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Letter

A repeated dose 28-day oral toxicity study of extract from cultured *Lentinula edodes* mycelia in Wistar rats

Yasuko Yoshioka, Makoto Tamesada and Hironori Tomi

Fundamental Research Dept., Central R & D Laboratory, Kobayashi Pharmaceutical Co., Ltd., 1-30-3 Toyokawa, Ibaraki-city, Osaka 567-0057, Japan

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ABSTRACT — To evaluate the toxicological safety of extract from cultured *Lentinula edodes* mycelia (L.E.M.), repeated doses (2,000 mg/kg/day) were administered to male and female Wistar rats for 28 days. No mortality or abnormality in the general status or appearance was observed in rats administered L.E.M extract. Body weight and food consumption decreased slightly, particularly in the case of male rats, although the degree of decrease was not as prominent toward the end of administration. Examination of hematology, serum biochemistry, absolute and relative organ weights, autopsy and histopathology revealed only a few statistically significant differences between the treatment and control groups; these differences suggested no clinically significant changes related to toxicity. Consequently, the no observed adverse effect level (NOAEL) of L.E.M. extract was considered to be more than 2,000 mg/kg/day under the conditions of the present study.

Key words: *Lentinula edodes* mycelia (L.E.M.), Safety

INTRODUCTION

Shiitake mushroom (*Lentinula edodes*) is a typical edible fungus in Japan and China and a common ingredient in cooking that has been artificial cultivated in Japan for centuries. A polysaccharide in the extract of *L. edodes* fruiting bodies was discovered to have antitumor activity and approved as a new pharmaceutical product called lentinan in 1985. On the other hand, the extract of *L. edodes* mycelia (L.E.M.), the stage prior to the fruiting body, has been reported to have various pharmacologic effects, including antitumor (Yamasaki *et al*., 2003; Itoh *et al*., 2002; Sugano *et al*., 1985), hepatoprotective (Itoh *et al*., 2009; Watanabe *et al*., 2006; Akamatsu *et al*., 2004; Kajimoto *et al*., 2000) and immunomodulatory (Nagashima *et al*., 2005; Ichikawa *et al*., 1991; Suzuki *et al*., 1990). L.E.M. extract has been used as a health food for at least 30 years in Japan.

In recent years, the opportunity to take health foods and supplements has increased as there is greater interest in preventive medicine and complementary and alternative medicine. Various health foods are now widely available, but the safety and efficacy have not yet been confirmed for many of them. In some cases, health problems have even resulted from consumption of such health foods. Evidence-based studies of the safety and efficacy of health foods are therefore becoming increasingly important.

In the present study, a repeated dose 28-day toxicity study of L.E.M. extract in male and female Wistar rats was carried out at a dose of 2,000 mg/kg/day, as a part of studies to evaluate of the toxicological safety of L.E.M. extract.

MATERIALS AND METHODS

Preparation of L.E.M. extract

For preparation of L.E.M extract, the mycelia of *L. edodes* were seeded in a solid medium composed of sugar-cane bagasse and defatted rice bran and cultured for several months until sufficient spread the mycelia. Then, the mycelia were extracted with hot water together with the medium and an extract was obtained further filtration, concentration, sterilization and lyophilization. It has a characteristic odor with a slight bitter taste. An aqueous suspension of L.E.M. extract powder (KOBAYASHI Pharmaceutical Co., Ltd., Osaka, Japan) was used as the test solution in this study.

Correspondence: Yasuko Yoshioka (E-mail: y.yoshioka@kobayashi.co.jp)

Animals and housing conditions

Twenty-five male and 25 female Wistar clean rats (body weight range: males, 73-83 g; females, 75-82 g) were acclimated and quarantined for 1 week prior to initiation of the experiment. The animals were housed in an environmentally controlled room in a barrier system maintained at a temperature of 20-25°C and relative humidity of 40-70% with 10 to 20 changes of air per hour, with a 12-hr light/dark cycle. The animals were allowed free access to laboratory chows and tap water.

