TOXICITY STUDIES OF A SYNTHETIC ANTIOXIDANT, 2, 2'-METHYLENEBIS (4-ETHYL-6-*TERT*-BUTYLPHENOL) IN RATS 1. ACUTE AND SUBCHRONIC TOXICITY

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Abstract.....The acute and subchronic toxicity studies on 2, 2'-methylenebis (4-ethyl-6-*tert*-butylphenol) (MBEBP) were conducted using male and female Wistar rats.

In acute toxicity test, the LD₅₀ values were estimated to be greater than 10 g/kg BW by oral and intraperitoneal administration in each sex.

In subchronic toxicity test, groups of 10 rats of each sex were fed a diet containing 0.2, 1.0 or 5.0 % of MBEBP and examined at 4 and 12 weeks. Body weight gain was significantly depressed at doses of 1.0 and 5.0 % in both sexes, but the depression in the 1.0 % group was severer than that in the 5.0 % group in males. Hematological analysis showed slight but significant decrease of hemoglobin in the 1.0 and 5.0 % groups of both sexes. Urine analysis showed no remarkable changes in all treated rats of both sexes. In biochemical analysis of serum, decrease of triglyceride level and cholinesterase activity, and increase of amylase activity were observed in treated rats. Histopathologically, testicular atrophy and decrease of spermatogenesis were observed in male rats fed 1.0 or 5.0 % MBEBP for 4 and 12 weeks and vacuolization of parathyroid gland cells was observed in female rats fed 1.0 and 5.0 % MBEBP for 12 weeks. In subchronic test, the lowest observable adverse effect levels for MBEBP toxicity were estimated to be 171 mg/kg BW/day in male rats and 180 mg/kg BW/day in female rats.

Key words : 2, 2'-methylenebis (4-ethyl-6-*tert*-butylphenol), acute toxicity test, subchronic toxicity test, testicular toxicity, parathyroid gland, rat.

INTRODUCTION

A variety of synthetic antioxidants have been used in rubber and plastics for protection against oxidative damages. 2, 2'-methylenebis (4-ethyl-6-*tert*-butylphenol) (MBEBP) is one of the phenolic antioxidants. It has been reported that after daily intragastric administration of MBEBP to rats (500 mg/kg BW/day) for 27 days, MBEBP was distributed to almost all tissues, especially to adipose tissue, lung, pancreas, liver and testis (Kohri et al., 1986). An LD₅₀ of MBEBP was reported to be approximately 50-100 mg/kg BW in the male mouse by intraperitoneal route (National Technical Information Service, 1961). MBEBP increased fetal resorption rate slightly in the rat (Telford et al., 1962) and has a weak lethal effect on fetal development but has no teratogenic effect in the rat (Tanaka et al., 1989). In biochemical study, MBEBP has been reported to decrease hepatic triglyceride level in rat (Takahashi and Hiraga, 1981). Meanwhile, complete toxicological studies (short-term or long-term test) have not been reported yet. Therefore, as a first step of the safety evaluation, we examined the acute and subchronic toxicities of MBEBP using male and female Wistar rats.

MATERIALS AND METHODS

Chemicals :

2, 2'-methylenebis (4-ethyl-6-*tert*-butylphenol) (MBEBP) was obtained from Ouchi Shinko Chemical Ind. Ltd. (Osaka, Japan). The chemical structure is shown in Fig. 1. MBEBP is white powder, insoluble in acetone, benzene, chloroform, ethylalcohol, etc (M. W. ; 368.61, RTECS No. ; SL9800000, CAS REGISTRY No. ; 88-24-2). As for the purity of MBEBP used in our experiment, it has been reported that any peak except MBEBP peak was not observed in HPLC or GC analysis (Kohri, 1986). All of reagents for hematological and biochemical analysis were purchased from Wako Pure Chemicals (Osaka, Japan).

Fig. 1. Chemical structure of 2, 2'-methylenebis (4-ethyl-6-*tert*-butylphenol).

Experimental animals and diets :

Specific pathogen-free Wistar male and female rats (4 weeks old) were obtained from SLC Co. (Hamamatsu, Japan) and acclimated for 1 week prior to initiation of the test. The basal diet powder was supplied by Funabashi Farm (Funabashi, Japan) and MBEBP was mixed into the diet prior to pelleting at the desired concentrations (0.2, 1.0 or 5.0 %) and kept at 4 °C until use.

Housing conditions :

The animal room was maintained at the temperature of 24 ± 1 °C and 55 ± 5 % humidity with a 12 h light/dark cycle. Rats were housed in plastic cages (5 rats/cage) using chip bedding in acute study or individually housed in the aluminum hanging cages in subchronic study.

Experimental design and conduct :

Acute toxicity test : Groups of 10 rats (average body weight : male 116 g, female : 95 g) of each sex were fasted for 16 h before administration. MBEBP suspended in olive oil (30 % w/v) was orally or intraperitoneally administered at doses of 5 and 10 g/kg BW to rats. Toxic signs and mortality were observed twice a day up to day 14 when the test was terminated and body weight was recorded at 3, 7, 10 and 14 days after the administration.

