



Subchronic Oral Dose Toxicity Study of *Enterococcus Faecalis* 2001 (EF 2001) in Mice

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Abstract

As a part of general toxicity studies of *Enterococcus Faecalis* 2001 (EF 2001) prepared using heat-treatment bacillus mort body EF 2001 in mice, this study examined the toxicity of EF 2001 in single and repeated administrations following the previous report in order to apply this product to preventive medicine. The safety of oral ingestion of EF 2001 was examined in 6-week-old male and female ICR mice with 1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day administrated by gavage of the maximum acceptable dose of EF 2001. The study was conducted using distilled water as a control following the methods for general toxicity studies described in the "Guidelines for Non-clinical Studies of Pharmaceutical Products 2002". As a control, 1) observation of general conditions, 2) measurement of body weight, 3) determination of food consumption, 4) determination of water consumption, 5) blood test and urinalysis and 6) pathological examination were performed for the administration of EF 2001. Mice received EF 2001 for 13 weeks and results were compared with those of the control group that received distilled water. The results of the above examinations revealed no significant differences between control and EF 2001 groups for both males and females. Thus, no notable toxicity was confirmed with single and repeated oral administrations of EF 2001. Oral administration in the above doses did not result in abnormal symptoms or death during the observation period. No abnormalities in blood cell count or organ weights were seen. Without any evidence of toxicity to cells and organs, EF 2001 is speculated to not adversely affect living organisms. The 50% lethal dose of EF 2001 with oral administration in mice is estimated to be greater than 5,000 mg/kg body weight/day for both male and female mice. Therefore, LD₅₀ value for animals was 5,000 mg/kg or more.

Key words: *Enterococcus Faecalis* 2001, Dose toxicity study, Body weight, Blood biochemistry, Organ weight, Survival rate

INTRODUCTION

In this study, we performed to obtain information on the toxicity test through the subchronic (90-day) oral (maximum 5,000 mg/kg body weight/day) experiment of *Enterococcus Faecalis* (EF-2001).

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Various previously intractable diseases have been overcome by the development of many new medicines. However, cancer is still a major cause of death. In the process of carcinogenesis, a multistep accumulation of gene mutations causes malignant transformation, and the probability of gene mutations is different depending on genetic and environmental factors. Individual differences are found in the susceptibility of cancer, and prevention of carcinogenesis is possible (1,2).

The hematopoietic system as well as the hematocytes is known to be sensitive to radiation, and low doses of radiation can induce damage. Radioprotective agents are those that are administered before exposure to ionizing radiation to reduce the damaging effects, including radiation induced lethality (1). Many synthetic or natural agents have

been investigated in the recent past years for their efficacy to protect against radiation injuries (3). Among the radioprotective compounds, estrogens have been extensively studied. Either estradiol, belonging to the natural estrogens, or the synthetic estrogens like diethylstilbestrol exerted radioprotective actions on radiation sickness of experimental mice including increasing the survival and accelerating the recovery of hematopoiesis (4). Moreover, estrogens also ameliorated hematopoietic suppression induced by cancer radiotherapy or chemotherapy in the clinic (5). However, the inherent toxicities of these agents at the radioprotective concentration warranted further search of a safer and effective radioprotector (6). In EF 2001, a naturally occurring β -glucan was found in *Enterococcus Faecalis* (7).

Enterococcus Faecalis is well known to exert radioprotective effect and anti-tumor effect *in vivo*, and these effects were reproduced in this study (8). To confirm the elucidative mechanisms by which *Enterococcus Faecalis* exerts these effects, the number of leukocyte and lymphocyte was monitored as a hemopoietic action. Furthermore, NK and LAK activity were measured as immunological parameters (9-11).

Many studies have demonstrated that EF 2001, as one of the most important phytoestrogens, had no toxicity on human health at the pharmacological concentration and possessed potential properties to act as both an estrogen and anti-estrogen, inhibit the activities of tyrosine kinase and DNA topoisomerase II, and improve the immune system (12). Consequently, it has gained increasing attention because of its association with beneficial effects for patients with breast cancer, prostate cancer, cardiovascular disease, high cholesterol levels and osteoporosis (13). Moreover, the isoflavone was an effective antioxidant, which could eliminate the free radicals and boost the antioxidant enzymes activities, so that it may provide protection against ultraviolet-B radiation when applied to the skin of hairless mice 1 hr before exposure (13). EF 2001 also reduced the frequency of micronucleated reticulocytes and increased survival of sublethally irradiated mice without exhibiting estrogenic actions on reproductive systems (14).

Hanaoka *et al.* reported that it is not affected by the toxicity test repeatedly feeding for 13 weeks using rats of lactic acid bacteria (*Lactobacillus salivarius* WB 21 strain) (15).

MATERIALS AND METHODS

Objective. Heat-treatment bacillus mort body (*Enterococcus Faecalis* 2001; EF 2001), from EF 2001, lacking fungal products and designated EF 2001.

EF 2001[®], a bacillus product, composed of heat-treatment bacillus mort body, dextrin and gelatin was supplied by Nihon BRM Co., Ltd (Tokyo, Japan).

To examine the toxicity of EF 2001 prepared by using the 1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day administrated by oral administration.

