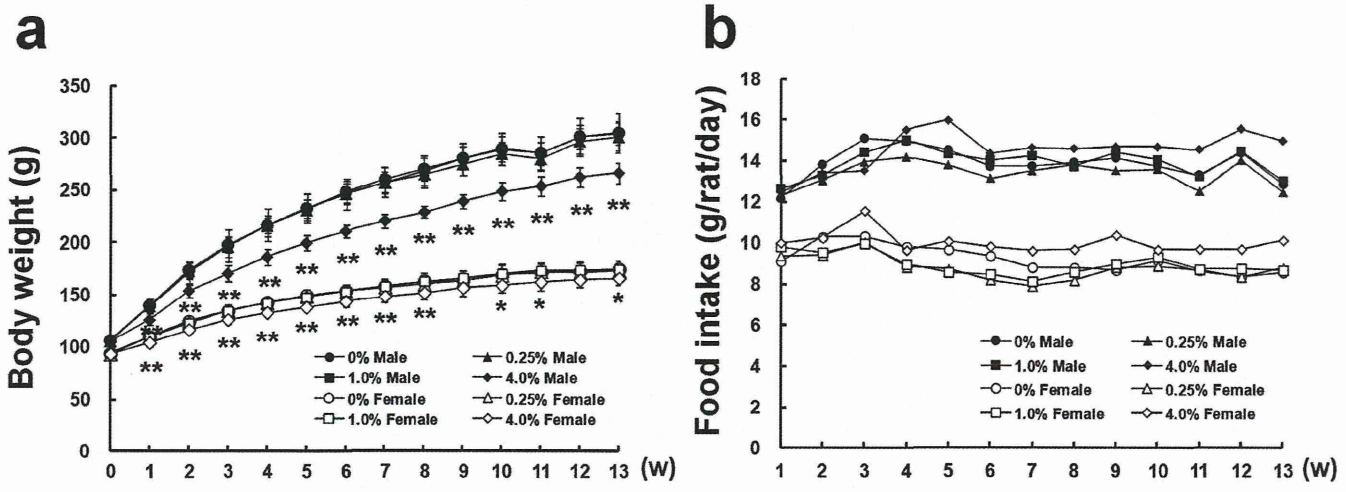

Fig. 2. Body weight (a) and daily food intake (b) data for male and female F344 rats treated with ferric citrate for 13 weeks. * and ** Significantly different from the controls at $p < 0.05$ and 0.01, respectively. Each group contained 10 animals.

3.2. Hematology and serum biochemistry

Data for hematology and serum biochemistry are summarized in Tables 2 and 3, respectively. Significant decreases of RBC and lymphocytes and increases of PLT and eosinophils were detected in 4.0% males and females (Table 2). In males, significant increases of MCV and MCH were observed in the 1.0% and 4.0% groups and with 4.0%, respectively. In serum biochemistry, increase of serum Fe levels and decreases of TP and transferrin were noted in 4.0% males and females (Table 3). Significant decrease of transferrin was also detected in 1.0% females. In males, increase of Na and decreases of AST and ALT were observed in the 4.0% group. In females, significant increase of IP and

Table 2
Hematology data for F344 rats treated with ferric citrate for 13 weeks.