Subchronic feeding study : Groups of 10 rats of each sex were given pellets containing either 0 (control), 0.2, 1.0 or 5.0 % MBEBP. The body weight and food consumption were recorded weekly. During the entire course of the study, the rats were daily observed. Five randomly chosen rats from each group were examined at 4 weeks as an intermittent study and the experiment was terminated at 12 weeks. All animals were studied for the following examination after 16 h starvation.

The blood for hematological study was collected from the orbital venous plexus under ether anesthesia 5 days before the sacrifice. The hematological study was conducted for red blood cells (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean hemoglobin concentration (MCH), mean corpuscular hemoglobin concentration (MCHC), red blood cell distribution width (RDW), platelets (PLT) and white blood cells (WBC) by a Coulter Counter model S-PLUS (Coulter Electronics Inc. USA) and for differential white blood cell by a Microx (Tateishi Electric Co., Japan).

The blood for serum biochemical study was collected from the orbital venous plexus under ether anesthesia in the morning at 4 and 12 weeks. The serum biochemical study was conducted for 24 items ; total protein (T-PRO), albumin (ALB), urea nitrogen (BUN), uric acid (UA), glucose (GLU), non-esterified fatty acids (NEFA), phospholipids (PL), triglycerides (T-GLY), total cholesterol (T-CHO), free cholesterol (F-CHO), alkaline phosphatase (ALP), amylase (AMY), cholinesterase (CHE), aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyl transpeptidase (γ -GTP), 2-hydroxybutyrate dehydrogenase (HBDH), leucine aminopeptidase (LAP), lactate dehydrogenase (LDH) and glucose-6-phosphate dehydrogenase (G6PDH) using a Gamsac Autoanalyzer Type 4

(Electro Nucleonics, USA) and calcium (Ca), inorganic phosphorus (Pi), sodium (Na), potassium (K), chloride (Cl) using Hitachi Electrolyte Analyzer 702 (Hitachi Ltd., Japan).

Fresh urine was examined for pH, protein, glucose, ketone body, occult blood, bilirubin and urobilin using Labstix-III (Miles-Sankyo Co. LTD., Japan) in the morning at weeks 4 and 12.

At autopsy, the weights of the brain, heart, lungs, liver, kidneys, spleen, adrenals, testes, ovaries, pituitary and thyroid gland were measured. The above-mentioned organs and salivary gland, esophagus, stomach, small and large intestine, pancreas, urinary bladder, seminal vesicle, epididymis, sciatic nerve, uterus, mesenteric lymph nodes, thymus, spinal cord, skeletal muscle, bone marrow (femur and sternum) were fixed in 10 % buffered formalin solution for routine histological processing. Paraffin sections were stained by hematoxylin and eosin for histopathological examination.

All statistical evaluations were made using analysis of variance (ANOVA) techniques. All statistical tests compared the treatment groups with the control groups at a two-sided significance level.

RESULTS

Acute toxicity :

No death occurred in rats administered MBEBP up to 10 g/kg by both oral and intraperitoneal administration. Therefore, the LD₅₀ values were estimated to be greater than 10 g/kg BW in both sexes by both routes. Decrease of spontaneous locomotor movement was observed immediately after the administration. Diarrhea was observed 1 h after the administration of MBEBP or vehicle alone (control). They disappeared within 2 days (control) or 4-6 days (MBEBP-treatment) after both administrations. In both sexes, body weight gain was significantly depressed in the rats administered 10 g/kg MBEBP (p. o.) at 3 days after the administration, however, in the 5 g/kg MBEBP treated group, significant depression of body weight gain was not observed. Significant depression of body weight gain was observed in the rats administered 5 or 10 g/kg MBEBP (i. p.) at 3 days after the administration in both sexes.

Subchronic feeding study :

General symptoms :

No death occurred and no toxicological signs except depression of body weight gains were observed. In each sex, significant depression of body weight gain was observed in the 5.0 and 1.0 % groups, but not in the 0.2 % group throughout the experiment. In male rats, depression of body weight was severer in the 1.0 % group than in the 5.0 % group, and in female rats, the degree of depression of body weight gain in the 1.0 % group was almost same as the 5.0 % group. Therefore, a dose-related reduction of body weight gains was not observed in the 1.0 and 5.0 %

Toxicity of MBEBP in rats

group in both sexes (Fig. 2 and 3). No remarkable changes in food consumption were observed in the control and MBEBP treated groups throughout the experimental period. Mean food consumption and mean MBEBP intake of rats fed MBEBP for 12 weeks are summarized in Table 1.

Fig. 2. Growth curve and food consumption of male rats fed MBEBP.

Fig. 3. Growth curve and food consumption of female rats fed MBEBP.

Hematological examination :

The results of hematological examination are shown in Table 2(a, b). Significant decrease of HGB was observed in male rats fed 1.0 or 5.0 % MBEBP at 12 weeks. In female rats, significant decrease of HGB was observed in the 0.2 or 1.0 % group at 4 weeks and in the 0.2, 1.0 or 5.0 % group at 12 weeks. The number of PLT was significantly increased in the 0.2 or 1.0 % group, but not in the 5.0 % group at 4 weeks in males. The number of RBC was significantly decreased in the 5.0 % group at 12 weeks in males. HCT was decreased in male rats fed 1.0 or 5.0 % MBEBP at 12 weeks. MCH was significantly decreased in the male rats fed 1.0 % and in the

Toxicity of MBEBP in rats

Table 1. Mean food consumption and mean MBEBP intake of rats fed MBEBP for 12 weeks.