Test animals and husbandry. Four-week-old male and female ICR mice were purchased from Clea Japan (Tokyo, Japan) and were housed in the laboratory animal room illuminated with 150-300 lux of light at our university. The examiners wore working clothes, head coverings, masks, gloves and other protective clothing. Other conventional conditions were employed, including room temperature of $22 \pm 3^\circ\text{C}$ and 60% relative humidity. Mice were administered with EF 2001 (1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day) access to consume food (EC-2, Clea Japan) and tap water. The study started after a 2-week acclimation period, and administration period is 90 days (7 days a week for 3 months).

Assignment and identification of animals. 20 male and 20 female mice were divided into control group (receiving distilled water) and EF 2001 group, forming a total of four groups of 10 mice each.

First, during the acclimation period, mice that were considered healthy were weighed and categorized into the groups divided by body weight at intervals of 5 g. Then, from each control group (receiving distilled water) and EF 2001 group, 10 male and 10 female mice that were close to the average weight for each sex were selected. Mice were identified by hair marking or identification cards.

Method for calculating number of deaths and survival rate. Survival rate was calculated over the 3 months of the administration period in intervals of 4 days using the following formula:

$$\begin{aligned} \text{Survival rate} \\ &= (\text{number of surviving mice}/\text{number of reared mice}) \\ &\times 100 (\%) \end{aligned}$$

Observed and examined items. Safety was evaluated following the "Guidelines for Non-clinical Studies of Pharmaceutical Products 2002", using distilled water as a control (16,17).

- **Observation of general conditions**

Mice were observed at least once daily for general symptoms and mortality from the starting day of administration for 3 months.

- After single administration, changes in symptoms were observed in detail for one day, observation and measurement were conducted for the following items.

- **Measurement of body weight**

All mice used in the study were weighed before the start of administration and at intervals of 1 week for 3 months after the start of administration.

- **Food and water consumption**

Food and water consumption were measured daily. Amounts were determined for each group and the average of 10 mice was calculated as food and water consumption for individual mice.

- **Hematological test and blood biochemistry**

Blood samples were collected before the study and on the last day of the study (3 months) and tested for the following items. Specifically, blood was drawn from the fundus and then from the heart for hematological tests (blood samples collected from the fundus and heart were combined and tested).

- a) Hematological test

1. Red blood cell count
2. White blood cell count
3. Platelet count
4. Hemoglobin
5. Hematocrit

- b) Blood biochemistry

1. Serum (plasma) protein
2. Albumin
3. Albumin/globulin (A/G) ratio
4. Protein fraction
5. Glucose
6. Cholesterol
7. Triglyceride
8. Bilirubin
9. Urea nitrogen
10. Creatinine
11. Transaminases (Alanine transaminase (ALT; Glutamic Pyruvic Transaminase (GPT)), Aspartate Transaminase (AST; Glutamic Oxaloacetic Transaminase (GOT))
12. Alkaline phosphatase

- **Urinalysis**

The following items that could be tested by test strips were examined (mice were forced to urinate directly onto test strips without urine collection): pH; protein; glucose; ketone bodies; and bilirubin.

- **Pathological examination**

Gross observation: All surviving mice were anesthetized with CO₂ 500 mg and sacrificed by abdominal incision followed by exsanguination. The external surface of the skin, oral cavities and eyes and then all internal organs and tissues were grossly observed. In this study, we used a method of putting animals in a container dedicated to euthanasia, “gradually raise the carbon dioxide concentration by 20% in 1 min, bring it to 100% in 5 min”.

Measurement of organ weight. In addition to actual measured organ weights (absolute weights), ratios compared to body weight (relative weight) were also calculated to clarify the implications of changes in organ weight.

The heart, lungs, liver, spleen, kidneys, adrenal glands, prostate, ovaries, brain, pituitary gland, salivary glands, thymus, thyroid glands, seminal glands and uterus were weighed. When no abnormalities were found in a) or b), histopathological observation was not conducted.

Methods for statistical analysis. Data was statistically processed using Statistical Analysis System software (Cary, NC, USA) and Labcat module (Innovative Programming Associates, Princeton, NJ, USA). This study has been approved by the Suzuka University of Medical Science Animal Research Ethics Committee (Ref: 07/625/37). In this research, the Ministry of Education, Culture, Sports, Science and Technology Notification No. 71 “Basic Guidelines on Implementation of Animal Experiments in Research Organizations” (June 1, 2006), Ministry of the Environment “Standards on breeding and storage of experimental animals and relief of pain” With reference to the reference.

RESULTS

Observation of food consumption. Observation of the general conditions of mice in the control group (receiving distilled water, male and female) and EF 2001 group (male and female) revealed no evidence of abnormalities in face washing or other movements, fur, skin sensitization or other conditions. As shown in Fig. 1, There is no difference according to elapsed time.

Changes in body weight among mice in each group. Fig. 2 show mean and standard error (SE) values of body weight of male and female mice in control and EF 2001 groups, and changes over time. The vertical axis of the plot represents body weight and the horizontal axis represents elapsed time. No irregular increase or decrease in body weight was seen in control or EF 2001 groups. No significant differences were identified between male and female mice in either group.

Hematology and biochemical results. Table 1, 2 shows mean and SE values of blood cell analysis and blood biochemistry results in male and female mice in control and EF 2001 groups.

No irregular increase or decrease in blood cell analysis of data was seen in control or EF 2001 groups. No significant differences were identified between male and female mice in either group.

