Fundamental Toxicological Sciences (Fundam. Toxicol. Sci.)
Vol.10, No.2, 69-82, 2023

The Japanese Society of Toxicology

Fundamental Toxicological Sciences

URL : http://www.fundtoxicolsci.org/index_e.html

Data Report

A 90-day repeated oral dose toxicity study of 2-Butylbenzo[d]isothiazol-3(2H)-one in rats

Makiko Kuwagata[1], Masaru Tsuboi[2], Toshime Igarashi[1], Mariko Tsurumoto[1], Takuya Nishimura[1], Yuhji Taquahashi[1] and Satoshi Kitajima[1]

[1]Division of Cellular and Molecular Toxicology, Center for Biological Safety and Research, National Institute of Health Sciences, 3-25-26, Tonomachi, Kawasaki-ku, Kawasaki, Kanagawa 210-9501, Japan
[2]Research Laboratories in Vitro and in Vivo, Higashimatsuyama Laboratories, Drug Safety Testing Center Co., Ltd., 88-75, Shingo, Higashi-Matsuyama, Saitama 355-0071, Japan

(Received January 30, 2023; Accepted February 7, 2023)

ABSTRACT — 2-Butylbenzo[d]isothiazol-3(2H)-one (BBIT, CAS No. 4299-07-4) is widely used as an industrial antiseptic and antifungal agent. To investigate its toxicological properties and determine the no-observed-adverse-effect level (NOAEL), a 90-day repeated oral toxicological study of BBIT was conducted in Crl:CD (SD) rats at doses of 0 (vehicle control, corn oil), 30, 90, or 270 mg/kg/day. There was no mortality or abnormal clinical signs related to treatment in any group. Slightly decreased body weight and food consumption were observed in the 270 mg/kg group in females. Increased urine volume and kidney weight, increased liver weight, and thickening of the forestomach mucosa in autopsy were observed in both sexes in the 270 mg/kg group. Histopathological examination revealed that hyperplasia of the squamous epithelium of the forestomach with parakeratosis and/or hyperkeratosis was observed in both sexes in all the BBIT-treated groups. Moreover, centrilobular hypertrophy of hepatocytes was observed in both sexes of the 270 mg/kg group. Similarly, increased depositions of eosinophilic bodies and/or hyaline droplets in the proximal tubules of the kidney were observed among the male in the 270 mg/kg group. Based on the forestomach changes, NOAEL was judged to be less than 30 mg/kg/day in both sexes under this study's conditions.

Key words: 2-Butylbenzo[d]isothiazol-3(2H)-one, A 90-day repeated oral toxicity study, Rats

INTRODUCTION

2-Butylbenzo[d]isothiazol-3(2H)-one (BBIT, Fig. 1) is widely used as an industrial antiseptic and antifungal agent. According to the GHS classification for health hazards (https://anzeninfo.mhlw.go.jp, https://www.nite.go.jp), BBIT can cause severe eye damage and eye irritation (Hazard Category 1, danger). Skin corrosion or irritation (Category 2) and skin sensitization (Category 1A) are classified as a warning by human and animal data. The 50% lethal doses (LD50) in rats were reported as 670 mg/kg in males and 784 mg/kg in females, resulting in a Category 4 in GHS classification: "Harmful if swallowed". Although several toxicological studies, such as developmental and/or reproductive toxicological studies, and repeated toxicological studies in experimental animals, were conducted, the data were insufficient for GHS classification; thus, BBIT is currently marked as unclassifiable in these fields. Therefore, we examined the effects of BBIT on toxicity during long-term exposure in rats in a 90-day repeated oral dose toxicity study to clarify this compound's toxicological properties and assess a no-observed-adverse-effect

Correspondence: Makiko Kuwagata (E-mail: kuwagata.m@nihs.go.jp)

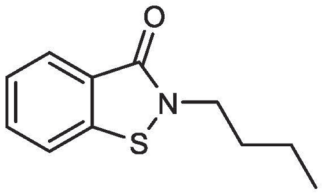


Fig. 1. Chemical structure of 2-Butylbenzo[d]isothiazol-3(2H)-one.

level (NOAEL).

MATERIALS AND METHODS

Test material

The test chemical, 2-Butylbenzo[d]isothiazol-3(2H)-one (BBIT, Fig. 1), manufactured by BLD Pharmatech Ltd. (Shanghai, China; Lot no BQY142; purity 99.8%), was a brown liquid, characterized via HPLC-UV.

Biochemical research-grade corn oil was purchased from Fujifilm Wako Pure Chemical Corporation (Osaka, Japan). We used corn oil as the vehicle control because BBIT is easily dissolved in corn oil.

Animals and treatment

Specific pathogen-free Crl: CD (SD) rats at five weeks of age were purchased from Jackson laboratories Japan, Inc. (Kanagawa, Japan) and were housed at the Animal Facility of the Drug Safety Testing Center Co., Ltd. (DSTC) (Saitama, Japan) under controlled temperature and relative humidity, with a 12 hr light/dark cycle (lights on at 06:00). The animals were individually accommodated in polycarbonate cages with Carefeeaz floor bedding (HAMRI Co., Ltd., Ibaraki, Japan). Pellet diet MF (Oriental Yeast Co., Ltd., Tokyo, Japan) and tap water filtrated through a 5-μm cartridge filter were given *ad libitum* to the animals. After quarantine, rats were randomly assigned to four groups (10 rats/group/sex) based on body weight. Each rat group received BBIT at 0 (vehicle control, corn oil), 30, 90, and 270 mg/kg/day orally using a stomach gavage tube once daily of 5 mL/kg body weight (Diehl *et al*., 2001) for 90 days (males) or 91 days (females), respectively.

The doses were based on the results of the 14-day dose-finding study in which BBIT was given orally at 100, 300, and 1000 mg/kg in rats. This preliminary study revealed that most rats in the 1000 mg/kg group died between treatment days 8 to 14. At autopsy, the stomach adhered to the surrounding tissues; whitish mucosa and/or reddish spots in the forestomach and gas retention in the digestive tract were observed. In the 300 mg/kg group, thickening of the forestomach mucosa was observed in both sexes. Moreover, the hematological analysis revealed that an increased reticulocyte ratio in both sexes was detected in the 300 and 1000 mg/kg groups. Considering the long-term treatment period in the main study, the highest dose was set at 270 mg/kg/day with a 10% reduction of the toxic dose, 300 mg/kg. The middle and low doses were set to 90 and 30 mg/kg/day, respectively, as one-third sequential dose reductions.

The animal study was approved by the Animal Care and Utilization Committee of the National Institute of Health Sciences, Japan, and the in-house animal care committee of the testing facility, DSTC. Animal husbandry, handling, and euthanasia were conducted according to the Japanese Act on Welfare and Management of Animals (2019), standards relating to the Methods of Destruction of Animals (Ministry of the Environments, 2007), measures relating to the Care and Keeping and Reducing Pain of Laboratory Animals (Ministry of the Environments, 2013), Basic Policies for the Conduct of Animal Experiments in Research Institutions Under the Jurisdiction of the Ministry of Health, Labor and Welfare (2015), and Regulations of Animal Experimental Ethics (DSTC, 2019).

A 90-day repeated oral dose toxicity study design

A 90-day repeated oral dose toxicity study of BBIT in rats was conducted according to the Guidelines for Designation of Food Additives and Revision of Standards for the Use of Food Additives (Ministry of Health, Laboratory, and Welfare of Japan; MHLW, 1996) in compliance with GLP standard in Standard Concerning Testing Laboratories Implementing Tests for New Chemical Substances, etc. (MHLW, 2011).