	Groups			
	Control	0.2%	1.0%	5.0%
Males				
mean food intake (g/kg BW/day)	80	86	126	102
mean MBEBP intake (mg/kg BW/day)	—	171	1258	5097
Females				
mean food intake (g/kg BW/day)	82	90	96	98
mean MBEBP intake (mg/kg BW/day)	—	180	958	4909

Values represent mean of 5-10 rats/group.

female rats fed 1.0 or 5.0 % MBEBP. MCHC was significantly decreased in the male rats fed 1.0 or 5.0 % MBEBP and in the female rats fed 1.0 % MBEBP at 12 weeks. RDW was significantly decreased in the male rats fed 5.0 % MBEBP at 12 weeks. The number of WBC was significantly increased in the female rats fed 1.0 and 5.0 % MBEBP at 12 weeks.

Biochemical examination in serum and urine :

Among 25 items examined in serum, only those with some changes are shown in Table 3 (a, b). Significant decreases of T-GLY level and CHE activity were observed in all treated male rats at 4 and 12 weeks. PL was decreased in all treated male rats at 4 weeks, however, in female rats, PL was significantly increased in the 1.0 and 5.0 % MBEBP treated groups at 4 weeks. T-CHO was significantly decreased in the 1.0 and 5.0 % MBEBP treated female rats at 4 and 12 weeks. F-CHO was significantly decreased in the all treated male rats at 4 weeks and significantly decreased in the 5.0 % MBEBP treated male rats at week 4. In both sexes, significant increase or increasing tendency of AMY activity were observed in the all treated group at 4 and 12 weeks. Significant increase of ALP was observed in the female rats treated with 1.0 or 5.0 % MBEBP at 12 weeks. LAP activity was significantly decreased in all treated females at 4 and 12 weeks. Significant increase of γ -GTP activity was observed in the female rats fed 1.0 or 5.0 % MBEBP at 4 and 12 weeks. LDH-P activity was significantly decreased in all treated female rats at 4 and 12 weeks. Ca was significantly decreased in the 5.0 % group in males at 4 weeks. Pi was significantly decreased in the 5.0 % group in males at 12 weeks. Na was significantly increased in the 1.0 and 5.0 % groups in females at 4 and 12 weeks. In these biochemical analysis, the degrees of change observed in the 1.0 and 5.0 %

groups were almost the same in male and female rats.

There were no changes in the biochemical parameters in urine of the MBEBP treated rats (data not shown).

Organ weight :

Absolute and relative weights of organs are shown in Table 4(a, b). In male rats, relative liver weight was significantly increased in the 0.2, 1.0 and 5.0 % groups at 4 and 12 weeks and the degree of increase observed in the 1.0 % group was highest at 4 and 12 weeks. Absolute testis weight was significantly decreased in the 1.0 % group at 4 and 12 weeks and in the 5.0 % group at 12 weeks. In female rats, absolute liver weight was significantly increased in the 1.0 and 5.0 % groups at 4 and 12 weeks and relative liver weight was increased in the 1.0 and 5.0 % at 4 weeks and in the 0.2, 1.0 and 5.0 % at 12 weeks. The degree of absolute and relative liver weight increase observed in the female 1.0 % group was highest at 4 and 12 weeks. Absolute weights of pituitary gland and ovary were significantly decreased in the female 1.0 and 5.0 % groups at 4 and 12 weeks.

Histopathological examination :

The major histopathological changes are listed in Table 5. In male rats, decreased spermatogenesis was observed in the 1.0 % group (slight : 1/5 at 4 weeks, marked and severe 5/5 at 12 weeks) and in the 5.0 % group (slight, mild and severe : 4/5 at 12 weeks). Testicular atrophy was observed in the 1.0 % group (marked and severe : 5/5 at 4 weeks ; mild, marked and severe : 5/5 at 12 weeks) and in the 5.0 % group (slight and mild : 3/5 at 4 weeks ; slight, marked and severe : 5/5 at 12 weeks) (Photo. 1-a, b). These testicular damages were not observed in the 0.2 % group and were related to the decrease of testis weights observed in the 1.0 and 5.0 % groups. In female rats, vacuolization of parathyroid gland cells was observed in the 1.0 and 5.0 % groups at 12 weeks (Photo. 2). No histopathological changes were observed in any other organs including liver and bone marrow in all treated groups.

Table 2.(a) Hematological examination in rats fed MBEBP for 4 weeks.