For both male and female animals, no significant differences were seen in any items of blood chemistry, comprising serum (plasma) protein, albumin, A/G ratio, protein fraction, glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine, transaminases (ASAT (GDT), ALAT (GPT)) and alkaline phosphatase between control and EF 2001 groups.

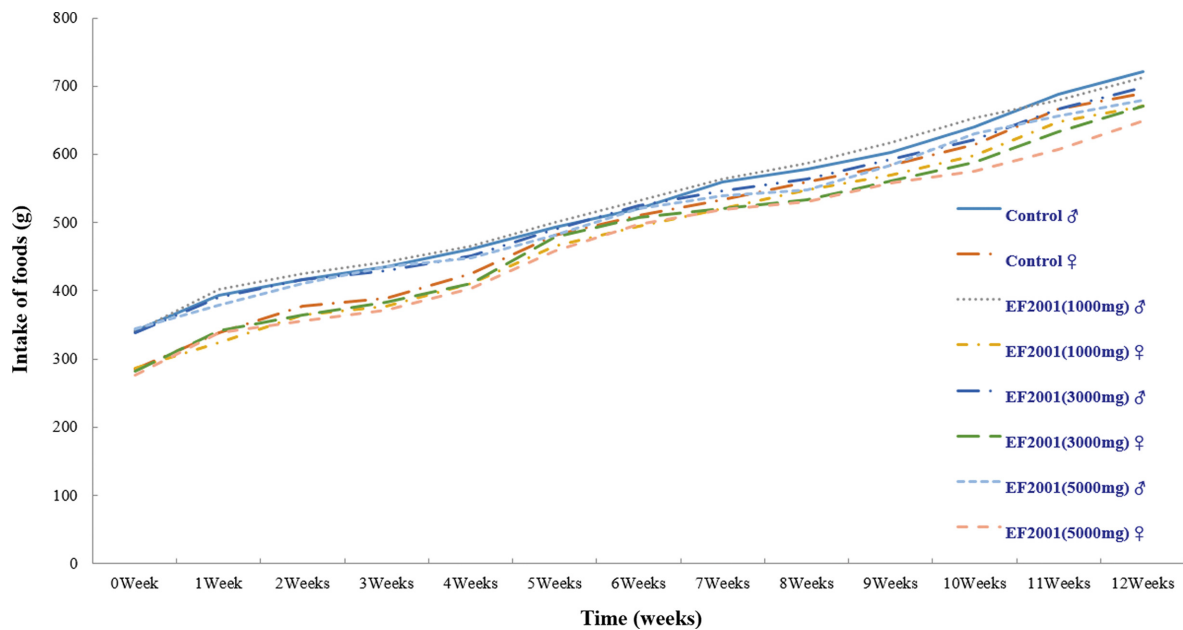


Fig. 1. The vertical axis of the plot represents food consumption and the horizontal axis represents elapsed time.

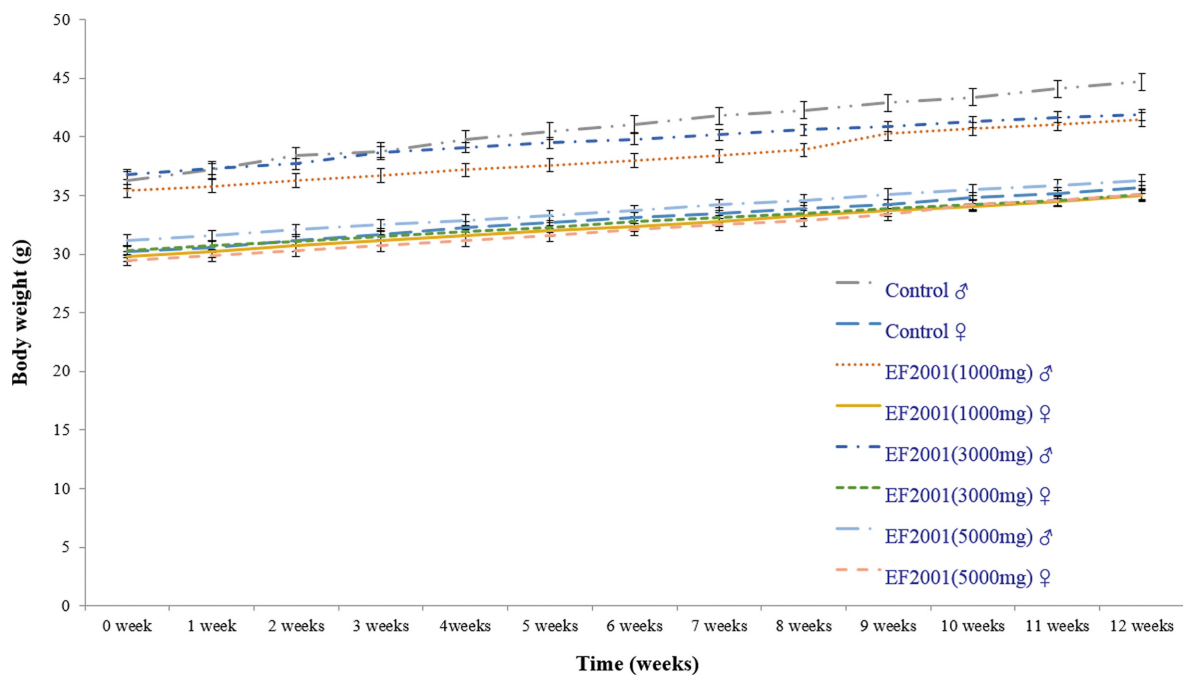


Fig. 2. Change in weight for ICR mice. Results represent the mean \pm SE ($n = 10$).