The start day of treatment was designated as treatment day 1 (T1). Animal conditions were observed daily. Body weight and 24-hr food consumption were measured every week from beginning the day before T1. The urinary examination was performed on the final week of administration. Five animals/groups/sex were placed in a metabolic cage, TG-781 (Tokyo Giken Service Co., Ltd., Tokyo, Japan), with food and water. Using a fresh urine sample obtained within 4 hr after excretion, qualitative parameters including urinary urobilinogen (Uro), protein (Pro), pH, occult blood (Ob), ketone body (Ket), bilirubin (Bil), urinary sugar (Glu), and specific gravity (SG), were examined using a Multistics SG (Siemens Healthcare Diagnostics K.K., Tokyo, Japan). The urinary volume (Vol), color, cloud (turbidity), odor, urinary sodium (Na), potassium (K), and chloride (Cl) levels of the 24-hr collected urine were assessed. Urinary electrolytes were determined using an automated analyzer, AU480 (Beckman Coulter, K.K., Tokyo, Japan). Moreover, the 24-hr sample was centri-

fuged to obtain urinary sediment for examining erythrocyte (EC), leukocyte (LC), epithelial cell (EpiC), crystal (Cry), casts, microorganisms (MO), and fatty globules (FG). Following urinary examination, eye appearance was observed, and light reflexes were examined in response to dark adaptation. Subsequently, a mydriatic, Mydrin® P (Santen Pharmaceutical Co., Ltd., Osaka, Japan), was administered to animals' eyes; the anterior part, optic media, and ocular fundus were then examined with a slit-lamp, SL-5 (Kowa Co., Ltd., Aichi, Japan), and a fundus camera, KOWA GENESIS-D (Kowa Co., Ltd.) during the final week of administration.

At autopsy, hematology and serum biochemistry were quantified via typical clinical methods. Before the autopsy, the animals fasted overnight. After being anesthetized with isoflurane, blood samples were acquired from the abdominal aorta. Blood samples were collected into EDTA-containing tubes for the hematology examination. Then, the whole blood was directly assayed. Red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cell (WBC), reticulocyte ratio (RET%), and differential leukocytes count were assessed using a multiple blood cells analyzer, XT-2000*i*V (Sysmex Corporation, Hyogo, Japan). Plasma was obtained after centrifugation with 3.2% sodium citrate and then assayed using an automated blood coagulation analyzer, CA-620 (Sysmex Corporation), for prothrombin time (PT) and activated partial thrombin time (APTT). After centrifugation, the serum samples were assayed using an automatic analyzer, AU480 (Beckman Coulter, Inc.), for the following: total bilirubin (T-Bil), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (γ-GTP), total protein (TP), albumin (Alb), globulin (Glb), total cholesterol (T-cho), triglyceride (TG), glucose (Glu), urea nitrogen (BUN), creatinine (Crea), sodium (Na), potassium (K), chloride (Cl), calcium (Ca), and inorganic phosphorus (IP).

Pathological examinations

All organs and tissues were carefully examined macroscopically to record all gross lesions. At autopsy, the heart, thymus, lungs, thyroid glands (after fixation), spleen, liver, kidneys, pituitary gland (after fixation), adrenal glands, testes, epididymides, uterus, ovaries, and brain were weighed. Lungs were immersed in fixatives following the injection fixation. Testis and epididymis were prefixed with the FSA solution, consisting of formaldehyde, sucrose, acetic acid, and methanol (Muto Pure Chemical Co., Ltd., Tokyo, Japan) along with 10% neutralized buffered formalin. The eyeball was fixed with Davidson's solution. The other organs and tissues were fixed in 10% neutralized buffered formalin. After fixation, paraffin-embedded sections were prepared and stained with hematoxylin and eosin for histopathological examination. If test material-related histopathological changes appeared in the 1000 mg/kg group, the relevant tissues from the lower dose groups were examined. In this study, histopathological examination was performed on liver, kidney, and stomach samples from all groups in both sexes, on the thyroid from all groups in males, and in all other tissues of the control and 1000 mg/kg groups. Results for male and female tissues are shown in Tables 7 and 8, respectively.

Statistical analysis

Statistical significance of the difference between the control and treated groups was estimated at ≤ 5% levels of probability via statistical tests using SAS 9.3 (SAS Institute Japan Ltd., Tokyo, Japan) and EXSUS 8.0 (EPS Corp., Tokyo, Japan).

Quantitative parameters, such as body weight, food consumption, urinary volume, urinary electrolytes, hematological parameters, blood chemical parameters, and organ weights, were first evaluated using Bartlett's test for equality of variances. When this test showed that the variances were homogeneous between groups ($P > 0.05$), a parametric one-way analysis of variance (ANOVA) was used to determine if any statistical differences existed among groups. When the results of one-way ANOVA were significant ($P \leq 0.05$), Dunnett's multiple comparisons were performed. When Bartlett's test showed that variances were heterogeneous between groups ($P \leq 0.05$), the data were evaluated by Kruskal-Wallis' nonparametric ANOVA. When the results were significant ($P \leq 0.05$), Steel's nonparametric test was applied. For histopathological findings, Fisher's exact and Wilcoxon's rank sum tests were used.

RESULTS

General conditions

No deaths or clinical signs were observed in any groups during the study period. Moreover, no ophthalmological effects of BBIT were seen. In females, body weight, and food consumption in the 270 mg/kg group were slightly lower than in the controls during the administration period. In males, there were no effects of BBIT on these parameters (Figs. 2 and 3).

Urinary analysis

Quantitative and qualitative urinary parameters are

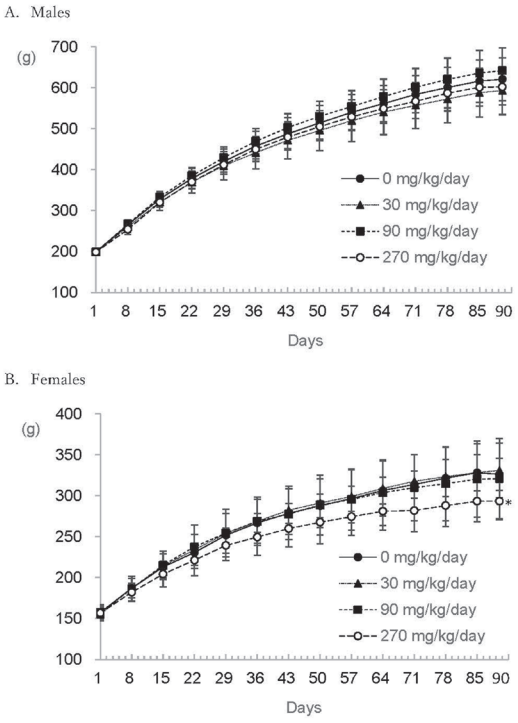


Fig. 2. Body weight of male (A) and female (B) rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days. *, Significantly different from the controls at $p < 0.05$.

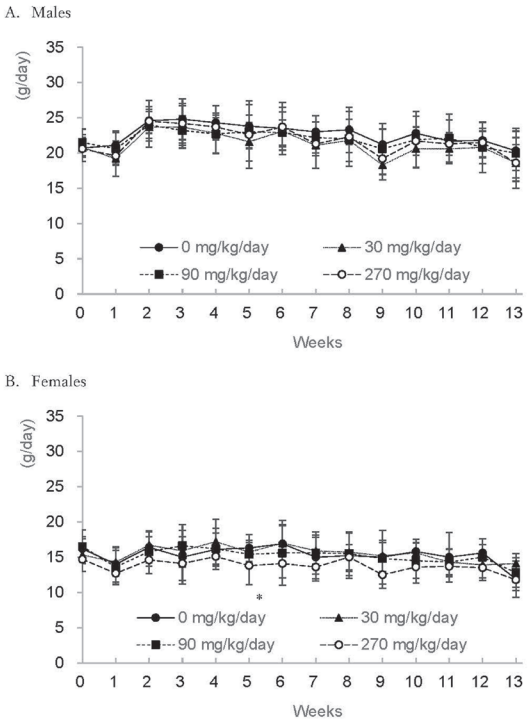


Fig. 3. Food intake of male (A) and female (B) rats treated orally with2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days. *, Significantly different from the controls at $p < 0.05$.

shown in Tables 1 and 2, respectively. Urine volume increased in the 270 mg/kg group and was statistically significant in females. Other urinary parameters were not influenced by BBIT treatment in either sex.