Repeated dose 28-day oral toxicity study

Twenty males and 20 females were chosen for this study after the period of acclimation and quarantine. The animals were assigned to 2 groups of 20 animals each (10 males and 10 females), and treated with the aqueous suspension of L.E.M. extract at a dose of 2,000 mg/kg BW or distilled water by gavage once daily for 28 days. During experimental periods, all animals were observed for mortality, appearance, and signs of intoxication once a day, and body weights were recorded on day 0 (day before administration), day 1 (initiation of treatment), and day 3, 7, 11, 14, 18, 21, and 28, and food intakes were recorded on days 1, 2, 7, 14, 21, and 28. After observation of external appearance on the day following the last dose, blood sampling from the abdominal aorta as well as autopsy were conducted under 4% chloral hydrate anesthesia after 16 hr starvation. The experimental protocol was approved by the Institutional Animal Ethical Committee and conducted according to OECD test guideline no. 407.

Hematology and biochemistry

Hematology was examined in all the animals that received oral administration for 28 days. Blood was treated with EDTA-2K as coagulant. Red blood cells (RBC), hemoglobin (Hb), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cells (WBC), neutrophils (NE), lymphocytes (LY), monocytes (MO), eoisinophils (EO), basophils (BA), and platelets (PLT) were measured using a Hemavet 950 (Drew Scientific Inc., Dallas, TX, USA). Prothrombin time (PT) and activated partial thromboplastin time (APTT) were measured using a single-channel coagulator, the BT-M1 Plus (RMD Mediaids Pvt. Ltd., New Delhi, INDIA). Reticulocytes (RET) were measured by counting using microscope.

Serum biochemistry was examined in all the animals that received oral administration for 28 days. Blood was left to stand for an hour and centrifuged at 3,000 rpm for 10 min to obtain serum. Asparatate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), creatinine kinase (CK), total protein (TP), albumin (ALB), globulin (GLB), albumin-globulin ratio (A/G), blood urea nitrogen (BUN), creatinine (Crea), total bilirubin (T-BIL), GLU, total cholesterol (CHO), triglyceride (TG), and inorganic phosphorus (P) were measured using the ALCYON-300 (BIOTECNICA, Varginha, Brazil). Calcium (Ca), potassium (K), sodium (Na), and chloride (Cl) were measured using an automatic analyzer (Model E-555, China).

Autopsy

Autopsy was conducted in all the animals that received oral administration for 28 days. Before autopsy, the overall condition of each animal was examined after under anesthesia. At autopsy, the position, external characteristics and cut surface of each organ and tissue including the mouth, nasal cavity and neck organs, organs of the chest, abdominal organs, pelvic cavity organs, the brain, pituitary gland etc. in all animals were examined macroscopically. The heart, lungs, liver, kidneys, adrenals, spleen, testes, epididymides, seminal vesicle, prostate gland, uterus, ovaries, salivary glands, and thyroid gland were removed, weighed, and compared with the body weight of the rats to determine relative organ weights.

Histopathology

Histopathology was conducted in all the animals that received oral administration for 28 days. The following organs and tissues of animals in each group were fixed in 10% neutral formalin, dehydrated using graded concentration of ethanol, embedded in paraffin, sectioned, and stained with hematoxylin-eosin: heart, lung, bronchus, thyroid, parathyroid gland, liver, kidney, adrenal gland, spleen, pancreas, salivary glands, parotid, sublingual gland, trachea, esophagus, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, bladder, prostate (male), testis (male), epididymis (male), seminal vesicle (male), ovary (female) and uterus (female). Histopathological examination was conducted, and the types and extents of lesions in all the animals were recorded.

Statistical analysis

All quantitative data such as body weight, food consumption, blood parameters, organ weight were represented by the mean value ± S.D. and analyzed statistically by means of Student's t-test. The final statistical significance of differences was determined at the level of $p < 0.05$ in comparison with the control group.