Parameter	Values for rats receiving MBEBP at dose levels (%) of:							
	0 (Control)	0.2%	Males			Females		
			1.0%	5.0%	0 (Control)	0.2%	1.0%	5.0%
RBC ($10^6/\mu\text{l}$)	8.05±0.25	7.46±0.49*	7.92±0.22	7.76±0.15	7.95±0.17	7.87±0.15	7.67±0.15	7.57±0.29
HGB (g/dl)	15.8±0.6	14.9±0.8	15.3±0.4	15.2±0.3	16.5±0.2	16.2±0.3	15.5±0.2**	15.2±0.5**
HCT (%)	41.5±1.1	39.1±2.0	40.4±1.2	39.6±0.9	41.9±1.1	41.7±0.7	39.7±0.6	39.3±1.7*
MCV (fl)	51.5±0.5	52.4±0.8	51.0±0.7	51.0±0.4	52.7±0.7	53.0±0.2	51.8±0.5	51.9±0.8
MCH (pg)	19.6±0.4	20.1±0.3	19.3±0.2	19.6±0.2	20.7±0.3	20.6±0.3	20.2±0.2	20.1±0.5
MCHC (g/dl)	38.0±0.5	38.3±0.4	37.9±0.1	38.5±0.5	39.3±0.4	38.9±0.3	39.1±0.5	38.7±0.8
RDW	9.58±1.24	9.12±0.91	7.92±0.47*	8.18±0.57*	6.18±1.37	6.35±0.78	6.84±0.36	6.46±0.74
PLT ($\text{X}10^3/\mu\text{l}$)	861±95	1066±155*	1068±64**	999±25	832±56	933±59	942±64	909±37
WBC ($\text{X}10^3/\mu\text{l}$)	11.4±1.3	10.6±0.4	9.9±0.9	9.1±1.5	8.23±1.35	9.40±0.70	9.06±1.22	9.18±0.84

RBC ; red blood cells, HGB ; hemoglobin, HCT ; hematocrit, MCV ; mean corpuscular volume, MCH ; mean hemoglobin concentration
MCHC ; mean corpuscular hemoglobin concentration, RDW ; red blood cell distribution width, PLT ; platelet, WBC ; white blood cells,
Values represent mean $\pm$ SD of 5 rats, Significantly different from control, *P<0.05, **P<0.01.

Table 2. (b) Hematological examination in rats fed MBEBP for 12 weeks.

Parameter	Values for rats receiving MBEBP at dose levels (%) of:							
	0 (Control)	0.2%	1.0%	5.5%	0 (Control)	0.2%	1.0%	5.0%
	Males				Females			
RBC ($10^6/\mu\text{l}$)	9.02±0.23	8.84±0.12	8.80±0.14	8.37±0.19**	8.00±0.17	7.39±0.45*	7.99±0.27	7.55±0.25
HGB (g/dl)	16.1±0.3	15.8±0.2	15.3±0.2**	15.0±0.3**	16.0±0.27	14.9±0.8*	14.9±0.5*	14.7±0.5**
HCT (%)	42.0±1.1	41.3±0.7	40.6±0.5*	40.0±0.9**	40.7±0.6	38.2±2.5	39.4±1.5	38.2±1.2
MCV (fl)	46.5±0.6	46.6±0.6	46.2±0.3	47.7±1.3	50.8±0.3	51.6±0.3*	49.3±0.6**	50.5±0.2
MCH (pg)	17.9±0.2	17.9±0.3	17.4±0.2*	17.8±0.4	20.1±0.3	20.3±0.4	18.7±0.1**	19.5±0.3*
MCHC (g/dl)	38.5±0.2	38.3±0.2	37.7±0.2**	37.4±0.4**	39.5±0.5	39.2±0.9	37.9±0.4**	38.6±0.5
RDW	10.9±0.6	9.9±0.9	8.6±1.3**	8.78±0.60**	7.52±1.05	5.92±0.40**	7.10±0.07	6.50±0.57
PLT ($\text{X}10^3/\mu\text{l}$)	760±19	805±66	804±148	906±59	833±93	884±50	846±64	919±111
WBC ($\text{X}10^3/\mu\text{l}$)	9.64±0.71	9.94±0.60	7.82±1.80	10.04±0.32	6.14±0.70	6.94±1.32	9.16±1.78*	9.30±0.30*

RBC ; red blood cells, HGB ; hemoglobin, HCT ; hematocrit, MCV ; mean corpuscular volume, MCH ; mean hemoglobin concentration
MCHC ; mean corpuscular hemoglobin concentration, RDW ; red blood cell distribution width, PLT ; platelet, WBC ; white blood cells,
Values represent mean $\pm$ SD of 5 rats. Significantly different from control, * $P < 0.05$, ** $P < 0.01$.

Table 3. (a) Biochemical examination of serum in rats fed MBEBP for 4 weeks.