Hematological observations and serum biochemistry

Tables 3 and 4 show the hematological and serum biochemical data, respectively. PT was significantly longer in the 90 and 270 mg/kg groups. The RET% slightly decreased in the 270 mg/kg group, but only in males. Decreased Alb fraction in the 90 and 270 mg/kg groups, an increased α2-globulin fraction in the 270 mg/kg group, and an increase in the β-globulin fraction in 90 and 270 mg/kg groups were observed in males, respectively. In addition, the ratio of albumin to globulin was significantly decreased in the 270 mg/kg group. Significantly changes were observed in some parameters, but degrees of changes were very subtle and/or not dose-dependent, suggesting that these changes were of low toxicological significance.

Pathological examinations

At autopsy, thickening of the forestomach mucosa was macroscopically observed in both sexes in all BBIT-treated groups. Data for organ weights are shown in Tables 5 (males) and 6 (females). The relative liver and kidney weights significantly increased in the 270 mg/kg group in both sexes. The relative thyroid weight was significantly increased in all the BBIT groups in males. Statistically significant increased absolute thyroid weight in males was observed only in the 90 mg/kg group. In females, the relative brain, heart, and adrenal glands' weight were increased in the 270 mg/kg group.

On histopathological assessment (Tables 7 and 8), treatment-related histopathological findings were noted in the forestomach, kidney, and liver. In the forestomach, hyperplasia of the squamous mucosa accompanied by hyperkeratosis and parakeratosis was observed in both sexes (Fig. 4); both degree and incidence were increased dose-dependent. The changes were statistically significant in the 90 and 270 mg/kg groups, for both sexes. Furthermore, an epidermal cyst was observed in two males and one female in the 270 mg/kg group. The centrilobular hypertrophy of hepatocytes was observed in the 270 mg/kg group in both sexes (Fig. 5); the degree and incidence were statistically significant. Eosinophilic bodies and hyaline droplets in the cortical proximal tubules

Table 1. Quantitative Urinary Parameters data of SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Dose (mg/kg/day)		0 [a)]	30	90	270
Male (No. of animals)		5	5	5	5
Vol	(mL)	9.3 ± 2.9	9.8 ± 2.2	10.7 ± 2.8	13.9 ± 3.5
Na	(mmol/L)	54 ± 39	56 ± 26	44 ± 19	31 ± 13
K	(mmol/L)	270.0 ± 80.4	238.6 ± 81.9	213.8 ± 58.1	156.7 ± 27.2
Cl	(mmol/L)	80 ± 51	71 ± 37	60 ± 25	38 ± 5
Na	(mmol/24 hr)	0.5 ± 0.3	0.5 ± 0.1	0.5 ± 0.3	0.4 ± 0.2
K	(mmol/24 hr)	2.4 ± 0.4	2.2 ± 0.4	2.2 ± 0.7	2.1 ± 0.2
Cl	(mmol/24 hr)	0.7 ± 0.4	0.6 ± 0.2	0.7 ± 0.3	0.5 ± 0.2
Female (No. of animals)		5	5	5	5
Vol	(mL)	7.3 ± 3.2	10.8 ± 4.4	6.4 ± 3.1	15.0 ± 5.3*
Na	(mmol/L)	116 ± 56	69 ± 23	125 ± 97	50 ± 16
K	(mmol/L)	331.7 ± 112.3	231.8 ± 75.1	373.3 ± 155.2	188.9 ± 67.1
Cl	(mmol/L)	149 ± 62	91 ± 26	170 ± 116	70 ± 20
Na	(mmol/24 hr)	0.8 ± 0.4	0.7 ± 0.1	0.6 ± 0.2	0.7 ± 0.3
K	(mmol/24 hr)	2.3 ± 0.8	2.3 ± 0.2	2.0 ± 0.2	2.6 ± 0.7
Cl	(mmol/24 hr)	1.0 ± 0.4	0.9 ± 0.2	0.9 ± 0.2	1.0 ± 0.3

Values; Mean ± SD.
a); vehicle control, corn oil.
*; Significantly different from the control at $p < 0.05$.

Table 2. Qualitative urinary parameters data of SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Item	Grade	Dose (mg/kg/day)							
		Male				Female			
		0 [a)]	30	90	270	0 [a)]	30	90	270
No. of animals examined		5	5	5	5	5	5	5	5
Uro	– (0.1)	5	5	5	5	5	5	5	5
Pro	– (Negative)	5	3	2	3	5	4	2	2
	± (Minimum)	0	2	2	2	0	1	2	3
	+ (30)	0	0	1	0	0	0	1	0
pH	7.0	1	0	0	1	3	4	3	3
	7.5	1	1	1	0	0	0	0	0
	8.0	1	3	0	1	0	0	1	1
	8.5	2	1	4	3	2	1	1	1
Ob	– (Negative)	5	5	5	5	5	5	5	5
Ket	– (Negative)	5	3	1	3	5	4	2	3
	± (5)	0	2	3	1	0	1	3	1
	+ (15)	0	0	1	1	0	0	0	1
Bil	– (Negative)	5	5	5	5	5	5	5	5
Glu	– (Negative)	5	5	5	5	5	5	5	5
SG	1.010	4	2	3	3	3	0	0	2
	1.015	0	3	2	0	0	1	3	2
	1.020	1	0	0	2	2	4	2	1
Color	Yellow	5	5	5	5	5	5	5	5
Cloud	Transparent to slightly turbid	5	5	5	5	5	5	5	5
Odor	No abnormal odor	5	5	5	5	5	5	5	5
EC	– (Not observed)	5	5	5	5	5	5	5	5
LC	– (Not observed)	5	5	5	5	5	5	5	5
EpiC	– (Not observed)	5	5	5	5	5	5	5	5
Cry	– (Not observed)	0	3	1	1	1	0	0	0
	1+ (A few in serveral field)	3	1	3	0	1	3	0	0
	2+ (A few in all fields)	1	1	1	2	3	2	2	2
	3 + (Many in all fields)	1	0	0	2	0	0	3	3
Casts	– (Not observed)	5	5	5	5	5	5	5	5
MO	– (Not observed)	5	5	5	5	5	5	5	5
FG	– (Not observed)	5	5	5	5	5	5	5	5

Data are presented as the number of animals of each grade.
a); vehicle control, corn oil.