RESULTS AND DISCUSSION

To evaluate the toxicological safety of L.E.M. extract, a repeated dose 28-day toxicity study of L.E.M. extract in male and female Wistar rats was carried out at a dose of 2,000 mg/kg/day

No mortality or abnormalities in appearance or behavior were observed in either male or female rats administered L.E.M. extract during the 28-day administration period.

Table 1 shows the changes in body weight and body weight gain. The body weights of male rats administered L.E.M. extract were significantly lower than those of the control group during the later period of administration, while the body weights of female rats transiently decreased during the middle of the administration period. Body weight gain was suppressed to a greater degree in males than in females; the degree of suppression in both males and females tended to be less toward the end of administration. Table 2 shows the changes in food consumption. Males administered L.E.M. extract consumed significantly less food during the administration period, although this decrease in food consumption was recovered toward the end of administration. In contrast, females administered L.E.M. extract showed no significant difference in food consumption, compared with the control group, indicating no effect by L.E.M extract administration. Taken these results together, L.E.M. extract could affect body weight gain by a mechanism associated with food consumption. However, such effects of L.E.M. extract on weight gain and food consumption appear to be weak, as these parameters tended to recover toward the end of the 28-day administration.

At the end of the 28-day administration, all hematological parameters were within the normal range, with no significant differences between rats administered L.E.M. extract and the control group (Table 3). Thus, administration of L.E.M. extract appeared to have negligible effects on hematological parameters. On serum biochemical examination, values of P and CHO in males administered L.E.M. extract differed significantly from those in the control group, while values of BUN, P and Ca were significantly elevated in females administered L.E.M. extract compared to the control group (Table 4). However, all of these values were within normal reference ranges (P: 0.85-5.07, CHO: 0.93-2.21, BUN: 5.01-10.57, Ca: 2.38-2.78). No statistically significant changes were found in the other parameters examined.

Autopsy was conducted for all animals at the end of the 28-day administration. On gross examination, no visible lesions in any organs or tissues were detected in either experimental group. As shown in Tables 5 and 6, the absolute and relative organ weights in all males were within normal ranges, with no significant differences between experimental groups. In contrast, some organs of female rats administered L.E.M. extract had significantly different absolute weights of the thyroid glands and adrenals and relative weights of the thyroid glands, adrenals, kid-

Table 1. Changes in body weight and body weight gain of rats orally administered L.E.M. extract for 28 days

Day	Control		L.E.M. extract (2,000 mg/kg/day)	
Males				
0	147.7 ± 7.8[a]		147.8 ± 7.7	
1	154.4 ± 8.1	(6.7)[b]	155.0 ± 7.7	(7.2)
3	167.9 ± 9.0	(13.5)	166.1 ± 7.2	(11.1)
7	200.8 ± 9.6	(32.9)	196.4 ± 8.4	(30.3)
11	235.9 ± 14.2	(35.1)	227.6 ± 11.1	(31.2)
14	262.2 ± 14.1	(26.3)	247.5 ± 12.5*	(19.9)
18	295.3 ± 19.7	(33.1)	270.0 ± 13.2**	(22.5)
21	308.9 ± 22.3	(13.6)	281.4 ± 15.5**	(11.4)
28	349.0 ± 30.6	(40.1)	324.3 ± 13.5**	(42.9)
Females				
0	138.1 ± 4.6		138.0 ± 4.7	
1	142.9 ± 5.1	(4.8)	139.0 ± 3.6	(1.0)
3	152.8 ± 7.6	(9.9)	149.2 ± 5.6	(10.2)
7	172.8 ± 8.1	(20.0)	164.8 ± 5.8*	(15.6)
11	193.8 ± 10.5	(21.0)	182.1 ± 8.1*	(17.3)
14	205.0 ± 11.4	(11.2)	194.4 ± 11.6	(12.3)
18	219.6 ± 14.2	(14.6)	210.6 ± 14.6	(16.2)
21	227.2 ± 11.2	(7.6)	216.9 ± 14.7	(6.3)
28	253.4 ± 14.4	(26.2)	242.0 ± 14.3	(25.1)

[a]Values are the mean body weight ± S.D. (g) of 10 rats. [b]Values show the body weight gain (g), the amount of increase from the means of the last body weight measurements. *$p < 0.05$, **$p < 0.01$ vs. control group.