Parameter	Values for rats receiving MBEBP at dose levels (%) of.						
	0 (Control)	0.2%	1.0%	5.0%	0 (Control)	0.2%	5.0%
	Males				Females		
T-PRO (g/dl)	6.63±0.07	6.90±0.16	6.87±0.25	6.83±0.18	6.20±0.17	6.49±0.13	6.47±0.20
ALB (g/dl)	4.23±0.08	4.33±0.07	4.33±0.11	4.35±0.07	4.04±0.10	4.19±0.06	4.15±0.07
NEFA (mequiv./l)	0.58±0.03	0.52±0.07	0.59±0.08	0.57±0.05	0.86±0.08	0.67±0.07	0.69±0.16
PL (mg/dl)	169±10	147±11*	149±9*	139±12**	174±7	187±7	220±14.3**
T-GLY (mg/dl)	199.6±41	86.6±9.8**	56.0±9.1**	53.3±15.9**	84.2±16.4	86.8±15.5	74.1±14.7
T-CHO (mg/dl)	96.3±12.4	82.8±10.0	86.7±8.7	78.7±7.9	120±10	144±14	168±27*
F-CHO (mg/dl)	49.1±4.9	39.9±3.7**	38.1±2.9**	32.5±3.9**	40.6±2.3	44.8±3.3	41.9±5.9
ALP (U/l)	511±36	485±32	444±36*	448±44*	325±53	316±32	351±15
AMY (U/l)	520±30	587±43*	604±38**	576±29	235±17	360±28**	443±38**
CHE (U/l)	688±142	346±60**	279±45**	282±61**	1306±114	975±187**	608±85**
γ-GTP (U/l)	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	0.01±0.00	1.21±0.94	4.64±0.47**
LAP (U/l)	47.4±2.7	44.7±2.1	45.9±2.9	45.7±2.8	47.5±1.5	52.1±2.2*	52.8±1.7**
LDH-P (U/l)	395±148	617±264	388±70	315±40	733±260	396±17.4*	323±167**
Ca (mg/dl)	9.56±0.26	9.43±0.19	9.01±0.55	9.04±0.19*	9.09±0.07	9.38±0.37	9.25±0.28
Pi (mg/dl)	6.71±0.46	6.81±0.24	6.37±0.29	6.44±0.53	5.43±0.58	5.36±0.57	5.38±0.47
Na (mequiv./l)	139±2	141.2±0.5	143±2**	143±1**	140±3	143±1	144±1*
Cl (mequiv./l)	100±1.0	100.8±0.5	102±1*	102±1**	101±0.82	102±1	102±1

T-PRO ; total protein, ALB ; albumin, NEFA ; non-esterified fatty acids, PL ; phospholipids, T-GLY ; triglycerides, T-CHO ; total cholesterol, F-CHO ; free cholesterol, ALP ; alkaline phosphatase, AMY ; amylase, CHE ; choline-esterase, γ-GTP ; γ-glutamyl transpeptidase ; LAP ; leucine aminopeptidase, LDH-P ; lactate dehydrogenase, Ca ; calcium, Pi ; phosphorus, Na ; sodium, Cl ; chloride
 Values represent means ± SD of 5 rats/group, Significantly different from control, *P<0.05, **P<0.01.

Table 3. (b) Biochemical examination of serum in rats fed MBEBP for 12 weeks.

Parameter	Values for rats receiving MBEBP at dose levels (%) of:							
	0 (Control)	0.2%	1.0%	5.0%	0 (Control)	0.2%	1.0%	5.0%
	Males				Females			
T-PRO (g/dl)	7.20±0.08	7.53±0.22*	7.48±0.19*	7.67±0.14**	6.76±0.11	7.01±0.26	7.31±0.18**	7.03±0.34
ALB (g/dl)	4.40±0.09	4.52±0.05	4.59±0.09**	4.54±0.09*	4.51±0.10	4.45±0.12	4.54±0.10	4.39±0.18
NEFA (mequiv./l)	0.87±0.12	0.70±0.10	0.65±0.13*	0.58±0.04**	0.76±0.08	0.81±0.11	0.68±0.07	0.60±0.12*
PL (mg/dl)	196±4	189±9	199±26	191±5	194±4	199±8	222±17**	208±9
T-GLY (mg/dl)	312±38	212±43**	99.3±40.5**	122±19**	88.0±13.8	86.2±15.5	94.7±16.1	59.7±11.5*
T-CHO (mg/dl)	114±8	118±11	134±39	100±7	121±5	134±11	178±25**	162±10*
F-CHO (mg/dl)	37.1±1.3	34.6±6.3	38.4±11.9	21.9±1.9*	39.4±1.5	42.4±3.4	49.3±10.0	36.2±3.7
ALP (U/l)	270±10	235±18	240±40	252±27	171±15	176±22	252±21**	231±35**
AMY (U/l)	525±36	635±32**	606±48**	657±28**	300±45	330±11	494±37**	441±16**
CHE (U/l)	971±73	684±109**	415±125**	486±83**	1916±120	1504±245*	778±123**	870±242**
γ-GTP (U/l)	0.01±0.00	0.01±0.00	0.61±0.85	0.30±0.41	0.01±0.00	0.26±0.55	6.66±1.36**	4.05±1.68**
LAP (U/l)	46.8±1.0	44.4±2.0	45.5±3.0	43.7±1.3	43.5±1.2	48.7±1.8*	51.9±2.7**	52.8±4.0**
LDH (U/l)	1008±399	1008±487	679±308	795±229	757±286	387±66*	433±113*	633±151
Ca (mg/dl)	10.1±0.2	10.2±0.3	10.2±0.4	10.3±0.3	9.97±0.38	9.85±0.25	9.77±0.23	9.97±0.39
Pi (mg/dl)	5.91±0.51	5.39±0.29	5.30±0.69	4.87±0.40*	4.31±0.68	3.89±0.58	4.55±0.35	4.42±0.69
Na (mequiv./l)	143±1	142±1	143±1	143±1	141.3±0.4	143±1	145±2.51*	145±2.0*
Cl (mequiv./l)	99.8±0.84	99.6±1.5	100±1	99.8±0.45	99.6±0.6	101.2±0.5	101±2	103±1**