Table 3. Hematology data of SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Dose (mg/kg/day)		0 [a)]	30	90	270
Male (No. of animals)		10	10	10	10
RBC	($\times 10^4$/μL)	885 ± 36	861 ± 29	870 ± 34	869 ± 25
HGB	(g/dL)	15.5 ± 0.4	15.3 ± 0.5	15.1 ± 0.3	15.2 ± 0.5
HCT	(%)	43.9 ± 1.3	43.1 ± 1.2	43.0 ± 1.2	43.6 ± 2.0
MCV	(fL)	49.7 ± 2.4	50.1 ± 1.9	49.5 ± 2.6	50.2 ± 2.5
MCH	(pg)	17.5 ± 0.8	17.8 ± 0.6	17.4 ± 0.7	17.5 ± 0.7
MCHC	(g/dL)	35.2 ± 0.3	35.5 ± 0.5	35.2 ± 0.6	34.8 ± 0.5
PLT	($\times 10^4$/μL)	121.4 ± 10.7	111.7 ± 12.4	115.9 ± 11.8	118.8 ± 8.9
RET	(%)	3.08 ± 0.21	2.75 ± 0.42	2.97 ± 0.42	2.61 ± 0.47*
WBC	($\times 10^2$/μL)	112.1 ± 33.5	108.9 ± 28.5	92.2 ± 9.2	109.9 ± 31.7
NEUT	(%)	15.3 ± 6.0	12.3 ± 4.2	15.6 ± 3.6	12.7 ± 3.4
LYMPH	(%)	80.4 ± 6.3	83.4 ± 4.8	78.4 ± 4.4	83.2 ± 3.7
MONO	(%)	3.2 ± 0.7	3.3 ± 0.9	4.8 ± 1.2**	3.0 ± 0.9
EO	(%)	1.1 ± 0.3	1.0 ± 0.3	1.2 ± 0.5	1.1 ± 0.4
BASO	(%)	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.0
NEUT	($\times 10^2$/μL)	17.9 ± 12.7	12.9 ± 4.1	14.4 ± 3.6	13.4 ± 3.6
LYMPH	($\times 10^2$/μL)	89.4 ± 23.0	91.4 ± 25.9	72.2 ± 8.0	92.1 ± 28.9
MONO	($\times 10^2$/μL)	3.6 ± 1.2	3.4 ± 0.7	4.5 ± 1.3	3.2 ± 0.9
EO	($\times 10^2$/μL)	1.2 ± 0.3	1.1 ± 0.4	1.1 ± 0.5	1.2 ± 0.6
BASO	($\times 10^2$/μL)	0.0 ± 0.0	0.0 ± 0.1	0.0 ± 0.1	0.0 ± 0.0
PT	(sec.)	10.7 ± 1.5	11.5 ± 1.2	13.6 ± 3.3*	13.3 ± 2.3*
APTT	(sec.)	17.5 ± 4.0	17.5 ± 1.6	19.3 ± 2.4	20.1 ± 2.3
Female (No. of animals)		10	10	10	10
RBC	($\times 10^4$/μL)	797 ± 38	765 ± 30	791 ± 24	763 ± 41
HGB	(g/dL)	15.3 ± 0.8	14.9 ± 0.4	15.1 ± 0.4	14.7 ± 0.6
HCT	(%)	42.4 ± 1.6	41.9 ± 0.8	42.1 ± 1.2	41.7 ± 1.7
MCV	(fL)	53.3 ± 1.5	54.9 ± 1.8	53.3 ± 1.5	54.7 ± 1.9
MCH	(pg)	19.2 ± 0.5	19.5 ± 0.5	19.0 ± 0.4	19.3 ± 0.6
MCHC	(g/dL)	36.0 ± 0.5	35.5 ± 0.6	35.8 ± 0.6	35.3 ± 0.5*
PLT	($\times 10^4$/μL)	101.2 ± 19.3	107.0 ± 8.1	108.4 ± 15.6	110.3 ± 21.2
RET	(%)	2.72 ± 0.47	3.21 ± 0.60	2.76 ± 0.45	3.07 ± 0.46
WBC	($\times 10^2$/μL)	83.0 ± 24.1	72.9 ± 20.4	75.9 ± 19.2	69.5 ± 8.2
NEUT	(%)	11.4 ± 3.4	11.5 ± 4.3	11.6 ± 2.7	12.1 ± 2.8
LYMPH	(%)	83.9 ± 4.0	83.6 ± 4.4	84.2 ± 3.1	83.6 ± 3.7
MONO	(%)	2.9 ± 0.7	3.3 ± 0.7	3.1 ± 0.7	3.0 ± 0.9
EO	(%)	1.7 ± 0.7	1.7 ± 0.3	1.1 ± 0.4*	1.3 ± 0.3*
BASO	(%)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
NEUT	($\times 10^2$/μL)	9.4 ± 4.2	8.2 ± 3.8	8.6 ± 2.4	8.4 ± 2.1
LYMPH	($\times 10^2$/μL)	69.6 ± 20.3	61.1 ± 18.4	64.3 ± 17.4	58.1 ± 7.1
MONO	($\times 10^2$/μL)	2.5 ± 1.1	2.4 ± 0.6	2.3 ± 0.5	2.1 ± 0.8
EO	($\times 10^2$/μL)	1.5 ± 0.7	1.2 ± 0.4	0.9 ± 0.3	0.9 ± 0.3
BASO	($\times 10^2$/μL)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PT	(sec.)	9.5 ± 0.2	9.4 ± 0.2	9.5 ± 0.2	9.4 ± 0.2
APTT	(sec.)	15.1 ± 1.3	15.6 ± 0.8	16.2 ± 1.0	16.4 ± 1.2

Values; Mean ± SD.

a); vehicle control, corn oil.

*, **; Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively.