Dose level (%)	0	0.25	1.0	4.0
Males				
No. of animals examined	10	10	10	10
WBC × 10^2/μl	38.8 ± 11.6	33.9 ± 6.9	31.7 ± 4.4	45.0 ± 11.1
RBC × 10^4/μl	908.0 ± 57.2	889.3 ± 70.0	849.1 ± 47.9	838.3 ± 48.5*
HGB g/dl	16.0 ± 1.0	15.6 ± 1.0	15.2 ± 0.8	16.1 ± 0.7
HCT %	49.5 ± 2.7	49.1 ± 2.7	47.4 ± 2.3	49.3 ± 2.1
MCV fl	54.6 ± 1.1	55.4 ± 1.4	55.9 ± 0.9*	58.9 ± 1.3**
MCH pg	17.7 ± 0.3	17.6 ± 0.4	17.9 ± 0.2	19.2 ± 0.5**
MCHC g/dl	32.3 ± 0.7	31.8 ± 0.5	32.1 ± 0.5	32.6 ± 0.6
PLT × 10^4/μl	68.2 ± 11.1	64.2 ± 11.5	65.5 ± 5.3	79.5 ± 4.7*
Differential cell count				
Band %	0.1 ± 0.2	0.3 ± 0.4	0.3 ± 0.3	0.3 ± 0.3
Seg %	28.2 ± 4.4	30.9 ± 8.4	31.7 ± 5.8	34.9 ± 3.8
Eosin %	1.3 ± 0.8	1.0 ± 0.5	1.3 ± 0.9	9.5 ± 7.0**
Baso %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymph %	70.2 ± 4.6	67.6 ± 8.3	66.4 ± 5.8	55.1 ± 7.7**
Mono %	0.3 ± 0.4	0.3 ± 0.4	0.3 ± 0.4	0.3 ± 0.4
Ebl (per 200 WBC)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.3
Females				
No. of animals examined	9[a]	10	10	10
WBC × 10^2/μl	21.1 ± 3.2	17.9 ± 5.7	17.2 ± 2.2	24.5 ± 6.2
RBC × 10^4/μl	811.1 ± 33.9	770.5 ± 98.0	781.7 ± 48.2	749.9 ± 34.1*
HGB g/dl	15.3 ± 0.6	14.5 ± 1.9	15.0 ± 1.0	14.4 ± 0.7
HCT %	46.8 ± 1.7	45.3 ± 5.3	46.2 ± 2.7	44.4 ± 1.6
MCV fl	57.7 ± 0.9	58.9 ± 1.7	59.1 ± 1.1	59.2 ± 1.3
MCH pg	18.8 ± 0.4	18.8 ± 0.7	19.2 ± 0.2	19.3 ± 0.3
MCHC g/dl	32.7 ± 0.6	31.9 ± 0.8	32.4 ± 0.6	32.5 ± 0.9
PLT × 10^4/μl	69.5 ± 8.3	69.7 ± 9.7	71.5 ± 7.1	80.6 ± 8.1*
Differential cell count				
Band %	0.2 ± 0.3	0.2 ± 0.2	0.1 ± 0.2	0.4 ± 0.5
Seg %	29.1 ± 2.8	29.2 ± 5.4	27.1 ± 4.5	32.7 ± 6.3
Eosin %	1.7 ± 0.8	0.9 ± 0.8	1.8 ± 2.1	10.2 ± 3.0*
Baso %	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Lymph %	68.2 ± 3.4	69.6 ± 5.6	70.8 ± 3.6	56.2 ± 8.4*
Mono %	0.8 ± 1.0	0.2 ± 0.3	0.3 ± 0.4	0.6 ± 0.5
Ebl (per 200 WBC)	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.6	0.0 ± 0.0

Values are mean ± S.D. * and ** Significantly different from the controls at $p < 0.05$ and < 0.01, respectively. [a] The number of effective animals was reduced to nine due to incidental nephroblastoma.

Table 3
Serum biochemistry data for F344 rats treated with ferric citrate for 13 weeks.