T-PRO ; total protein, ALB ; albumin, NEFA ; non-esterified fatty acids, PL ; phospholipids, T-GLY ; triglycerides, T-CHO ; total cholesterol, F-CHO ; free cholesterol, ALP ; alkaline phosphatase, AMY ; amylase, CHE ; choline-esterase, γ-GTP ; γ-glutamyl transpeptidase ; LAP ; leucine aminopeptidase, LDH-P ; lactate dehydrogenase, Ca ; calcium, Pi ; phosphorus, Na ; sodium, Cl ; chloride
 Values represent means ± SD of 5 rats/group. Significantly different from control, *P<0.05, **P<0.01.

Table 4. (a) Body and organ weights in rats fed MBEBP for 4 weeks.

Values for rats receiving MBEBP at dose level (%) of:								
Organ	0 (Control)	0.2%	Males		Females			
Body weights	221±11	213±15	163±15**	172±16**	158±8	139±9	119±6**	126±5
Absolute organ weights								
Brain (g)	1.76±0.04	1.72±0.05	1.67±0.04*	1.68±0.04*	1.65±0.03	1.62±0.07	1.61±0.03	1.60±0.02
Heart (g)	0.74±0.04	0.66±0.02	0.55±0.07**	0.60±0.07**	0.52±0.02	0.45±0.02**	0.42±0.04**	0.44±0.03**
Lung (g)	0.88±0.08	0.76±0.03	0.65±0.03	0.74±0.15	0.65±0.03	0.67±0.17	0.58±0.04	0.55±0.14
Liver (g)	7.72±0.66	8.01±0.79	6.98±0.63**	7.04±0.64	4.78±0.73	5.48±1.47	5.87±0.67**	5.83±0.33**
Kidney (g)	1.60±0.13	1.55±0.13	1.32±0.11**	1.40±0.11**	1.17±0.07	1.17±0.09	0.98±0.11**	1.03±0.04
Spleen (g)	0.48±0.02	0.46±0.04	0.35±0.03**	0.35±0.03**	0.37±0.02	0.31±0.03**	0.26±0.01**	0.28±0.02**
Testis (g)	2.63±0.12	2.60±0.13	2.23±0.24**	2.45±0.14	—	—	—	—
Ovary (mg)	—	—	—	—	69.4±13.0	51.8±7.6	44.0±6.3*	45.8±14.7*
Pituitary (mg)	6.6±0.9	6.0±1.0	5.8±1.3	6.4±0.5	10.4±2.3	7.2±0.8	6.6±0.5*	6.6±1.1*
Thyroid (mg)	17.4±5.7	16.6±2.3	14.0±3.5	14.0±4.7	14.8±3.3	14.4±4.8	13.6±3.5	13.6±1.1
Adrenal (mg)	40.6±0.9	39.8±4.5	35.6±6.2	39.2±4.3	52.0±6.4	51.8±6.4	49.2±4.9	52.6±4.8
Relative organ weights								
Brain (%)	0.79±0.04	0.81±0.04	1.03±0.09**	0.99±0.10**	1.04±0.04	1.18±0.05**	1.36±0.05**	1.27±0.05**
Heart (%)	0.33±0.01	0.31±0.02	0.34±0.02	0.35±0.03	0.33±0.02	0.33±0.01	0.35±0.02	0.35±0.02
Lung (%)	0.39±0.03	0.36±0.02	0.40±0.02	0.43±0.08	0.41±0.02	0.49±0.11	0.49±0.34	0.44±0.11
Liver (%)	3.47±0.14	3.75±0.12*	4.27±0.10**	4.09±0.17**	3.02±0.32	3.94±0.93	4.94±0.34**	4.62±0.10**
Kidney (%)	0.72±0.03	0.73±0.03	0.81±0.04*	0.82±0.07**	0.74±0.04	0.85±0.02**	0.83±0.06**	0.81±0.01*
Spleen (%)	0.22±0.03	0.21±0.01	0.21±0.02	0.21±0.02	0.23±0.01	0.22±0.02	0.22±0.01	0.22±0.01
Testis (%)	1.19±0.03	1.22±0.04	1.37±0.18	1.43±0.14	—	—	—	—
Ovary (X10 ⁻³ %)	—	—	—	—	44.2±9.3	37.4±4.8	37.1±4.2	36.0±10.6
Pituitary (X10 ⁻³ %)	3.0±0.4	2.8±0.4	3.6±0.9	3.7±0.3	6.6±1.3	5.2±0.4	5.6±0.7	5.2±0.9
Thyroid (X10 ⁻³ %)	7.8±2.3	7.8±0.9	8.5±1.9	8.1±2.6	9.4±2.3	10.3±3.0	11.5±2.9	10.8±0.7
Adrenal (X10 ⁻³ %)	18.3±0.8	18.8±3.0	21.8±3.0	22.8±2.4*	33.1±5.0	37.3±3.2	41.6±4.3	41.7±3.7

Values represent mean ± SD of 5 rats/group. Significantly different from control, *P<0.05, **P<0.01.