Table 4. Blood Chemistry data of SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Dose (mg/kg/day)		0 [a)]	30	90	270
Male (No. of animals)		10	10	10	10
T-bil	(mg/dL)	0.07 ± 0.01	0.06 ± 0.01**	0.06 ± 0.01**	0.05 ± 0.01**
AST	(IU/L)	115 ± 118	90 ± 20	78 ± 18	71 ± 15
ALT	(IU/L)	47 ± 46	34 ± 4	30 ± 6	33 ± 5
γ-GTP	(IU/L)	1 ± 1	1 ± 1	1 ± 1	1 ± 1
ALP	(IU/L)	333 ± 115	371 ± 105	284 ± 47	339 ± 154
TP	(g/dL)	6.3 ± 0.3	6.3 ± 0.1	6.3 ± 0.2	6.4 ± 0.4
A/G		1.18 ± 0.07	1.13 ± 0.05	1.12 ± 0.06	1.09 ± 0.07**
Alb	(g/dL)	3.4 ± 0.1	3.3 ± 0.1	3.3 ± 0.1	3.3 ± 0.1
Glb	(g/dL)	2.9 ± 0.2	2.9 ± 0.1	3.0 ± 0.1	3.1 ± 0.3
T-cho	(mg/dL)	70 ± 11	58 ± 15	66 ± 9	72 ± 13
TG	(mg/dL)	91 ± 41	51 ± 17*	78 ± 31	68 ± 24
Glu	(mg/dL)	239 ± 28	232 ± 28	229 ± 31	234 ± 30
BUN	(mg/dL)	16.6 ± 3.2	15.9 ± 2.6	15.2 ± 2.2	14.9 ± 1.8
Crea	(mg/dL)	0.30 ± 0.04	0.32 ± 0.04	0.35 ± 0.06	0.33 ± 0.04
Na	(mmol/L)	143 ± 1	144 ± 1	144 ± 1*	146 ± 1**
K	(mmol/L)	4.8 ± 0.3	4.9 ± 0.2	4.9 ± 0.4	5.1 ± 0.3
Cl	(mmol/L)	102 ± 1	103 ± 1	103 ± 1	104 ± 1*
Ca	(mg/dL)	10.2 ± 0.2	10.0 ± 0.2	10.1 ± 0.1	10.3 ± 0.5
IP	(mg/dL)	6.0 ± 0.6	6.1 ± 0.4	6.0 ± 0.4	6.5 ± 0.6
Mg	(mg/dL)	1.88 ± 0.12	1.94 ± 0.11	1.91 ± 0.12	2.01 ± 0.10
PL	(mg/dL)	115 ± 14	93 ± 17*	108 ± 16	111 ± 17
Serum protein electrophoresis					
Alb	(%)	51.4 ± 1.7	49.5 ± 1.7	49.3 ± 1.8*	48.7 ± 2.0**
α1	(%)	24.0 ± 2.5	24.7 ± 1.9	24.3 ± 2.5	23.9 ± 2.1
α2	(%)	4.5 ± 0.7	4.5 ± 0.7	4.5 ± 1.0	5.5 ± 0.7*
β	(%)	3.6 ± 0.2	3.6 ± 0.3	4.0 ± 0.4*	4.0 ± 0.5*
γ	(%)	16.5 ± 1.6	17.7 ± 1.1	17.8 ± 1.6	17.9 ± 1.0
Female (No. of animals)		10	10	10	10
T-bil	(mg/dL)	0.09 ± 0.02	0.10 ± 0.03	0.10 ± 0.02	0.08 ± 0.02
AST	(IU/L)	98 ± 46	88 ± 12	93 ± 41	90 ± 19
ALT	(IU/L)	34 ± 20	32 ± 7	30 ± 17	29 ± 5
γ-GTP	(IU/L)	0 ± 1	0 ± 0	0 ± 0	0 ± 0
ALP	(IU/L)	155 ± 55	156 ± 43	105 ± 11*	125 ± 45
TP	(g/dL)	6.7 ± 0.6	6.8 ± 0.2	6.9 ± 0.3	6.9 ± 0.5
A/G		1.30 ± 0.06	1.33 ± 0.08	1.35 ± 0.07	1.31 ± 0.13
Alb	(g/dL)	3.8 ± 0.4	3.9 ± 0.2	4.0 ± 0.1	3.9 ± 0.3
Glb	(g/dL)	2.9 ± 0.2	2.9 ± 0.1	3.0 ± 0.2	3.0 ± 0.3
T-cho	(mg/dL)	75 ± 16	84 ± 18	89 ± 15	85 ± 13
TG	(mg/dL)	56 ± 32	58 ± 31	64 ± 32	33 ± 15
Glu	(mg/dL)	178 ± 16	181 ± 25	179 ± 14	164 ± 16
BUN	(mg/dL)	20.1 ± 3.3	19.0 ± 6.2	18.6 ± 3.4	19.1 ± 4.5
Crea	(mg/dL)	0.39 ± 0.05	0.41 ± 0.07	0.39 ± 0.04	0.42 ± 0.07
Na	(mmol/L)	142 ± 1	144 ± 1*	144 ± 1*	145 ± 2**
K	(mmol/L)	4.5 ± 0.2	4.5 ± 0.3	4.6 ± 0.3	4.5 ± 0.3
Cl	(mmol/L)	105 ± 1	106 ± 2	106 ± 1	106 ± 2
Ca	(mg/dL)	10.5 ± 0.4	10.5 ± 0.2	10.6 ± 0.3	10.5 ± 0.4
IP	(mg/dL)	4.8 ± 0.7	4.7 ± 0.6	4.9 ± 0.6	5.1 ± 0.5
Mg	(mg/dL)	2.04 ± 0.15	2.16 ± 0.11	2.08 ± 0.11	2.31 ± 0.16**
PL	(mg/dL)	140 ± 28	148 ± 24	159 ± 21	148 ± 23
Serum protein electrophoresis					
Alb	(%)	57.8 ± 1.9	57.7 ± 2.3	58.2 ± 1.3	56.0 ± 3.3
α1	(%)	16.8 ± 1.3	18.4 ± 1.6	17.7 ± 1.6	18.4 ± 2.0
α2	(%)	4.4 ± 0.5	3.5 ± 0.4**	3.9 ± 0.4	4.4 ± 0.5
β	(%)	2.6 ± 0.5	2.7 ± 0.4	2.5 ± 0.5	2.4 ± 0.3
γ	(%)	18.5 ± 1.5	17.8 ± 2.3	17.6 ± 0.7	18.9 ± 1.9

Values; Mean ± SD.

a); vehicle control, corn oil.

*, **; Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively.

Table 5. Organ weights of SD male rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Dose (mg/kg/day)	0 [a)]	30	90	270
Male (No. of animals)	10	10	10	10
Body weight (g)	602.8 ± 53.6	577.1 ± 56.3	622.7 ± 53.3	582.6 ± 45.4
Absolute (g)				
Brain	2.1557 ± 0.0782	2.1728 ± 0.1121	2.1813 ± 0.1149	2.1409 ± 0.0743
Pituitary	0.0148 ± 0.0016	0.0150 ± 0.0014	0.0149 ± 0.0011	0.0148 ± 0.0016
Heart	1.6691 ± 0.1277	1.5815 ± 0.1419	1.6454 ± 0.2174	1.5789 ± 0.1013
Thymus	0.3095 ± 0.0807	0.3216 ± 0.0912	0.3180 ± 0.0696	0.3326 ± 0.0839
Lungs	1.6265 ± 0.1530	1.6175 ± 0.1668	1.6309 ± 0.1659	1.5977 ± 0.1360
Thyroids	0.0417 ± 0.0082	0.0505 ± 0.0086	0.0537 ± 0.0084**	0.0505 ± 0.0075
Spleen	0.7754 ± 0.0967	0.7525 ± 0.1210	0.7293 ± 0.0901	0.7375 ± 0.1374
Liver	17.3431 ± 1.4445	17.1486 ± 2.3094	18.2997 ± 2.4156	19.8542 ± 3.2914
Kidneys	3.6674 ± 0.4195	3.4863 ± 0.2221	3.5278 ± 0.3755	3.8635 ± 0.3956
Salivary glands	0.7849 ± 0.0840	0.7239 ± 0.0578	0.7819 ± 0.1193	0.7781 ± 0.0509
Adrenals	0.0563 ± 0.0072	0.0545 ± 0.0086	0.0583 ± 0.0077	0.0583 ± 0.0140
Prostate	1.5023 ± 0.2578	1.5011 ± 0.2334	1.5162 ± 0.2536	1.7092 ± 0.2571
Testes	3.4476 ± 0.3119	3.5622 ± 0.3465	3.5690 ± 0.1759	3.4105 ± 0.2803
Seminal vesicle	1.8285 ± 0.2682	1.7465 ± 0.3934	1.7077 ± 0.2115	1.7315 ± 0.2143
Rerative (%)				
Brain	0.3602 ± 0.0344	0.3787 ± 0.0277	0.3523 ± 0.0318	0.3694 ± 0.0300
Pituitary	0.0025 ± 0.0003	0.0026 ± 0.0004	0.0024 ± 0.0001	0.0025 ± 0.0003
Heart	0.2777 ± 0.0178	0.2748 ± 0.0179	0.2637 ± 0.0191	0.2718 ± 0.0180
Thymus	0.0513 ± 0.0128	0.0555 ± 0.0150	0.0510 ± 0.0106	0.0572 ± 0.0147
Lungs	0.2703 ± 0.0189	0.2805 ± 0.0150	0.2622 ± 0.0202	0.2746 ± 0.0176
Thyroids	0.0069 ± 0.0010	0.0088 ± 0.0014*	0.0087 ± 0.0014*	0.0087 ± 0.0016*
Spleen	0.1291 ± 0.0154	0.1305 ± 0.0189	0.1173 ± 0.0118	0.1265 ± 0.0203
Liver	2.8804 ± 0.1069	2.9686 ± 0.2357	2.9303 ± 0.1759	3.3913 ± 0.3232**
Kidneys	0.6090 ± 0.0499	0.6078 ± 0.0536	0.5661 ± 0.0268	0.6626 ± 0.0336*
Salivary glands	0.1305 ± 0.0116	0.1261 ± 0.0106	0.1264 ± 0.0225	0.1343 ± 0.0136
Adrenals	0.0094 ± 0.0012	0.0095 ± 0.0016	0.0094 ± 0.0011	0.0101 ± 0.0027
Prostate	0.2512 ± 0.0498	0.2609 ± 0.0370	0.2446 ± 0.0424	0.2949 ± 0.0491
Testes	0.5757 ± 0.0714	0.6187 ± 0.0425	0.5781 ± 0.0675	0.5893 ± 0.0751
Seminal vesicle	0.3061 ± 0.0567	0.3054 ± 0.0748	0.2749 ± 0.0327	0.2984 ± 0.0406

a); vehicle control, corn oil.
*, **; Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively.

of the kidney were observed only in males, regardless of the dosage. They were also observed in the control group. However, in the 270 mg/kg group, the degree and incidence of this finding were statistically significant (Fig. 6). Several lesions in other tissues were sporadically detected, but no significant treatment-dependent alterations were apparent.