Measurement of organ weight. Table 3 shows mean and SE values for organ weight of male and female mice in the control and EF 2001 groups. For both male and female animals, no significant differences were observed in any of the organs weighed, comprising lungs, heart, liver, stomach, kidneys, spleen, intestine, adrenal glands, prostate, brain, pituitary gland, salivary glands, thymus,

thyroid gland, seminal glands, testicles, uterus and ovaries between the control and EF 2001 group.

Urinalysis results. Table 4 summarizes the results of urinalysis for mice in each group. Neither male nor female animals demonstrated any significant difference in protein, glucose, occult blood, urobilinogen, ketone bodies or

Table 1. Blood cell analysis for each group of mice The results represent the mean \pm S.E. (n = 10).

| Male mice/Groups | | CBA | CAA | EFBA1000mg | EFAA1000mg | EFBA3000mg | EFAA3000mg | EFBA5000mg | EFAA5000mg |
|----------------------------|------|-------|-------|------------|------------|------------|------------|------------|------------|
| WBC [$10^3/\mu\text{L}$] | Mean | 133.8 | 133.7 | 131.2 | 142.7 | 130.2 | 142.5 | 131.2 | 130.5 |
| | SE | 42.8 | 12.1 | 28.6 | 5.9 | 29.6 | 6.7 | 30.2 | 22.4 |
| RBC [$10^4/\mu\text{L}$] | Mean | 845.2 | 857.6 | 836.2 | 864.5 | 835.7 | 862.5 | 833.9 | 855.4 |
| | SE | 36.5 | 52.1 | 38.6 | 10.4 | 38.9 | 11.2 | 36.1 | 24.5 |
| HGB [g/dL] | Mean | 15.6 | 15.3 | 16.5 | 15.7 | 15.7 | 15.4 | 15.1 | 15.6 |
| | SE | 3.2 | 1.8 | 3.2 | 0.5 | 3.1 | 0.6 | 2.8 | 1.1 |
| HCT [%] | Mean | 44.2 | 43.1 | 43.1 | 45.1 | 42.5 | 44.8 | 43.6 | 43.8 |
| | SE | 8.6 | 0.8 | 7.1 | 0.6 | 6.8 | 0.8 | 5.1 | 0.8 |
| MCV [fL] | Mean | 51.6 | 51.2 | 51.4 | 51.2 | 50.4 | 50.7 | 50.2 | 50.7 |
| | SE | 1.3 | 1.2 | 1.4 | 1.2 | 1.5 | 1.4 | 2.1 | 1.9 |
| MCH [pg] | Mean | 17.5 | 16.8 | 16.9 | 18.1 | 17.4 | 17.5 | 16.9 | 16.8 |
| | SE | 0.8 | 0.6 | 1.1 | 0.4 | 1.2 | 0.5 | 1.1 | 0.7 |
| MCHC [g/dL] | SE | 35.4 | 33.5 | 33.9 | 34.2 | 34.5 | 33.9 | 34.1 | 34.2 |
| | Mean | 1.6 | 0.8 | 1.5 | 0.5 | 1.4 | 0.7 | 1.5 | 1.5 |
| PLT [$10^4/\mu\text{L}$] | SE | 112.6 | 118.5 | 113.8 | 121.2 | 114.5 | 120.9 | 116.8 | 118.9 |
| | Mean | 8.7 | 3.4 | 8.4 | 5.8 | 9.1 | 6.3 | 8.6 | 10.8 |
| Female mice/Groups | | CBA | CAA | EFBA1000mg | EFAA1000mg | EFBA3000mg | EFAA3000mg | EFBA5000mg | EFAA5000mg |
| WBC [$10^2/\mu\text{L}$] | Mean | 130.2 | 133.6 | 131.2 | 132.5 | 132.5 | 140.2 | 129.8 | 131.2 |
| | SE | 16.4 | 12.9 | 15.4 | 25.1 | 32.1 | 34.2 | 28.5 | 13.9 |
| RBC [$10^4/\mu\text{L}$] | Mean | 849.8 | 851.2 | 851.6 | 856.8 | 842.6 | 851.4 | 851.2 | 849.7 |
| | SE | 29.6 | 78.6 | 36.5 | 85.5 | 33.7 | 10.5 | 78.1 | 66.8 |
| HGB [g/dL] | Mean | 13.6 | 13.9 | 13.5 | 13.4 | 14.5 | 15.2 | 13.6 | 13.5 |
| | SE | 1.5 | 0.9 | 1.2 | 1.1 | 2.4 | 0.8 | 1.4 | 1.3 |
| HCT [%] | Mean | 35.6 | 34.6 | 35.9 | 35.2 | 37.8 | 36.5 | 36.8 | 35.7 |
| | SE | 4.3 | 4.9 | 6.3 | 9.5 | 6.3 | 1.9 | 8.6 | 5.9 |
| MCV [fL] | Mean | 51.2 | 50.4 | 50.9 | 51.2 | 50.4 | 46.8 | 51.2 | 50.7 |
| | SE | 1.5 | 1.3 | 10.5 | 11.8 | 1.7 | 0.4 | 7.6 | 4.7 |
| MCH [pg] | Mean | 19.1 | 19.2 | 18.9 | 19.1 | 19.2 | 17.5 | 18.9 | 18.7 |
| | SE | 1.2 | 1.6 | 1.3 | 1.3 | 0.5 | 0.6 | 1.3 | 1.9 |
| MCHC [g/dL] | SE | 35.9 | 35.2 | 36.1 | 36.2 | 37.5 | 36.9 | 36.4 | 36.1 |
| | Mean | 3.1 | 1.4 | 5.6 | 2.8 | 1.2 | 0.9 | 4.6 | 4.9 |
| PLT [$10^4/\mu\text{L}$] | SE | 112.6 | 112.4 | 114.2 | 113.8 | 109.8 | 116.8 | 114.5 | 113.8 |
| | Mean | 6.1 | 13.5 | 14.8 | 15.8 | 8.7 | 5.4 | 15.7 | 25.5 |