Dose level (%)	0	0.25	1.0	4.0
Males				
No. of animals examined	10	10	10	10
TP (g/dl)	6.3 ± 0.2	6.3 ± 0.2	6.4 ± 0.1	6.1 ± 0.2**
A/G	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1	1.8 ± 0.1
Alb (g/dl)	4.0 ± 0.1	4.1 ± 0.1	4.1 ± 0.2	3.9 ± 0.1
Bil (mg/dl)	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Glucose (mg/dl)	170.2 ± 16.2	178.7 ± 19.6	174.9 ± 22.2	151.6 ± 18.1
TG (mg/dl)	87.5 ± 23.6	94.7 ± 20.4	102.2 ± 32.2	82.9 ± 16.4
T-Chol (mg/dl)	58.3 ± 4.3	58.4 ± 5.5	61.1 ± 4.2	57.7 ± 3.6
BUN (mg/dl)	20.7 ± 2.1	20.8 ± 3.1	20.6 ± 2.1	21.6 ± 2.7
Cre (mg/dl)	0.28 ± 0.02	0.28 ± 0.03	0.28 ± 0.02	0.26 ± 0.03
Na (mEq/l)	141.0 ± 0.5	141.8 ± 0.6	141.6 ± 2.0	142.4 ± 1.1**
Cl (mEq/l)	100.7 ± 1.2	101.5 ± 0.7	100.7 ± 1.6	100.5 ± 0.8
K (mEq/l)	4.6 ± 0.2	4.5 ± 0.2	4.5 ± 0.2	4.4 ± 0.2
Ca (mg/dl)	9.8 ± 0.3	10.0 ± 0.3	9.9 ± 0.4	9.9 ± 0.5
IP (mg/dl)	5.1 ± 0.4	4.9 ± 0.5	5.0 ± 0.6	6.3 ± 1.2
AST (IU/l)	81.3 ± 5.6	78.2 ± 8.3	85.6 ± 9.8	72.0 ± 3.5*
ALT (IU/l)	53.4 ± 5.2	56.1 ± 7.2	60.6 ± 6.9*	41.7 ± 4.8**
ALP (IU/l)	410.0 ± 41.4	429.4 ± 67.4	386.2 ± 32.2	371.1 ± 34.9
γ-GTP (IU/l)	<3	<3	<3	<3
Transferrin (mg/dl)	148.0 ± 3.3	148.6 ± 5.1	150.5 ± 5.5	141.5 ± 4.7*
Fe (μg/dl)	113.4 ± 14.3	125.1 ± 11.1	109.0 ± 9.9	165.6 ± 48.1**
Females				
No. of animals examined	9[a]	10	10	10
TP (g/dl)	6.2 ± 0.1	6.3 ± 0.3	6.2 ± 0.3	5.6 ± 0.2**
A/G	2.1 ± 0.2	2.0 ± 0.1	2.0 ± 0.1	2.0 ± 0.1
Alb (g/dl)	4.2 ± 0.1	4.2 ± 0.2	4.1 ± 0.1	3.8 ± 0.1**
Bil (mg/dl)	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
Glucose (mg/dl)	109.0 ± 19.7	137.5 ± 22.5	127.6 ± 31.2	111.5 ± 24.6
TG (mg/dl)	30.0 ± 6.3	46.5 ± 25.7	42.0 ± 36.0	41.9 ± 19.5
T-Chol (mg/dl)	74.3 ± 12.9	83.9 ± 10.5	82.4 ± 8.7	68.5 ± 4.9
BUN (mg/dl)	17.3 ± 1.4	19.4 ± 2.7	18.6 ± 2.9	20.0 ± 2.0
Cre (mg/dl)	0.31 ± 0.04	0.30 ± 0.02	0.30 ± 0.03	0.31 ± 0.02
Na (mEq/l)	142.7 ± 0.9	142.5 ± 1.4	142.5 ± 1.4	142.0 ± 1.2
Cl (mEq/l)	103.1 ± 0.6	103.1 ± 1.2	103.1 ± 1.1	101.5 ± 1.1**
K (mEq/l)	4.2 ± 0.2	3.9 ± 0.2*	4.1 ± 0.2	4.2 ± 0.1
Ca (mg/dl)	9.8 ± 0.2	9.9 ± 0.3	9.6 ± 0.3	9.5 ± 0.3
IP (mg/dl)	4.1 ± 0.3	4.3 ± 0.9	4.6 ± 1.0	6.6 ± 1.2**
AST (IU/l)	96.9 ± 34.3	72.3 ± 6.4	76.9 ± 7.4	74.3 ± 3.3
ALT (IU/l)	43.4 ± 10.1	39.6 ± 5.5	39.5 ± 4.1	37.1 ± 7.4
ALP (IU/l)	320.6 ± 75.2	288.1 ± 48.8	288.5 ± 36.3	284.8 ± 27.1
γ-GTP (IU/l)	<3	<3	<3	<3
Transferrin (mg/dl)	152.8 ± 5.6	149.7 ± 4.4	145.6 ± 4.9*	137.9 ± 6.8**
Fe (μg/dl)	249.4 ± 36.9	288.8 ± 37.1	272.1 ± 33.4	308.4 ± 57.3*

Values are mean ± S.D. * and ** Significantly different from the controls at $p < 0.05$ and < 0.01, respectively. [a] The number of effective animals was reduced to nine due to incidental nephroblastoma.

decreases of Alb and Cl were found in the 4.0% group. Increase of ALT in 1.0% males and decrease of K levels in 0.25% females were also detected, without any dose-dependence.