DISCUSSION

In this study, adverse effects of BBIT were found in body weight, food consumption, forestomach, liver, and kidney. Body weight and food consumption were slightly lower in females in the 270 mg/kg group compared to the controls. Moreover, females in the 270 mg/kg group exhibited increased relative weights of the brain, heart, and adrenal glands. However, the higher weights were likely to be indirect changes brought about by the low body weight, and were judged not to be toxicological concerns. None of these organs were accompanied by any unusual macroscopic and microscopic lesions.

The thickening of the forestomach mucosa was macroscopically observed in all the BBIT groups. Histopathologically, the forestomach mucosa showed squamous hyperplasia accompanied by hyperkeratosis and parakeratosis. By contrast, no effects were observed in the glandular stomach. It has been reported that oral administration of irritant chemicals in rats often leads to erosion,

Table 6. Organ weights of SD female rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Dose (mg/kg/day)	0 [a)]	30	90	270
Female (No. of animals)	10	10	10	10
Body weight (g)	319.0 ± 18.9	321.8 ± 33.2	310.0 ± 46.8	283.2 ± 22.7**
Absolute (g)				
Brain	1.9745 ± 0.1237	1.9394 ± 0.0839	1.9903 ± 0.0744	1.9921 ± 0.0959
Pituitary	0.0189 ± 0.0038	0.0184 ± 0.0022	0.0190 ± 0.0022	0.0206 ± 0.0046
Heart	1.0283 ± 0.0814	1.0238 ± 0.1176	1.0045 ± 0.1156	0.9870 ± 0.0459
Thymus	0.2842 ± 0.0908	0.3169 ± 0.1008	0.2718 ± 0.0866	0.2553 ± 0.0637
Lungs	1.2399 ± 0.1018	1.2367 ± 0.0866	1.1454 ± 0.1881	1.1943 ± 0.1076
Thyroids	0.0298 ± 0.0077	0.0334 ± 0.0032	0.0350 ± 0.0052	0.0284 ± 0.0067
Spleen	0.5044 ± 0.0523	0.5328 ± 0.0868	0.5228 ± 0.0515	0.4911 ± 0.0604
Liver	8.4206 ± 1.0226	8.7550 ± 0.7861	8.4904 ± 0.9995	9.4881 ± 0.9188
Kidneys	2.0438 ± 0.1341	1.9833 ± 0.1441	1.9869 ± 0.1868	2.1047 ± 0.3644
Salivary glands	0.5223 ± 0.0575	0.5138 ± 0.0587	0.5193 ± 0.0573	0.5050 ± 0.0509
Adrenals	0.0704 ± 0.0064	0.0730 ± 0.0102	0.0694 ± 0.0096	0.0773 ± 0.0069
Uterus	0.6076 ± 0.1245	0.7322 ± 0.1712	0.6359 ± 0.1543	0.6595 ± 0.1641
Ovaries	0.1281 ± 0.0214	0.1308 ± 0.0218	0.1243 ± 0.0175	0.1360 ± 0.0264
Rerative (%)				
Brain	0.6204 ± 0.0450	0.6079 ± 0.0616	0.6528 ± 0.0826	0.7063 ± 0.0500**
Pituitary	0.0060 ± 0.0013	0.0058 ± 0.0009	0.0062 ± 0.0010	0.0073 ± 0.0015
Heart	0.3226 ± 0.0216	0.3182 ± 0.0205	0.3262 ± 0.0261	0.3498 ± 0.0224*
Thymus	0.0885 ± 0.0252	0.0975 ± 0.0240	0.0868 ± 0.0199	0.0897 ± 0.0197
Lungs	0.3889 ± 0.0256	0.3862 ± 0.0280	0.3718 ± 0.0512	0.4220 ± 0.0234
Thyroids	0.0093 ± 0.0022	0.0104 ± 0.0010	0.0114 ± 0.0017	0.0100 ± 0.0020
Spleen	0.1581 ± 0.0128	0.1653 ± 0.0194	0.1707 ± 0.0220	0.1734 ± 0.0162
Liver	2.6389 ± 0.2836	2.7264 ± 0.1392	2.7557 ± 0.2139	3.3534 ± 0.2251**
Kidneys	0.6414 ± 0.0359	0.6190 ± 0.0400	0.6478 ± 0.0683	0.7401 ± 0.0903**
Salivary glands	0.1638 ± 0.0158	0.1601 ± 0.0149	0.1698 ± 0.0254	0.1785 ± 0.0146
Adrenals	0.0221 ± 0.0019	0.0228 ± 0.0034	0.0229 ± 0.0053	0.0274 ± 0.0028**
Uterus	0.1903 ± 0.0358	0.2313 ± 0.0642	0.2104 ± 0.0636	0.2329 ± 0.0556
Ovaries	0.0403 ± 0.0068	0.0412 ± 0.0084	0.0409 ± 0.0082	0.0482 ± 0.0100

Values; Mean ± SD.

a); vehicle control, corn oil.

*, **; Significantly different from the control at $p < 0.05$ and $p < 0.01$, respectively.

ulcer, and/or squamous hyperplasia of the forestomach (Oishi, 2008). Therefore, the forestomach mucosal changes in this study were thought to be caused by the potentially irritating character of BBIT. However, in the preliminary study, animals exposed to 100 mg/kg for 14 days did not show any forestomach lesions, indicating that the development of forestomach lesions likely depends on the duration of the treatment.

Increased relative liver weights and centrilobular hypertrophy of hepatocytes were observed in both sexes in the 270 mg/kg group. Hepatocytes in the central lobules are rich in drug-metabolizing enzymes such as cytochrome P450 and glutathione-S-transferase and often enlarged upon induction of these enzymes by certain drugs (Harada *et al*., 2017). Therefore, the changes observed in this study might be due to the induction of drug-metabolizing enzymes by BBIT. In contrast, no changes indicative of hepatic damage, such as hepatocyte necrosis or elevation of AST and ALT, were observed (Yoshida *et al*., 2015), suggesting that BBIT only mildly affected liver function and did not lead to injury.