Control Before Administration; CBA, Control After Administration; CAA, EF2001 (1,000 mg) Before Administration; EFBA1000 mg, EF2001 (1,000 mg) After Administration; EFAA1000 mg, EF2001 (3,000 mg) Before Administration; EFBA3000 mg, EF2001 (3,000 mg) After Administration; EFAA3000 mg, EF2001 (5,000 mg) Before Administration; EFBA5000 mg, EF2001 (5,000 mg) After Administration; EFAA5000 mg. WBC, white blood cells ($\times 10^3/\text{mL}$); RBC, red blood cells ($\times 10^4/\text{mL}$); HGB, hemoglobin concentration (g/dL); HCT, hematocrit (%); MCV, mean corpuscular volume (fL); MCH, mean corpuscular hematocrit (pg); MCHC, mean corpuscular hematocrit concentration (g/dL); PLT, platelets ($10^4/\mu\text{L}$).

WBC: [$10^3/\mu\text{L}$], RBC: [$10^4/\mu\text{L}$], HGB: [g/dL], HCT: [%], MCV: [fL], MCH: [pg], MCHC: [g/dL], PLT: [$10^4/\mu\text{L}$].

bilirubin between control and EF 2001 groups. However, pH values indicated that urine tended to be slightly more alkaline in the EF 2001 group compared with that in the control group, although no significant difference was identified.

Survival rate. Fig. 3 shows survival rates of male and female mice in the control and EF 2001 groups. For both

male and female animals, survival rates were 100% in both control and EF 2001 groups. No deaths occurred during the study.

DISCUSSION

The mice received administration of EF 2001 prepared using by gastric tube at a dose of 500 mg/kg/day and were

Table 2. Blood biochemistry results

| Groups | | CM | CF | EM1000mg | EF1000mg | EM3000mg | EF3000mg | EM5000mg | EF5000mg |
|---|------|-------|-------|----------|----------|----------|----------|----------|----------|
| Serum CRP (plasma protein (mg/dL)) | Mean | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| | SE | 0.02 | 0.009 | 0.005 | 0.008 | 0.006 | 0.005 | 0.003 | 0.004 |
| Albumin (g/dL) | Mean | 4.1 | 4.0 | 4.1 | 4.3 | 4.2 | 4.1 | 4.3 | 4.0 |
| | SE | 0.09 | 0.06 | 0.05 | 0.06 | 0.07 | 0.09 | 0.05 | 0.08 |
| Albumin/globulin ratio (A/G ratio: 1.2~2.0) | Mean | 1.6 | 1.5 | 1.6 | 1.5 | 1.5 | 1.4 | 1.6 | 1.3 |
| | SE | 0.08 | 0.06 | 0.05 | 0.04 | 0.04 | 0.05 | 0.05 | 0.03 |
| Bilirubin (mg/dL) | Mean | 2.8 | 3.0 | 2.7 | 2.6 | 2.6 | 2.7 | 2.5 | 2.6 |
| | SE | 0.33 | 0.37 | 0.31 | 0.21 | 0.37 | 0.27 | 0.33 | 0.21 |
| BUN (9~21 mg/dL) | Mean | 13.7 | 13.1 | 13.8 | 13.7 | 14.9 | 13.9 | 13.8 | 12.8 |
| | SE | 0.66 | 0.71 | 1.23 | 0.72 | 1.12 | 0.65 | 1.16 | 0.62 |
| Creatinine (mg/dL) | Mean | 0.8 | 0.6 | 0.8 | 0.5 | 0.7 | 0.4 | 0.7 | 0.3 |
| | SE | 0.075 | 0.15 | 0.12 | 0.14 | 0.14 | 0.12 | 0.21 | 0.61 |
| ALP (IU/l) | SE | 147.3 | 148.1 | 145.6 | 144.6 | 147.1 | 147.9 | 143.6 | 140.6 |
| | Mean | 8.25 | 9.62 | 8.91 | 12.6 | 9.41 | 6.20 | 14.32 | 7.5 |
| Glucose (ml/dL) | SE | 107.6 | 106.1 | 108.6 | 103.5 | 106.7 | 106.6 | 108.4 | 107.6 |
| | Mean | 1.57 | 1.45 | 2.64 | 6.7 | 1.54 | 0.87 | 6.8 | 9.7 |