Table 2. Changes of food consumptions in rats orally administered L.E.M. extract for 28 days

day	Male		Female	
	Control	L.E.M.extract (2,000 mg/kg/day)	Control	L.E.M.extract (2,000 mg/kg/day)
0	21.8 ± 0.6	21.0 ± 0.4**	20.4 ± 0.2	20.2 ± 0.4
2	19.8 ± 0.2	18.9 ± 0.3**	19.2 ± 0.4	18.9 ± 0.3
7	23.4 ± 0.2	22.6 ± 0.6**	20.3 ± 1.2	19.3 ± 1.4
14	28.6 ± 0.2	26.5 ± 0.5**	21.7 ± 1.4	21.3 ± 0.7
21	31.0 ± 0.8	25.9 ± 1.4**	20.4 ± 1.7	21.3 ± 0.7
28	32.5 ± 4.5	30.1 ± 0.1	25.2 ± 3.0	25.4 ± 3.6

Values are the mean ± S.D. (g) of 10 rats. **$p < 0.01$ vs. control group.

Table 3. Hematological parameters in rats orally administered L.E.M. extract for 28 days

	Male		Female	
	Control	L.E.M.extract (2,000 mg/kg/day)	Control	L.E.M.extract (2,000 mg/kg/day)
WBC (10^9/l)	8.5 ± 0.80	8.6 ± 0.52	9.0 ± 0.80	8.8 ± 0.63
NE (%)	17.4 ± 6.66	16.2 ± 8.94	16.7 ± 8.11	19.5 ± 5.67
LY (%)	78.2 ± 7.83	79.2 ± 8.06	78.5 ± 8.37	77.6 ± 5.78
MO (%)	4.2 ± 1.96	4.3 ± 1.47	4.3 ± 2.24	2.7 ± 1.80
EO (%)	0.11 ± 0.21	0.23 ± 0.40	0.33 ± 0.45	0.11 ± 0.12
BA (%)	0.08 ± 0.12	0.10 ± 0.16	0.11 ± 0.16	0.07 ± 0.09
RBC (10^{12}/l)	6.1 ± 0.50	6.5 ± 0.45	6.2 ± 0.33	6.1 ± 0.83
Hb (g/dl)	11.6 ± 0.72	11.8 ± 0.74	11.7 ± 0.48	11.4 ± 0.70
HCT (%)	36.0 ± 1.61	36.6 ± 1.79	35.9 ± 1.51	36.0 ± 1.41
MCV (fl)	59.2 ± 5.99	56.2 ± 3.93	57.9 ± 3.82	60.0 ± 8.90
MCH (Pg)	19.0 ± 1.61	18.1 ± 1.38	18.9 ± 1.36	18.8 ± 2.31
MCHC (g/dl)	32.1 ± 1.30	32.1 ± 0.84	32.6 ± 0.97	31.6 ± 1.25
PLT (10^9/l)	1025 ± 76	975 ± 183	967 ± 85	1063 ± 140
RET (%)	4.8 ± 0.77	4.9 ± 0.72	4.7 ± 0.58	4.3 ± 0.44
PT (S)	14.6 ± 0.40	14.2 ± 0.51	14.4 ± 0.49	14.4 ± 0.58
APTT (S)	25.2 ± 5.30	25.5 ± 5.66	23.5 ± 4.35	22.8 ± 3.60

Values are the mean ± S.D. of 10 rats.