3.3. Organ weights

The data for organ weights are shown in Table 4. In males, absolute heart weights were decreased in the 4.0% group compared to the controls. In addition, relative weights of brain, spleen, adrenals, kidneys, and testes were significantly increased in 4.0% males. In females, decrease of absolute and relative heart weights, increase of absolute and relative spleen weights and increase of relative liver weights were observed in the 4.0% group. Absolute weights of liver were also increased in 0.25% females without dose-dependence.

3.4. Histopathological findings

At necropsy, one female rat in the control group had severe enlargement of the right kidney due to a histologically characteristic nephroblastoma. Although nephroblastoma is rare in F344 rats (Montgomery and Seely, 1990), it was considered to be an incidental case, because the rat was one of the untreated controls and no other animals had similar lesions. The animal was therefore excluded from the effective numbers for the hematology, serum biochemistry, organ weight and histopathology.

Histopathological findings are summarized in Table 5. Treatment-related pathological findings were noted in the colon, mesenteric lymph node, spleen and bone marrow (Fig. 3). There was moderate to severe colitis in all 4.0% animals (Fig. 3c-d). In the affected colons, numbers of eosinophils as well as neutrophils and macrophages had infiltrated into the lamina propria and submucosa. Absorptive epithelial cells of colonic mucosa were replaced by hyperplastic mucous cells with frequent mitotic figures. Eosinophilic infiltration was also observed occasionally in the submucosa of stomach, duodenum and cecum in 4.0% males and females with a lower incidence than in the colon. Mesenteric lymph nodes of all animals of 4.0% groups were macroscopically enlarged and histologically showed dilated sinuses featuring aggregation of plasmacytes and hemosiderin deposits. Infiltration of eosinophils was noted not only in the sinuses of mesenteric lymph nodes but also in the surrounding adipose tissue (Fig. 3e). In spleens of 4.0% males and females, the degree of hemosiderin deposition was higher than in

Table 4
Organ weight data for F344 rats treated with ferric citrate for 13 weeks.