Table 4. (b) Body and organ weights in rats fed MBEBP for 12 weeks.

Values for rats receiving MBEBP at dose level (%) of:								
Organ	0 (Control)	0.2%	Males		Females			
			190±30**	252±30**	191±9	166±10**	151±11**	155±7**
Absolute organ weights								
Body weights	311±22	296±15						
Brain (g)	1.82±0.06	1.79±0.06	1.72±0.06	1.78±0.07	1.70±0.01	1.71±0.02	1.68±0.05	1.68±0.02
Heart (g)	0.86±0.04	0.81±0.04	0.59±0.09**	0.74±0.07*	0.56±0.04	0.54±0.03	0.52±0.04	0.49±0.03
Lung (g)	1.07±0.12	0.96±0.07	0.75±0.08**	0.87±0.07**	0.74±0.04	0.72±0.05	0.69±0.07	0.70±0.04
Liver (g)	8.84±0.71	10.33±0.76	8.36±1.14	9.59±1.26	4.74±0.29	5.20±0.33	6.86±0.31**	6.16±0.59**
Kidney (g)	1.98±0.17	1.95±0.12	1.41±0.20**	1.69±0.24	1.27±0.05	1.26±0.08	1.16±0.05	1.17±0.11
Spleen (g)	0.62±0.06	0.54±0.04	0.36±0.05**	0.46±0.05**	0.38±0.02	0.34±0.05	0.31±0.03**	0.31±0.03**
Testis (g)	2.86±0.12	2.79±0.08	1.18±0.16**	2.03±0.69**	—	—	—	—
Ovary (mg)	—	—	—	—	68.2±9.9	58.4±10.9	45.2±7.8**	47.0±9.3**
Pituitary (mg)	8.2±0.8	7.4±0.9	6.2±0.4*	7.8±1.6	11.6±0.9	11.2±1.6	6.8±1.3**	7.8±2.0**
Thyroid (mg)	16.8±3.3	21.8±2.2	18.6±6.5	22.4±6.6	16.8±3.3	17.2±2.9	16.6±5.3	16.0±3.4
Adrenal (mg)	43.6±7.0	43.8±6.5	39.2±4.4	41.6±4.3	49.0±3.7	52.0±7.1	49.0±3.2	49.8±8.4
Relative organ weights								
Brain (%)	0.59±0.02	0.60±0.01	0.92±0.12**	0.71±0.06**	0.89±0.04	1.03±0.05**	1.12±0.05**	1.09±0.06**
Heart (%)	0.28±0.01	0.27±0.01	0.31±0.02**	0.30±0.02	0.29±0.02	0.32±0.01	0.35±0.01	0.32±0.03
Lung (%)	0.35±0.06	0.33±0.01	0.40±0.03	0.35±0.02	0.39±0.02	0.43±0.02	0.46±0.03**	0.45±0.04*
Liver (%)	2.84±0.15	3.49±0.15**	4.42±0.26**	3.80±0.09**	2.48±0.08	3.14±0.26**	4.55±0.18**	3.97±0.33**
Kidney (%)	0.64±0.03	0.66±0.02	0.74±0.05**	0.67±0.02	0.66±0.02	0.76±0.06*	0.77±0.05*	0.76±0.05*
Spleen (%)	0.20±0.02	0.18±0.01	0.19±0.01	0.18±0.01	0.20±0.01	0.20±0.03	0.20±0.01	0.20±0.02
Testis (%)	0.92±0.07	0.94±0.05	0.63±0.08**	0.79±0.20	—	—	—	—
Ovary (X10 ⁻³ %)	—	—	—	—	35.8±5.9	35.1±5.8	29.8±3.7	30.3±6.0
Pituitary (X10 ⁻³ %)	2.7±0.4	2.5±0.2	3.3±0.6	3.1±0.7	6.1±0.5	6.8±1.3	4.5±0.9	5.0±1.2
Thyroid (X10 ⁻³ %)	5.5±1.4	7.4±0.8	9.7±3.3*	8.8±1.9	8.8±1.5	10.4±1.7	10.9±3.1	10.3±2.2
Adrenal (X10 ⁻³ %)	14.0±1.4	14.8±1.8	20.8±2.1**	16.5±1.1	25.6±1.2	31.2±2.6	32.5±2.2*	32.2±6.1*

Values represent mean ± SD of 5 rats/group, Significantly different from control, * P<0.05, ** P<0.01.

Table 5. Summary of histological examination in rats fed MBEBP.