Table 4. Serum biochemical parameters in rats orally administered L.E.M. extract for 28 days

	Male		Female	
	Control	L.E.M.extract (2,000 mg/kg/day)	Control	L.E.M.extract (2,000 mg/kg/day)
AST (U/l)	88.0 ± 10.4	86.0 ± 15.5	95.9 ± 11.6	87.2 ± 11.7
ALT (U/l)	32.2 ± 5.53	30.2 ± 1.91	31.1 ± 3.45	28.9 ± 4.58
ALP (U/l)	131.5 ± 17.5	135.1 ± 16.4	117.6 ± 21.4	108.2 ± 4.58
TP (g/l)	60.0 ± 3.12	60.3 ± 1.96	61.7 ± 1.75	60.8 ± 1.64
ALB (g/l)	27.9 ± 0.82	28.3 ± 1.10	28.6 ± 0.89	28.3 ± 1.02
GLB (g/l)	32.2 ± 3.61	32.0 ± 2.03	33.2 ± 1.72	32.5 ± 2.06
A/G	0.88 ± 0.11	0.89 ± 0.07	0.86 ± 0.06	0.87 ± 0.08
T-BIL (μmol/l)	3.62 ± 0.50	3.23 ± 0.53	3.37 ± 0.31	3.18 ± 0.33
BUN (mmol/l)	7.27 ± 0.82	6.98 ± 0.78	6.21 ± 0.50	7.66 ± 1.10**
Crea (μmol/l)	80.4 ± 2.88	80.1 ± 5.47	84.7 ± 7.54	83.0 ± 2.95
GLU (mmol/l)	7.42 ± 1.04	7.33 ± 0.66	6.08 ± 0.76	5.96 ± 1.12
TG (mmol/l)	0.88 ± 0.44	0.73 ± 0.17	0.56 ± 0.37	0.30 ± 0.12
CHO (mmol/l)	1.79 ± 0.23	1.43 ± 0.15**	1.72 ± 0.21	1.57 ± 0.16
CK (U/l)	365.7 ± 41.1	348.9 ± 74.3	334.1 ± 72.9	354.4 ± 58.0
P (mmol/l)	2.70 ± 0.42	3.36 ± 0.85*	2.42 ± 0.39	3.12 ± 0.67*
K (mmol/l)	4.50 ± 0.29	4.39 ± 0.85	4.23 ± 0.31	4.29 ± 0.30
Na (mmol/l)	134.6 ± 2.11	136.9 ± 0.85	135.8 ± 1.5	135.2 ± 2.28
Cl (mmol/l)	95.5 ± 1.84	97.2 ± 0.85	96.6 ± 2.4	97.3 ± 1.23
Ca (mmol/l)	2.45 ± 0.02	2.47 ± 0.85	2.44 ± 0.03	2.48 ± 0.02**

Values are the mean ± S.D. of 10 rats. *$p < 0.05$, **$p < 0.01$ vs. control group.

neys, uterus and ovaries, compared with those of the control group. Despite these differences, no abnormalities were noted in their general condition or histopathology, suggesting no considerable signs of toxicity due to the observed differences in organ weights.

Histopathology of 29 organs per rat was also conducted for all animals at the end of the 28-day administration. The lungs in a few rats of both experimental groups exhibited slight increases in inter-alveolar distance and mild infiltration of lymphocytes and mononuclear cells; such observations occurred at the same frequency and to the same extent for both rat groups, suggesting they were spontaneous lesions characteristic of rats. No pathological alterations were observed in the other organs examined. Thus, no toxicity-related pathological changes resulted in the organs of L.E.M. extract-administered rats.