Dose level (%)	0	0.25	1.0	4.0
Males				
No. of animals examined	10	10	10	10
Body weight (g)	298.1 ± 11.4	293.7 ± 10.5	298.5 ± 17.8	265.2 ± 11.2**
Absolute (g)				
Brain	1.907 ± 0.050	1.934 ± 0.036	1.902 ± 0.052	1.888 ± 0.050
Thymus	0.183 ± 0.012	0.170 ± 0.029	0.198 ± 0.040	0.173 ± 0.032
Lung	0.915 ± 0.126	0.878 ± 0.048	0.886 ± 0.118	0.825 ± 0.077
Heart	0.828 ± 0.043	0.837 ± 0.025	0.850 ± 0.049	0.760 ± 0.039**
Spleen	0.601 ± 0.035	0.606 ± 0.031	0.599 ± 0.046	0.636 ± 0.033
Liver	6.867 ± 0.363	6.940 ± 0.522	6.977 ± 0.459	6.476 ± 0.427
Adrenals	0.034 ± 0.005	0.036 ± 0.003	0.037 ± 0.004	0.035 ± 0.003
Kidneys	1.746 ± 0.068	1.727 ± 0.092	1.747 ± 0.102	1.678 ± 0.098
Testes	2.956 ± 0.173	2.964 ± 0.095	2.926 ± 0.130	2.919 ± 0.093
Relative (%)				
Brain	0.641 ± 0.032	0.659 ± 0.020	0.639 ± 0.037	0.713 ± 0.030**
Thymus	0.061 ± 0.005	0.058 ± 0.009	0.066 ± 0.014	0.065 ± 0.011
Lung	0.308 ± 0.046	0.299 ± 0.021	0.296 ± 0.028	0.311 ± 0.030
Heart	0.278 ± 0.016	0.285 ± 0.007	0.285 ± 0.006	0.287 ± 0.007
Spleen	0.202 ± 0.009	0.206 ± 0.008	0.201 ± 0.008	0.240 ± 0.009**
Liver	2.304 ± 0.088	2.363 ± 0.156	2.338 ± 0.096	2.441 ± 0.097
Adrenals	0.011 ± 0.002	0.012 ± 0.001	0.013 ± 0.001	0.013 ± 0.001**
Kidneys	0.586 ± 0.024	0.588 ± 0.020	0.586 ± 0.029	0.633 ± 0.029**
Testes	0.993 ± 0.065	1.010 ± 0.041	0.983 ± 0.061	1.102 ± 0.050**
Females				
No. of animals examined	9[a]	10	10	10
Body weight (g)	165.4 ± 5.4	167.5 ± 8.3	168.0 ± 5.6	161.7 ± 5.6
Absolute (g)				
Brain	1.795 ± 0.055	1.788 ± 0.052	1.772 ± 0.071	1.740 ± 0.105
Thymus	0.148 ± 0.022	0.149 ± 0.017	0.160 ± 0.018	0.145 ± 0.018
Lung	0.619 ± 0.105	0.612 ± 0.058	0.606 ± 0.054	0.620 ± 0.060
Heart	0.562 ± 0.036	0.563 ± 0.031	0.544 ± 0.026	0.508 ± 0.025**
Spleen	0.378 ± 0.035	0.402 ± 0.042	0.388 ± 0.024	0.438 ± 0.038**
Liver	3.732 ± 0.143	3.916 ± 0.161*	3.751 ± 0.164	3.877 ± 0.142
Adrenals	0.038 ± 0.004	0.038 ± 0.002	0.038 ± 0.004	0.037 ± 0.004
Kidneys	1.096 ± 0.041	1.092 ± 0.048	1.074 ± 0.055	1.082 ± 0.045
Ovaries	0.051 ± 0.014	0.046 ± 0.005	0.049 ± 0.010[b]	0.049 ± 0.009
Relative (%)				
Brain	1.087 ± 0.056	1.069 ± 0.037	1.056 ± 0.058	1.078 ± 0.085
Thymus	0.089 ± 0.013	0.089 ± 0.010	0.095 ± 0.012	0.090 ± 0.010
Lung	0.375 ± 0.063	0.366 ± 0.031	0.361 ± 0.035	0.384 ± 0.039
Heart	0.340 ± 0.024	0.336 ± 0.011	0.324 ± 0.020	0.315 ± 0.013*
Spleen	0.229 ± 0.020	0.239 ± 0.018	0.231 ± 0.014	0.271 ± 0.025**
Liver	2.258 ± 0.092	2.339 ± 0.079	2.234 ± 0.112	2.399 ± 0.070**
Adrenals	0.023 ± 0.002	0.023 ± 0.002	0.022 ± 0.003	0.023 ± 0.003
Kidneys	0.663 ± 0.027	0.652 ± 0.023	0.639 ± 0.035	0.670 ± 0.039
Ovaries	0.031 ± 0.008	0.028 ± 0.003	0.029 ± 0.007[b]	0.030 ± 0.005

Values are mean ± S.D. * and ** Significantly different from the controls at $p < 0.05$ and < 0.01, respectively. [a] and [b] The number of effective animals was reduced to nine due to incidental nephroblastoma and failure of tissue sampling, respectively.

the control groups (Fig. 3f-g). Hematopoietic cells in the bone marrow were increased in 4.0% males and females. Although several lesions in other organs were sporadically detected, no significant treatment-dependent alteration in their incidences was apparent.

4. Discussion

In the present 13-week subchronic toxicity study, there were no significant changes in clinical signs. Significant reduction of body weight gain observed in 4.0% males and females was considered to be a treatment-related effect because daily food intake was not decreased. Hematological analysis demonstrated that significant decrease of RBC and increase of PLT in both sexes and increases of MCV and MCH in males were induced by 4.0% ferric citrate, suggesting a toxicologic influence of treatment. In addition, differential cell counts of leukocytes clearly revealed that there was remarkable eosinophilia of peripheral blood in 4.0% males and females.