Control Male; CM, Control Female; CF, EF2001 (1,000 mg) Male; EM1000mg, EF2001 (1,000 mg) Female; EF1000mg, EF2001 (3,000 mg) Male; EM3000mg, EF2001 (3,000 mg) Female; EF3000mg, EF2001 (5,000 mg) Male; EM5000mg, EF2001 (5,000 mg) Female; EF5000mg. CRP; C-reactive protein, BUN; Blood urea nitrogen, ALP; Alkaline phosphatase.

also allowed to drink EF 2001. EF 2001 was forcibly administered in 1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day, resulting in additional ingestion of approximately 6~7 g/day/mouse. Each mouse thus ingested approximately 150 g/kg/day or more (calculated assuming a body weight of 40 g) of EF 2001 in total in this study. We examined the toxicity of EF 2001 by 1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day administered and by comparing groups receiving the above amount of EF 2001 and control groups receiving distilled water. The results are discussed below.

Observation of food consumption. For both single and repeated administration and for both male and female mice, no notable differences in general symptoms were seen between EF 2001 and control groups.

Changes in body weight. Body weight steadily increased in each group without irregular increases or decreases. No significant difference was observed between control and EF 2001 groups, indicating an absence of any effect of EF 2001 on body weight. Based on these results, we speculate that daily ingestion of EF 2001 prepared using Doctor EF 2001 would not affect body weight in humans.

In the present study, a subchronic (90-day) toxicity test was conducted, and no change was observed in the body weight of the EF 2001 administration group as compared with the control group (18). The reason for this is that EF 2001 is not toxic and is considered to be a dead cell (19,20).

Effect on food and water consumption. Mean daily water consumption was 6~7 mg/day/mouse in both control and EF 2001 groups when mice were allowed to drink water. Also, administration is only forced administration. EF 2001 was forcibly administered in 1,000 mg/kg, 3,000 mg/kg and 5,000 mg/kg body weight/day in addition to daily administration (500 mg/kg body weight/day) in the morning and evening by gavage. For both male and female mice, no difference in food consumption was noted between the control and EF 2001 groups. The EF 2001 group thus did not specifically show any effect of EF 2001 on food or water consumption relative to the control group.

Changes in blood cell count and blood biochemistry. For all items determined by blood cell counts, no significant differences were observed between control and EF 2001 groups. We therefore speculate that daily ingestion of EF 2001 would not affect blood cells, indicating an absence of cytotoxicity. For both male and female mice, no significant difference was detected between the EF 2001 and control groups when blood biochemistry was examined over time (Table 2). Ingestion of EF 2001 did not result in significant differences in serum (plasma) protein, albumin, A/G ratio, protein fraction, glucose, cholesterol, triglyceride, bilirubin, urea nitrogen, creatinine. No toxicity was evident in terms of blood biochemistry.

Effects on organ weight. None of the organs demonstrated significant differences in weight between control and EF 2001 groups or any macroscopic abnormality. For

Table 3. Organ weight

| Organ/Groups | Lung (g) | Heart (g) | Liver (g) | Stomach (g) | Kidney (g) | Spleen (g) | Intestine (g) | Adrenal gland (g) | Prostate (g) |
|--------------------|----------|-----------|-----------|-------------|------------|------------|---------------|-------------------|--------------|
| Control ♂ | 0.12 | 0.21 | 2.24 | 0.62 | 0.75 | 0.06 | 4.02 | 0.058 | 0.076 |
| SE | 0.087 | 0.07 | 0.136 | 0.132 | 0.169 | 0.04 | 0.261 | 0.002 | 0.004 |
| Control ♀ | 0.06 | 0.17 | 1.57 | 0.46 | 0.51 | 0.05 | 3.36 | 0.057 | N |
| SE | 0.049 | 0.078 | 0.11 | 0.128 | 0.054 | 0.05 | 0.261 | 0.001 | N |
| EF2001 (1000 mg) ♂ | 0.13 | 0.22 | 2.25 | 0.66 | 0.78 | 0.07 | 4.11 | 0.061 | 0.081 |
| SE | 0.053 | 0.05 | 0.151 | 0.143 | 0.172 | 0.05 | 0.266 | 0.003 | 0.003 |
| EF2001(3000 mg) ♂ | 0.17 | 0.22 | 2.19 | 0.79 | 0.67 | 0.05 | 3.91 | 0.058 | 0.084 |
| SE | 0.05 | 0.06 | 0.104 | 0.197 | 0.046 | 0.05 | 0.171 | 0.001 | 0.001 |
| EF2001 (5000 mg) ♂ | 0.16 | 0.21 | 2.15 | 0.81 | 0.65 | 0.05 | 3.89 | 0.054 | 0.079 |
| SE | 0.04 | 0.07 | 0.12 | 0.184 | 0.051 | 0.06 | 0.188 | 0.004 | 0.001 |
| EF2001 (1000 mg) ♀ | 0.08 | 0.16 | 1.51 | 0.65 | 0.48 | 0.05 | 3.25 | 0.054 | 0.06 |
| SE | 0.01 | 0.04 | 0.061 | 0.051 | 0.04 | 0.03 | 0.231 | 0.001 | 0.001 |
| EF2001 (3000 mg) ♀ | 0.07 | 0.15 | 1.44 | 0.60 | 0.46 | 0.04 | 3.16 | 0.058 | N |
| SE | 0.05 | 0.05 | 0.066 | 0.045 | 0.049 | 0.04 | 0.196 | 0.001 | N |
| EF2001 (5000 mg) ♀ | 0.06 | 0.05 | 1.53 | 0.65 | 0.04 | 0.04 | 3.21 | 0.062 | 0.001 |
| SE | 0.01 | 0.02 | 0.042 | 0.039 | 0.034 | 0.01 | 0.27 | 0.002 | 0.001 |