In conclusion, a 28-day repeated dose of L.E.M. extract at 2,000 mg/kg/day to Wistar rats induced no abnormalities in external appearance or behavior. Although there was slight reduction in body weight and food consumption, particularly in male rats, the level of reduction recovered toward the end of administration. No toxicologically significant effect was observed on hematological and biochemical examinations, absolute and relative organ weights, macroscopic findings and histological findings at the end of administration. Taken together, the no observed

Table 5. Absolute organ weights in rats orally administered L.E.M. extract for 28 days

	Male		Female	
	Control	L.E.M.extract (2,000 mg/kg/day)	Control	L.E.M.extract (2,000 mg/kg/day)
Thyroid gland	0.16 ± 0.05	0.14 ± 0.04	0.11 ± 0.02	0.16 ± 0.03**
Heart	1.39 ± 0.28	1.22 ± 0.17	1.04 ± 0.18	1.11 ± 0.17
Lung	1.65 ± 0.32	1.51 ± 0.28	1.47 ± 0.31	1.24 ± 0.20
Liver	10.8 ± 1.52	10.8 ± 2.47	7.89 ± 0.49	7.77 ± 1.02
Kidneys[a]	2.64 ± 0.30	2.57 ± 0.24	1.66 ± 0.26	1.83 ± 0.21
Adrenals[a]	0.07 ± 0.05	0.08 ± 0.02	0.08 ± 0.01	0.11 ± 0.02**
Spleen	0.78 ± 0.10	0.81 ± 0.16	0.61 ± 0.10	0.65 ± 0.13
Testes ▪ epididymides[a,b]	4.52 ± 0.40	4.42 ± 0.70	-	-
Seminal vesicle	0.71 ± 0.33	0.86 ± 0.29	-	-
Prostate gland	0.49 ± 0.15	0.48 ± 0.10	-	-
Uterus ▪ ovaries[a,b]	-	-	0.83 ± 0.19	0.97 ± 0.17
Salivary glands[a]	1.03 ± 0.10	0.95 ± 0.09	0.79 ± 0.09	0.80 ± 0.12

Values are the mean ± S.D. (g) of 10 rats. Values are represented as the mean ± S.D. (g) of 10 rats. [a]: The right and left organs were weighed together. [b]: Organs described in parallel was weighed together. **$p < 0.01$ vs. control group.

Table 6. Relative organ weights in rats orally administered L.E.M. extract for 28 days

	Male		Female	
	Control	L.E.M.extract (2,000 mg/kg/day)	Control	L.E.M.extract (2,000 mg/kg/day)
Thyroid gland	0.046 ± 0.017	0.043 ± 0.011	0.042 ± 0.009	0.066 ± 0.012**
Heart	0.40 ± 0.08	0.38 ± 0.06	0.41 ± 0.081	0.46 ± 0.063
Lung	0.48 ± 0.10	0.46 ± 0.08	0.59 ± 0.149	0.51 ± 0.061
Liver	3.09 ± 0.23	3.33 ± 0.73	3.12 ± 0.125	3.21 ± 0.334
Kidneys[a]	0.76 ± 0.06	0.79 ± 0.06	0.66 ± 0.104	0.76 ± 0.052*
Adrenals[a]	0.021 ± 0.014	0.024 ± 0.006	0.032 ± 0.006	0.044 ± 0.006**
Spleen	0.22 ± 0.03	0.25 ± 0.04	0.24 ± 0.036	0.27 ± 0.040
Testes ▪ epididymides[a,b]	1.30 ± 0.12	1.37 ± 0.23		
Seminal vesicle	0.21 ± 0.11	0.27 ± 0.09	-	-
Prostate gland	0.14 ± 0.05	0.15 ± 0.03	-	-
Uterus ▪ ovaries[a,b]	-	-	0.33 ± 0.072	0.397 ± 0.057*
Salivary glands[a]	0.30 ± 0.04	0.29 ± 0.03	0.31 ± 0.028	0.33 ± 0.045

Values are the mean of absolute weight (g) / 100 g B.W. ± S.D. for group of 10 rats. [a]: The right and left organs were weighed together. [b]: Organs described in parallel was weighed together. *$p < 0.05$, **$p < 0.01$ vs. control group.

adverse effect level (NOAEL) of L.E.M. extract was considered to be more than 2,000 mg/kg/day under the conditions of the present study.

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