In serum biochemistry, decreases of transferrin and TP and increase of Fe levels were observed in both sexes treated with 4.0% ferric citrate. Transferrin is known to play a major role in iron transportation in blood plasma (Gkouvatsos et al., 2012). While increase of serum Fe was considered to be a result of excessive iron intake, the decrease of transferrin may reflect poor nutritional status including low TP levels and reduction of body weight gain. Increase of serum Na levels in 4.0% males and decreases of Alb and Cl in 4.0% females were considered to have no toxicological significance, because there were no abnormalities in related parameters and no significant histopathological changes were noted in the liver and kidney. Although significant decrease of K in 0.25% females, decrease of AST in 4.0% males and fluctuation of ALT in 1.0% and 4.0% males were detected, the lack of any dose-relation suggests no association with test substance exposure.

Serum IP levels were significantly increased in 4.0% females and showed a tendency for increase in 4.0% males, although there was no statistical significance. There was no fluctuation of serum Ca or significant development of histopathological lesions in related organs/tissues such as the thyroid, parathyroid, bone, skeletal muscle and kidney. However, it was considered that the change of IP levels should be evaluated as an adverse effect, because the marked increase compared to the controls demonstrated clear

Table 5
Histopathological findings for F344 rats treated with ferric citrate for 13 weeks.

		Male				Female			
	Dose level (%)	0	0.25	1.0	4.0	0	0.25	1.0	4.0
Organs and findings	No. of animals	10	10	10	10	9[a]	10	10	10
Liver	Microgranuloma	3	–	–	3	5	–	–	4
Spleen	Increased hemosiderosis	0	0	0	10**	0	0	0	6*
Kidney	Regenerative tubules	4	–	–	2	0	–	–	0
	Inflammation, interstitial, focal	1	–	–	1	0	–	–	0
	Hyaline casts	1	–	–	2	0	–	–	0
	Mineralization	0	–	–	0	6	–	–	1
Heart	Mononuclear cell infiltration, focal	8	–	–	8	2	–	–	0
Lung	Mineralization	6	–	–	7	6	–	–	5
	Osseous metaplasia	0	–	–	1	1	–	–	0
Stomach	*Inflammation with eosinophilic infiltration*	0	0	0	3	0	0	0	2
Duodenum	Inflammation with eosinophilic infiltration	0	0	0	1	0	0	0	0
Cecum	Inflammation with eosinophilic infiltration	0	0	0	4	0	0	0	1
Colon	Inflammation with eosinophilic infiltration	0	0	0	10**	0	0	0	10**
	Mucosal hyperplasia	0	0	0	10**	0	0	0	10**
Mesenteric lymph node	Infiltration of eosinophils	0	0	0	10**	0	0	0	10**
	Plasmacytosis	0	0	0	7**	0	0	0	7**
	Hemosiderosis	0	0	1	10**	0	0	0	9**
Pancreas	Mononuclear cell infiltration, focal	1	–	–	1	1	–	–	1
	Atrophy, focal	1	–	–	2	1	–	–	0
Parotid gland	Mononuclear cell infiltration, focal	1	–	–	0	0	–	–	3
	Basophilic cell foci	2	–	–	3	0	–	–	1
Testis	Degeneration/atrophy, tubular, unilateral	1	–	–	0	–	–	–	–
Epididymis	Cell debris, luminal, unilateral	1	–	–	0	–	–	–	–
Prostate	Inflammation, mild	1	–	–	2	–	–	–	–
Spinal cord	Inclusion cyst	0	–	–	1	0	–	–	0
Pituitary gland	Cyst, pars distalis	0	–	–	1	1	–	–	0
Bone marrow	*Increased hematopoiesis*	0	0	0	8**	0	0	0	9**
Eye	Retinal atrophy	1	–	–	0	0	–	–	0
Harderian gland	Mononuclear cell infiltration, focal	1	–	–	0	2	–	–	1

–; Not evaluated. * and ** Significantly different from the controls at $p < 0.05$ and < 0.01, respectively. [a] The number of effective animals was reduced to nine due to incidental nephroblastoma.

dose-dependence. Ferric citrate is known as an absorbent of phosphorus for patients of chronic kidney disease. Iida et al. recently reported that dietary administration of 3.0% ferric citrate induced significant decrease of serum phosphorus levels in both normal rats and chronic renal failure model rats (Iida et al., 2013). They described no lesions associated with eosinophilic infiltration, while 4.0% ferric citrate treatment caused severe eosinophilia and eosinophilic colitis in the present study. Thus, the administration doses and the presence of induced lesions may explain the difference of serum phosphorus, although further investigation is required to clarify the detailed mechanisms.