In rats, induction of hepatic drug-metabolizing enzymes causes excess metabolism of thyroid hormone, T4, leading to a feedback enhancement of the thyroid-stimulating hormone action from the pituitary gland. In this study, the thyroid weights were increased in males in all the BBIT groups. However, no histopathological changes were evident, indicating hyperfunction of the thyroid or the pituitary gland was not detected in any BBIT groups. In addition, the BBIT did not influence thyroid hormone

Table 7. Histopathological findings of SD male rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Organ: Findings	0 mg/kg/day (Control)					30 mg/kg/day					90 mg/kg/day					270 mg/kg/day				
	-	+	++	+++	P	-	+	++	+++	P	-	+	++	+++	P	-	+	++	+++	P
Lung (Bronchus)	n = 10															n = 10				
Cell infiltration, lymphocyte and eosinophil, perivascular	9	1														10				
Heart	n = 10															n = 10				
Accumulation, mononuclear cell, ventricular wall	8	2														7	3			
Focal degeneration, myocardial fiber, ventricular wall	10															9	1			
Liver	n = 10					n = 10					n = 10					n = 10				
Hypertrophy, hepatocyte, centrilobular	10					10					10					1	9			**, ††
Vacuolar change, hepatocyte, perilobular	5	5				5	4	1			7	3				4	6			
Focal necrosis, hepatocyte	9		1			10					10					10				
Accumulation, mononuclear cell, Glisson and lobule	5	5				9	1				7	3				8	2			
Spleen	n = 10															n = 10				
Deposit/accumulation, hemosiderin, vascular wall, hilum, partial	9	1														10				
Kidney	n = 10					n = 10					n = 10					n = 10				
Eosinophilic body and hyaline droplet, tubular, cortex	5	3	2			4	5	1			5	4	1				2	8		*, ††
Basophilic change, tubular, cortex	9	1				10					9	1				9	1			
Infarct, cortex	10					10					10					9	1			
Cystic dilatation, tubular, medulla	10					10					7	3				10				
Stomach	n = 10					n = 10					n = 10					n = 10				
Squamous cell hyperplasia, mucosa, with parakeratosis and hyperleratosis, forestomach	10					7	2	1			1	8	1		**, ††			10		**, ††
Epidermal cyst, forestomach	10					10					10								2	
Pituitary	n = 10					n = 10					n = 10					n = 10				
Craniopharyngeal duct remnant	9	1														9	1			
Cyst, anterior lobe, single	9	1														9	1			
Pseudocyst, anterior lobe, single	10															9	1			
Thyroid	n = 10					n = 10					n = 10					n = 10				
Accumulation, mononuclear cell, perivascular, unilateral	9	1				10					10					10				
Ultimobranchial remnant	9	1				8	2				9	1				5	5			
Ectopic thymus	10					10					10					9	1			
Testis	n = 10															n = 10				
Atrophy, seminiferous tubule	9	1														9	1			
Prostate	n = 10															n = 10				
Infiltration, lymphocyte, interstitium	4	6														6	3	1		

Data are presented as the number of animals showing each finding.

Grade: - No change/Not observed, + Slight, ++ Moderate, +++ Marked, P: Present.

*, **; Significantly different from the control at $p < 0.0\,5$ and $p < 0.01$ in the Fisher's exact test, respectively.

†† ; Significantly different from the control at $p < 0.01$ in the Wilcoxon's rank sum test.

Cerebrum, Cerebellum, Medulla oblongata, Spinal cord, Submandibular gland, Sublingual gland, Parotid gland, Submandibular and mesenteric lymphnodes, Thymus, Tongue, Aorta, Esophagus, Trachea, Pancreas, Stomach, Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum, Adrenal gland, Parathyloid, Eye ball, Harderian gland, Sciatic nerve, Skin (mammary gland), Thigh muscle, Testis, Epididymis, Seminal vesicle, Urinary bladder, Femur and Sternum (bone marrow), Nasal cavity, and Zymbal's gland were not observed any changes related to the treatment of 2-Butylbenzo[d]isothiazol-3(2H)-one.

Table 8. Histopathological findings of SD female rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days.

Organ: Findings	0 mg/kg/day (Control)						30 mg/kg/day						90 mg/kg/day						270 mg/kg/day					
		-	+	++	+++	P		-	+	++	+++	P		-	+	++	+++	P		-	+	++	+++	P
Lung (Bronchus)	n = 10																		n = 10					
Foamy cell, alveolar lumen		9	1																	10				
Heart	n = 10																		n = 10					
Accumulation, mononuclear cell, ventricular wall		10																		9	1			
Liver	n = 10						n = 10						n = 10						n = 10					
Hypertrophy, hepatocyte, centrilobular		10						10						10							10			**, ††
Vacuolar change, hepatocyte, perilobular		10						7	3					8	2					9	1			
Accumulation, mononuclear cell, Glisson and lobule		7	3					7	3					7	3					10				
Spleen	n = 10																		n = 10					
Increase, extramedullary hematopoiesis		8	2																	8	2			
Kidney	n = 10						n = 10						n = 10						n = 10					
Infarct, cortex		10						10						10						9	1			
Mineralization, medulla		9	1					10						10						10				
Cystic dilatation, tubular, medulla		10						10						9	1					10				
Pyelitis, suppurative, unilateral		10						9	1					10						10				
Stomach	n = 10						n = 10						n = 10						n = 10					
Squamous cell hyperplasia, mucosa, forestomach		10						8	2					1	9			**, ††				10		**, ††
Epidermal cyst, fore stomach		10						10						10									1	
Thyroid	n = 10																		n = 10					
Ultimobranchial remnant		8	2																	7	3			
Ectopic thymus		10																		9	1			
Uterus	n = 10																		n = 10					
Epidermal cyst, cervix		10																		9			1	
Nasal cavity (nasoturbinate)	n = 10																		n = 10					
Inflammatory change, mucosa, nasoturbinae, partial		10																		9	1			

Data are presented as the number of animals showing each finding.
Grade: - No change/Not observed, + Slight, ++ Moderate, +++ Marked, P: Present.
**; Significantly different from the control at $p < 0.01$ in the Fisher's exact test.
†† ; Significantly different from the control at $p < 0.01$ in the Wilcoxon's rank sum test.
Cerebrum, Cerebellum, Medulla oblongata, Spinal cord, Sublingual gland, Sublingual gland, Parotid gland, Submandibular and Mesenteric lymphnodes, Thymus, Tongue, Aorta, Esophagus, Trachea, Pancreas, Duodenum, Jejunum, Ileum, Cecum, Colon, Rectum, Pituitary, Adrenal gland, Parathyroid, Eye ball, Harderian gland, Sciatic nerve, Skin (mammary gland), Thigh muscle, Ovary, Oviduct, Uterus, Vagina, Urinary bladder, Femur and Sternum (bone marrow), and Zymbal's gland were not observed any changes related to the treatment of 2-Butylbenzo[d] isothiazol-3(2H)-one.

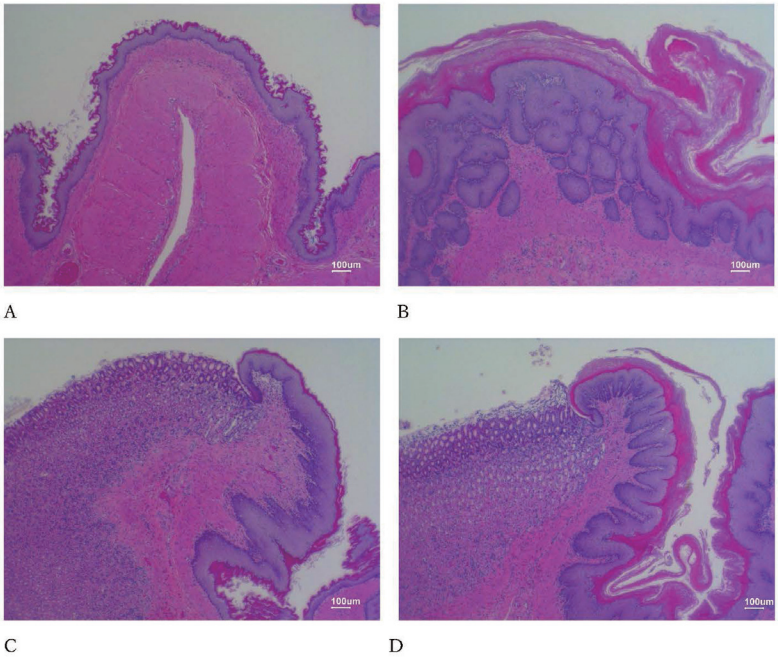


Fig. 4. Representative findings of the forestomach of SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days. Hematoxylin and eosin stains. Hyperplasia of the squamous mucosa accompanied by hyperkeratosis and parakeratosis was observed in male (B) and female (D) in the 270 mg/kg, whereas the control males (A) and females (C) did not exhibit similar changes.