| Organ/Groups | Brain (g) | Pituitary gland (g) | Salivary gland (g) | Thymus (g) | Thyroid gland (g) | Seminal gland (g) | Testicle (g) | Uterus (g) | Ovary (g) |
|--------------------|-----------|---------------------|--------------------|------------|-------------------|-------------------|--------------|------------|-----------|
| Control ♂ | 0.641 | 0.0019 | 0.540 | 0.036 | 0.0354 | 0.6411 | 0.3251 | N | N |
| SE | 0.021 | 0.0001 | 0.013 | 0.001 | 0.0001 | 0.021 | 0.011 | N | N |
| Control ♀ | 0.56516 | 0.0018 | 0.4991 | 0.0351 | 0.0339 | N | N | 0.35 | 0.033 |
| SE | 0.031 | 0.0001 | 0.015 | 0.0012 | 0.0002 | N | N | 0.011 | 0.001 |
| EF2001 (1000 mg) ♂ | 0.653 | 0.002 | 0.58 | 0.041 | 0.0372 | 0.716 | 0.3832 | 0.241 | 0.041 |
| SE | 0.021 | 0.001 | 0.02 | 0.001 | 0.0001 | 0.002 | 0.001 | 0.011 | 0.001 |
| EF2001 (3000 mg) ♂ | 0.6361 | 0.0018 | 0.5383 | 0.0351 | 0.0332 | 0.6421 | 0.3391 | N | N |
| SE | 0.028 | 0.0001 | 0.023 | 0.002 | 0.0001 | 0.04 | 0.02 | N | N |
| EF2001 (5000 mg) ♂ | 0.648 | 0.0021 | 0.6121 | 0.0412 | 0.0351 | 0.710 | 0.387 | 0.081 | 0.048 |
| SE | 0.031 | 0.001 | 0.0012 | 0.0018 | 0.0012 | 0.031 | 0.0018 | 0.021 | 0.014 |
| EF2001 (1000 mg) ♀ | 0.612 | 0.0017 | 0.5143 | 0.0384 | 0.0341 | 0.061 | 0.3211 | 0.061 | 0.041 |
| SE | 0.031 | 0.001 | 0.051 | 0.001 | 0.0001 | 0.011 | 0.0012 | 0.0018 | 0.0124 |
| EF2001 (3000 mg) ♀ | 0.5663 | 0.0018 | 0.4987 | 0.0357 | 0.0338 | N | N | 0.32 | 0.030 |
| SE | 0.026 | 0.0002 | 0.016 | 0.001 | 0.0001 | N | N | 0.020 | 0.0003 |
| EF2001 (5000 mg) ♀ | 0.5312 | 0.0021 | 0.0471 | 0.0314 | 0.0317 | 0.028 | 0.2891 | 0.0571 | 0.0354 |
| SE | 0.014 | 0.007 | 0.0121 | 0.001 | 0.002 | 0.002 | 0.003 | 0.002 | 0.002 |

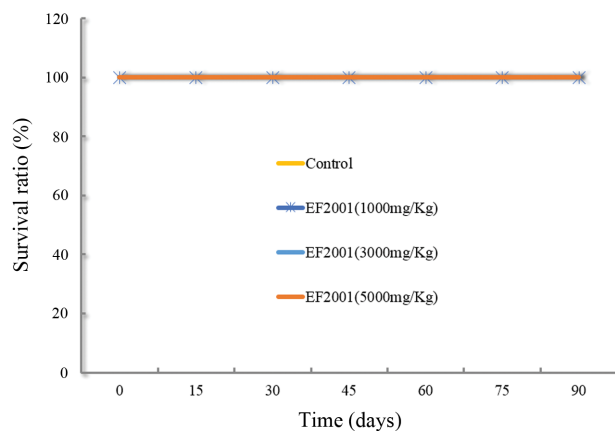
| Organ/Groups | Lung (g) | Heart (g) | Liver (g) | Stomach (g) | Kidney (g) | Spleen (g) | Intestine (g) | Adrenal gland (g) | Prostate (g) |
|----------------|----------|-----------|-----------|-------------|------------|------------|---------------|-------------------|--------------|
| Control male | 0.12 | 0.21 | 2.24 | 0.62 | 0.75 | 0.06 | 4.02 | 0.058 | 0.076 |
| ± SE | 0.087 | 0.07 | 0.136 | 0.132 | 0.169 | 0.049 | 0.261 | 0.002 | 0.004 |
| Control female | 0.06 | 0.17 | 1.57 | 0.46 | 0.51 | 0.05 | 3.36 | 0.0579 | N |
| ± SE | 0.049 | 0.078 | 0.11 | 0.128 | 0.054 | 0.05 | 0.261 | 0.0017 | N |
| EF 2001 male | 0.17 | 0.22 | 2.19 | 0.79 | 0.67 | 0.05 | 3.91 | 0.0583 | 0.0848 |
| ± SE | 0.05 | 0.06 | 0.104 | 0.197 | 0.046 | 0.05 | 0.171 | 0.0019 | 0.001 |
| EF 2001 female | 0.07 | 0.15 | 1.44 | 0.60 | 0.46 | 0.04 | 3.16 | 0.0581 | N |
| ± SE | 0.05 | 0.05 | 0.066 | 0.045 | 0.049 | 0.049 | 0.196 | 0.0015 | N |