The significant increase of relative spleen weights observed in 4.0% males and females was considered to be a toxicologic change corresponding to excessive hemosiderosis in the red pulp. Although absolute and relative heart weights were significantly decreased in 4.0% females, no histopathological changes were observed. In dogs and rats, it has been reported that inhibitors of angiotensin converting enzyme (ACE) can induce decrease of heart weights through reduction of circulatory blood flow (Greaves, 2012). In addition, like cardiac dysfunctions such as arrhythmia and tachycardia and the mitochondria injury caused by anthracycline antibiotics, cardiotoxicity without any morphological changes at the light microscopic level is also known (MacKenzie and Alison, 1990). Since the heart weights in 4.0% females were markedly decreased in a dose-dependent manner, it is appropriate that this should be evaluated as toxicity of ferric citrate. Similarly, decrease of absolute heart weights in 4.0% males was estimated as an adverse effect, since no difference of relative heart weights was considered to be due to the reduction of body weight gain. On the other hand, increases of relative weights of brain, adrenals, kidneys and testes in 4.0% males were considered to be associated with the reduction of body weight gain. While significant increase of relative liver weights was noted in 4.0% females, there were no treatment-related histological findings in the liver.

On histopathological assessment, gastroenteritis with mild to severe infiltration of eosinophils was observed in 4.0% males and females, especially in the colon. While eosinophilic gastrointestinal disorders (EGIDs) are known as human diseases that primarily affect the gastrointestinal tract with eosinophilic infiltration in the absence of known causes (Rothenberg, 2004), the occurrence of eosinophilic gastroenteritis is rare in rodent models. A previous study demonstrated that iron lactate, a food additive with a similar structure to ferric citrate, caused eosinophilic gastroenterocolitis in F344 rats on dietary administration for 3 months (Narama et al., 1999). The colitis was characterized by infiltration of eosinophils and mucosal hyperplasia, decrease of RBC and increases of PLT and eosinophil ratio, in line with the present study. The authors also demonstrated that eosinophilic infiltration was induced in the stomach, duodenum, cecum and colon by 2.5% and 5.0% iron lactate and in the jejunum, ileum and rectum by 5.0%. Interestingly, eosinophilic enteritis induced by 4.0% ferric citrate was mainly located not only in the colon but also in several cases in the stomach, duodenum and cecum, suggesting that ferric citrate and iron lactate might have similar target organs and dose-reactivity.

The histopathological changes here observed in the colon and mesenteric lymph node can be considered due to the ferric citrate treatment. Allergic reaction to dietary components is hypothesized as one of the causes of EGIDs in humans. In rodents, vitamin E and selenium deficiency could induce eosinophilic enteritis and eosinophilia in SD rats (Hong and Chow, 1988). Although there is a possibility that nutritional changes associated with iron overdosing might be one of the factors causing eosinophilic colitis, further investigations are needed to clarify the detailed mechanisms and human relevance of the eosinophilic infiltration in