		Values for rats receiving MBEBP at dose levels (%) of:						
		0 (Control)	0.2%	1.0%	5.0%	0 (Control)	0.2%	5.0%
		4 weeks			12 weeks			
Testis								
atrophy	±	0	0	1	0	0	0	2
	+	0	0	0	0	0	0	1
	++	0	0	0	0	0	3	0
	+++	0	0	0	0	0	2	1
spermatogenic arrest								
	±	0	0	0	2	0	0	2
	+	0	0	0	1	0	1	2
	++	0	0	3	0	0	1	0
	+++	0	0	2	0	0	3	1
calcification								
	±	0	0	0	0	0	0	1
	+	0	0	0	0	0	1	2
interstitial edema								
	±	0	0	0	0	0	0	1
	+	0	0	0	0	0	4	3
Epididymis								
hypospermia								
	±	0	0	0	2	0	0	2
	+	0	0	3	1	0	1	2
	++	0	0	2	0	0	1	0
	+++	0	0	0	0	0	3	1
interstitial edema								
	±	0	0	0	0	0	0	0
	+	0	0	0	0	0	2	0
Parathyroid gland								
vacuolization								
	±	0	0	0	0	0	0	2
	+	0	0	0	0	0	0	1

5 rats/ group, ± : slight, + : mild, ++ : marked, +++ : severe

Photo. 1. Effect of MBEBP on rat seminiferous tubules. Atrophy of seminiferous tubules, severe epithelial degeneration and interstitial edema in male rat fed 1.0 % MBEBP in diet for 12 weeks. H. E. stain, Magnification, (a) $\times 10$, (b) $\times 50$.

Toxicity of MBEBP in rats

Photo. 2. Vacuolization of parathyroid gland cells in female rats fed 1.0 % MBEBP for 12 weeks. H. E. stain, Magnification, $\times 100$.

DISCUSSION

It was found that MBEBP had a low acute toxicity when administered orally or intraperitoneally to male and female Wistar rats. Death was not observed in treated animals up to 10g/kg BW. Compared to acute toxicity study in mice (National Technical Information Service, 1961), Wistar rats seem to be much less sensitive to MBEBP than mice. However, the reason for this apparent difference in the acute toxicity remains unclear.

In subchronic feeding study, the interesting toxicological finding is the suppression of body weight gain. Dose-dependent suppression of body weight gains was not observed, especially, in male rats in the 1.0 and 5.0 % groups. We confirmed the concentrations of MBEBP in the diet were correct, because they were 1701, 9175 and 44330 ppm in the 0.2, 1.0 and 5.0 % diets, respectively (recovery rate of MBEBP was 90 %) (Takagi et al., 1987). However, dose-dependent distribution of MBEBP in male rats was not observed in adipose tissue, liver and testis except digestive tract after 4 weeks administration of MBEBP (1.0 % and 5.0 % in diet) (Takagi et al., 1987; Takagi, 1988). Therefore, it seems likely that inhibition of MBEBP absorption from digestive tract might be induced in the 5.0 % group, because MBEBP inhibits ATP synthesis by the uncoupling action on mitochondria (Takagi, unpublished data). The reason for the dose dependent MBEBP distribution in digestive tract seemed to be adherence of MBEBP to the mucosal surface. Alternatively, increase of MBEBP

metabolic ratio and/or excretion ratio might be more strongly induced in the 5.0 % group than 1.0 % group.

In hematological tests, small but significant decreases of HGB, RBC, HCT, MCHC were observed. However, anemia-related histopathological findings such as extramedullary hematopoiesis were not observed.

In biochemical tests, significant decrease of serum T-GLY was observed in the 0.2, 1.0 and 5.0 % MBEBP treated male rats. It has been reported that the administration of MBEBP at a level of 0.42 % in the diet for 1 week decreased liver T-GLY level in male SD rat (Takahashi and Hiraga, 1981). Therefore, the decrease of serum T-GLY level might be resulted from decrease of T-GLY content in the liver. Decreased levels of serum PL and F-CHO in male rats may also be resulted from reduced production of hepatic lipoprotein synthesis due to the low hepatic triglyceride. However, histopathological change was not observed in the livers of rats treated with MBEBP.

Absolute testis weight was significantly decreased in the 1.0 % group at 4 and 12 weeks and in the 5.0 % group at 12 weeks. Histopathologically, testicular atrophy and decrease of spermatogenesis were observed in both 1.0 and 5.0 % groups. Testicular atrophy has not been reported for some phenolic antioxidants such as butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT) (Hiraga et al., 1970). On the other hand, 2, 2'-methylenebis (4-methyl-6-*tert*-butylphenol) has been reported to cause testicular atrophy (Stasenкова et al., 1977). Therefore, not an antioxidative action but MBEBP molecule itself or some metabolic product might play an important role for causing testis damage. Absolute weights of pituitary gland and ovary were significantly decreased in females of the 1.0 and 5.0 % groups at 4 and 12 weeks without any corresponding histopathological changes. Vacuolization of parathyroid gland cells was only observed in female rats with no changes in serum Ca levels, which seems to imply that the changes are not biologically significant at this stage. The mechanism of sex specific vacuolization of parathyroid gland is not known. However, sex hormones may not be related to this change, because no histological changes in uterus, ovary and pituitary were observed. Therefore, it is supposed that MBEBP or its metabolite would directly induce vacuolization of parathyroid gland and MBEBP metabolism in female rats would be qualitatively and/or quantitatively different from that in male rats.

In conclusion, MBEBP administration to rat for 12 weeks induced testicular damage in males (1.0 and 5.0 % groups) and vacuolization of parathyroid gland cells in females (1.0 and 5.0 % groups). In biochemical test, decrease of serum cholinesterase activity was observed in all treated groups. Therefore, LOAEL was calculated to be 171 mg/kg BW/day in males or 180 mg/kg BW/day in females.

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