| Organ/Groups | Brain (g) | Pituitary gland (g) | Salivary gland (g) | Thymus (g) | Thyroid gland (g) | Seminal gland (g) | Testicle (g) | Uterus (g) | Ovary (g) |
|----------------|-----------|---------------------|--------------------|------------|-------------------|-------------------|--------------|------------|-----------|
| Control male | 0.636 | 0.001 | 0.538 | 0.035 | 0.0333 | 0.6366 | 0.3248 | N | N |
| ± SE | 0.031 | 0.0001 | 0.023 | 0.001 | 0.0001 | 0.030 | 0.012 | N | N |
| Control female | 0.5606 | 0.0017 | 0.4927 | 0.0347 | 0.0324 | N | N | 0.34 | 0.032 |
| ± SE | 0.035 | 0.0001 | 0.017 | 0.0012 | 0.0002 | N | N | 0.012 | 0.002 |
| EF 2001 male | 0.6361 | 0.0018 | 0.5383 | 0.0351 | 0.0332 | 0.6421 | 0.3391 | N | N |
| ± SE | 0.028 | 0.0001 | 0.023 | 0.002 | 0.0001 | 0.04 | 0.02 | N | N |
| EF 2001 female | 0.5618 | 0.0017 | 0.4927 | 0.0345 | 0.0323 | N | N | 0.35 | 0.033 |
| ± SE | 0.027 | 0.0003 | 0.017 | 0.0011 | 0.0001 | N | N | 0.022 | 0.0004 |

The results represent mean ± SE (n = 10), N = none.

Table 4. Results of urine tests

| Male mice | Protein | Glucose in urine | Occult blood | Urobilinogen | Ketone bodies | Bilirubin | pH |
|------------------|---------|------------------|--------------|--------------|---------------|-----------|---------|
| Control | - | - | - | ± | - | - | 6 ± 2.4 |
| EF2001 (1000 mg) | - | - | - | ± | - | - | 8 ± 2.5 |
| EF2001 (3000 mg) | - | - | - | ± | - | - | 7 ± 3.4 |
| EF2001 (5000 mg) | - | - | - | ± | - | - | 7 ± 5.6 |
| Female mice | Protein | Glucose in urine | Occult blood | Urobilinogen | Ketone bodies | Bilirubin | pH |
| Control | - | - | - | ± | - | - | 6 ± 2.1 |
| EF2001 (1000 mg) | - | - | - | ± | - | - | 8 ± 2.2 |
| EF2001 (3000 mg) | - | - | - | ± | - | - | 7 ± 5.7 |
| EF2001 (5000 mg) | - | - | - | ± | - | - | 7 ± 1.8 |

**Fig. 3.** This graph shows the survival rate (%).

this reason, no histopathological observation was conducted for any organ. We speculated that daily ingestion of EF 2001 would not be associated with toxicity to any particular organs. Active oxygen is produced in energy production in various organs, including liver and muscle tissues. Hydrogen molecules in EF 2001 are known to be quickly absorbed and distributed throughout the body and have also been reported to selectively eliminate active oxygen (5). Instead of being toxic to organs, EF 2001 is expected to protect organs from the excessive presence of harmful reactive oxygen species and thus effectively prevent diseases associated with these species (7,12).

In this study, EF2001 has effects on the increase of immunological activity, anti-tumor effects and indigestion effects, but no effect on body weight and organ weight.

Effect on urine. Neither control nor EF 2001 groups showed any changes in items of urinalysis, such as protein, glucose in urine, occult blood, urobilinogen, ketone bodies or bilirubin. A tendency towards a difference in pH was seen between control and EF 2001 groups, but was not statistically significant. We considered that this was partially because the EF 2001 prepared by gastric tube used in this study was alkaline, with a pH of 7.6 to 8.6.

Survival rate. Survival rates for male and female mice in both control and EF 2001 groups were 100%, without any evidence of fatal toxicity. Compare the control group and the administration group and compare the sprinting by *t*-test (14).

For the mortality rate, dead animals were not observed during the test period in the 5,000 mg/kg administration group. The LD₅₀ value for animals was 5,000 mg/kg or more and it was impossible to measure. For general symptoms, no abnormal symptoms were observed during the observation period in all test groups. Regarding body weight change, there was normal weight gain during the administration period in all test groups. Furthermore, there was no anatomical physiological abnormality. As for the autopsy findings, all the internal organs were observed macroscopically, but no abnormality in the organs was observed in all the groups. Therefore, in this study, it was judged that there was no toxicity due to administration of EF 2001 of 5,000 mg/kg.

ACKNOWLEDGMENTS

The authors would like to thank the Nihon BRM research center, Nihon BRM Co., Ltd. for providing Grant code (Jpn-BRM-07-13- 03) to accomplish this research.

Received May 18, 2017; Revised September 29, 2017; Accepted October 20, 2017

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