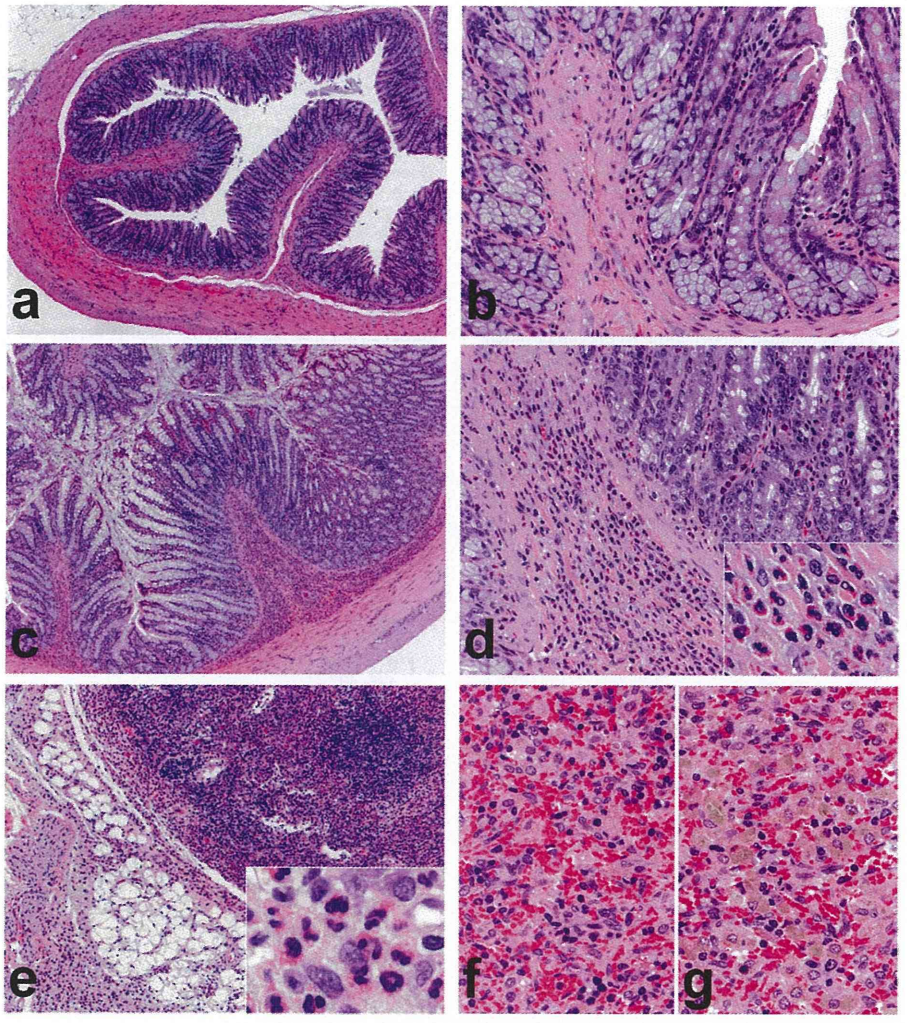

Fig. 3. Histopathological findings of F344 rats treated with ferric citrate. Original magnifications: ×40 (a, c and e) and ×200 (b, d, f and g). (a and b) Colon of a control male. (c and d) Colon of a male rat treated with 4.0% ferric citrate. Note mucosal hyperplasia with proliferation of mucous epithelial cells (c) and severe infiltration of inflammatory cells in the lamina propria and submucosa (d). Infiltration of eosinophils is prominent (d, insert). (e) Mesenteric lymph node (upper right) and surrounding adipose tissue (lower left) of a male rat treated with 4.0% ferric citrate. Note severe infiltration of eosinophils (insert). (f) Spleen of a control male. (g) Spleen of a male rat treated with 4.0% ferric citrate. Note increased deposition of hemosiderin in the red pulp.

gastrointestinal tissues induced by ferric citrate and relation with iron metabolism.

Increased hemosiderosis in the red pulp was observed in the spleens of 4.0% males and females, while it has been shown that 2.5% and 5.0% iron lactate treatment caused hemosiderosis in multiple organs such as the liver, bone marrow, kidney and testis as well as spleen (Narama et al., 1999). Like ferritin in the liver, hemosiderin has an important role for iron storage in the spleen. Thus, it is suggested that the increased hemosiderosis in the spleen was associated with excessive iron accumulation through intake of ferric citrate. The increased hematopoiesis observed in bone marrow of 4.0% males and females was considered to be associated with the reduced RBC.

5. Conclusions

In conclusion, the present 13-week subchronic toxicity study demonstrated that 4.0% ferric citrate treatment caused eosinophilic enteritis, especially in the colon, eosinophilic infiltration in the mesenteric lymph nodes, increased hemosiderosis in the spleen and increased hematopoiesis in the bone marrow of both males and females. Based on the results, the NOAEL for ferric citrate was estimated to be 1.0% (595.9 mg/kg bw/day for males and 601.4 mg/kg bw/day for females) in the present study.

Conflict of interest

The authors declare that there are no conflicts of interest.

Transparency document

The Transparency document associated with this article can be found in the online version.

Acknowledgements

We thank Ayako Saikawa and Yoshimi Komatsu for expert technical assistance in processing histological materials. This study was supported by a grant for Research on Food Sanitation from the Ministry of Health, Labour and Welfare of Japan.

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