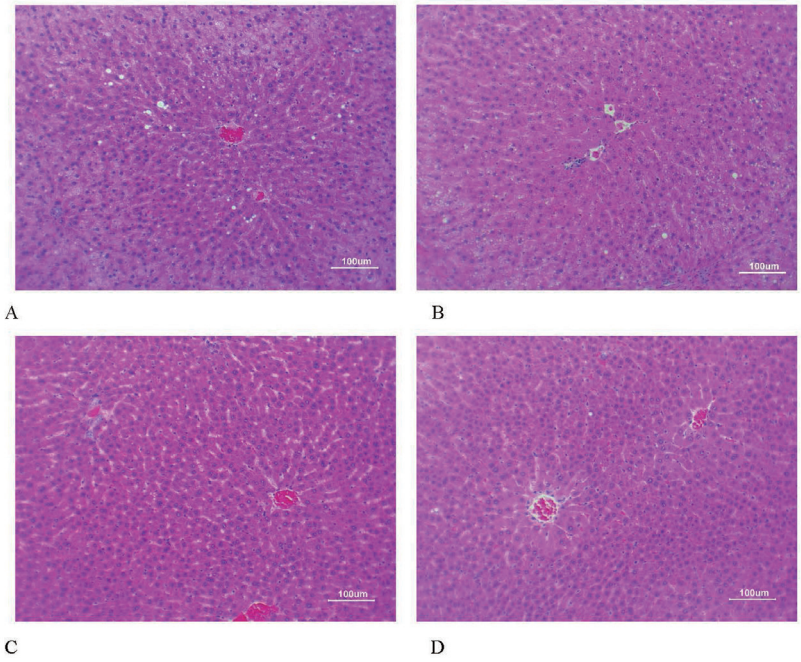
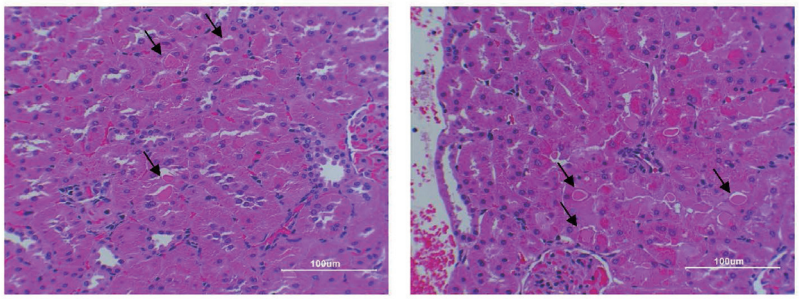


Fig. 5. Representative findings of the liver in SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days. Hematoxylin and eosin stains. Hypertrophy of hepatocytes in the centrilobular area was observed in male (B) and female (D) in the 270 mg/kg group. In contrast, similar changes were not observed in male (A) and female (C) in the control group.



A B

Fig. 6. Representative findings of the kidneys of male SD rats treated orally with 2-Butylbenzo[d]isothiazol-3(2H)-one for 90 days at 0 mg/kg (A) or 1000 mg/kg (B). Hematoxylin and eosin stains. Arrows showed the alternative renal epithelial cells resulting from eosinophilic bodies and hyaline droplets in males. These findings were observed in only males, regardless of dose, including the control group (A). However, in males in the 1000 mg/kg group, the frequency and degree of the deposits tended to increase (B).

homeostasis, which worked together among the pituitary, thyroid, and liver.

Serum protein electrophoresis in males revealed a decrease in the Alb fractionation ratio, an increase in α2-globulin, and increased β-fractionation ratios. A functional alteration of protein synthesis in the liver may partially cause these changes. Altered protein composition in serum was not considered a toxicological concern because there were no significant changes in the amount of serum Alb and TP. The changes were not observed in females who showed similar changes in liver weight and histopathology.

Increases in kidney weight and urine volume were observed in both sexes in the 270 mg/kg group. However, no degenerative renal histopathological changes or increases in renal damage markers, such as BUN and creatinine, were observed. In this study, water intake was not measured. Therefore, although these changes were likely caused by treatment with BBIT, the changes are not considered to be adverse stage at this time. Eosinophilic bodies and hyaline droplets in proximal renal tubules were observed at all the doses but only in males. Considering the male-specific significant increase in the α2 protein ratio and histopathological characteristics, the lesions might be derived from α2-microglobulin, an adult male rat-specific protein (Greaves, 2012; Hamamura *et al*., 2017). Various chemicals are reported to evoke α2-microglobulin nephropathy, characterized by similar increases in the eosinophilic bodies and hyaline droplets (Dietrich and Swenberg, 1991; Greaves, 2012; Hamamura *et al*., 2017). The frequency and grade of this finding significantly increased in males in the 270 mg/kg group. However, nephropathy induced by α2-microglobulin in male rats cannot be extrapolated to humans (Doi *et al*., 2007).

PT was slightly prolonged in the 90 and 270 mg/kg groups in males. Generally, PT is prolonged after severe hepatic injury because many coagulation factors are synthesized in the liver and during anemia. However, in this study, BBIT affected the liver function mildly, not severely, in both sexes. Moreover, no secondary effects, such as hemorrhagic signs, were observed. Thus, PT prolongation was considered a BBIT-related changes but not an adverse event.

In conclusion, toxicity effects of 2-Butylbenzo[d]isothiazol-3(2H)-one were observed in body weight, food intake, stomach, and kidneys. Based on the thickening mucosa and hyperplasia in the forestomach, the NOAEL was judged to be less than 30 mg/kg/day in both sexes under this study's condition.

ACKNOWLEDGMENTS

The authors thank the staff of DSTC for their expert technical assistance in conducting the animal study. This study was supported by MHLW, Japan.

Conflict of interest---- The authors declare that there is no conflict of interest.

REFERENCES

Diehl, K.H., Hull, R., Morton, D., Pfister, R., Rabemampianina, Y., Smith, D., Vidal, J.M. and van de Vorstenbosch, C. (2001): A good practice guide to the administration of substances and removal of blood, including routes and volumes. J. Appl. Toxicol., **21**, 15-23.

Dietrich, D.R. and Swenberg, J.A. (1991): The presence of alpha 2u-globulin is necessary for d-limonene promotion of male rat kidney tumors. Cancer Res., **51**, 3512-3521.

Doi, A., Hill, G., Seely, J., Hailey, J.R., Kissling, G. and Bucher, J.R. (2007): α2u-Globulin nephropathy and renal tumors in National Toxicology Program studies. Toxicol. Pathol., **35**, 533-540.

Greaves, P. (2012): Urinaly Tract, Kidney In *Histopathology of Preclinical Toxicity studies*, pp. 537-614. London, U.K.: Academic Press.

Hamamura, M., Oshikata, T., Katoku, K., Tsuchitani, M. and Yamaguchi, R. (2017): Two types of deposits, hyaline droplets and eosinophilic bodies, associated with α_{2u}-globulin accumulation in the rat kidney. J. Toxicol. Pathol., **30**, 275-282.

Harada, T., Nakae, D., Tamano, S. and Ueda, M. (2017): Liver In *Toxicologic Histopathology*, ed. Y Oishi, pp. 216-254. Tokyo, Japan: Nishimura Shoten.

Oishi, Y. (2008): Toxicology of the gastrointestinal tract. Folia Pharmacol. Jpn., **131**, 373-377.

Yoshida, M., Umemura, T., Kojima, H., Inoue, K., Takahashi, M., Uramaru, N., Kitamura, S., Abe, K., Tohkin, M., Ozawa, S. and Yoshinari, K. (2015): Basic Principles of Interpretation of Hepatocellular Hypertrophy in Risk Assessment in Japan. Food Hyg. Saf. Sci., **56**, 42-48.