原著

Protective Effects of *Enterococcus Faecalis* 2001 (EF 2001) against Radiation-induced Leukocytes Damage in Mice

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Abstract: Radiation protection from death and decrease damage leukocyte recovery by oral administrations consecutively of EF 2001 (*Enterococcus Faecalis* 2001), 200 mg/kg b.w., once a day, before whole-body x-rays irradiation was confirmed by tests with C3H mice, meanwhile, its radioprotective actions compared to immunological enhancement. Based on the studies of survival, behavior of hematograms, and numbers of lymphocytes, whole body following irradiation, it was demonstrated that EF 2001 was an effective radioprotector. The survival of irradiated mice protected by EF 2001 was significantly increased and statistically higher than that of mice pre-treated with oral administration. After administration of EF 2001, promoted recovery of leukocyte and lymphocytes counts were observed in mice pre-treated with EF 2001. All above-mentioned results were similar to those in mice protected by EF 2001, but the protecting actions of EF 2001 on promoting recovery of nucleated cells and leukocyte counts were significantly higher than those of EF 2001. It could be deduced that the uncertain radioprotective action against death is induced by a possible process of enhanced regeneration of the leukocyte stem cells due to not only strengthened radioresistance and increased numbers of remained leukocyte cells, but also enhanced post-irradiation repair or promoted proliferation of the leukocyte stem cells. This effect of EF 2001 may have some therapeutic implications for radiation-induced injuries. Showing in this paper. In addition, we think that indicating the activation of cell-mediated immune responses.

Keywords: Radiation protection, Lymphocyte, CD4, CD8, CD16, *Enterococcus Faecalis*

Introduction

The hematopoietic system as well as the hematocytes is known to be sensitive to radiation, and low doses of radiation can induce damage[1)]. Radioprotective agents are those 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[2)]. Among the radioprotective compounds, estrogens have been extensively studied. Either estradiol, belonging to the natural estrogens, or the synthetic estrogens such as diethylstilbestrol exerted radioprotective actions on radiation sickness of experimental animals increasing the surviving fraction and accelerating the recovery of hematopoiesis[3)]. Moreover, estrogens also ameliorated hematopoietic suppression induced by caner radiotherapy or chemotherapy in clinical crials[4)]. However, the inherent toxicities of these agents at the radioprotective concentration warranted further search of a safer and effective radioprotector[5)]. EF 2001 (*Enterococcus faecalis* 2001; EF 2001), a naturally occurring β-glucan found in *Enterococcus faecalis*[6)]. Many studies have demonstrated that EF 2001, as one of the most important enterococcus faecalis, had no toxicity on human health at the pharmacological concentration and possessed potential properties to act as both an immune and endocrine, inhibit the activities of tyrosine kinase and DNA topoisomerase II and improve the immune system[7)]. Consequently, it has gained increasing attention because of its association with beneficial effects for persons with immune compromise[8)]. Moreover, the β-glucan was an effective antioxidant, which could eliminate the free radicals

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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 h before exposure[9]. EF 2001 also reduced the frequency of micronucleated reticulocytes and increased survival of sublethally irradiated mice without exhibiting immune actions on immuno systems[10]. The purpose of the tests reported here was to study *in vivo* radioprotection of EF 2001 on hematopoietic recovery contributing to increase survival of sublethally irradiated mice[11].

Material and Methods

Animals

Male C3H/Hej mice purchased from Japan SLC (Shizuoka, Japan) were used at 7 weeks of age. Mice were housed with controlled lightning (12L: 12D) and food and water were given *ad libitum.* All mice were acclimated to laboratory conditions for 1 week before experimentation. This study is an experiment that was approved by the animal ethics committee of Suzuka university of medical science in 2010.

Test material

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).

Radio-protective effect

Mice were orally medicated with EF 2001 suspended in physiological saline at a dose of 200 mg/kg/day for two weeks at one day intervals. The vehicle-control mice received an equivalent volume of physiological saline. After the final injection, mice were exposed to X-ray radiation. Whole body radiation exposure was carried out at a dose of 2 Gy (a dose rate of 1.12 Gy/min) using a X-ray irradiation device (MG226/4.5, Phillips, Inc. Tokyo). Body weight and the number of surviving animals were daily monitored.

Leukocyte and lymphocyte counts

Mice were orally medicated with EF 2001 suspended in physiological saline at a dose of 200 mg/kg. The vehicle-control mice received an equivalent volume of physiological saline. After the injection, blood samples were obtained from caudal vein into heparinized tubes (coated with lithium heparin at 14 IU of heparin/mL of blood) at given time points for measuring leukocyte and lymphocyte counts using an automated hematology analyzer (Celltac-α MEK-6318, Nihonkouden Co., Ltd. Tokyo).

Measurement of CD4, CD8, and CD16 positive T lymphocytes in peripheral blood

Lymphocytes were separated by the gravity centrifugation method[6]. Lymphocyte separating solution (5 mL; sodium hypaque, Ficoll 400; specific gravity, 1.0875 ± 0.0005 at 25 °C) was added into a 15-ml sample tube, on to which 5 ml of cell suspension was carefully loaded. After centrifugation at room temperature (15-20 °C) at 500 g for 20 min, plasma in the supernatant was collected to extract lymphocyte subsets. After addition of PBS (pH 7.2, without Ca2+ or Mg2+) supplemented with 10% inactivated FBS (heat-inactivated at 56 °C for 30 min), and red blood cell lysing solution, the mixture was centrifuged at room temperature at 400 g (3,000 rpm) for 10 min.

The supernatant was collected and the cells were resuspended and washed twice in PBS containing FBS. Lymphocytes were resuspended in PBS prior to analysis.

Flow cytometry reagents for lymphocyte subset measurement were added into the lymphocyte suspension in PBS, and the mixture was stained for immunofluorescence for about 30 min at 4 °C in a dark room. After the reaction, the solution was rinsed three times with PBS, and CD4, CD8, and CD16 subsets were analyzed by a FACS Caliber flow cytometer (Becton Dickinson).

To analyze T lymphocyte subsets, Multicolor

Flowcytometry (FCS) System (Santa Cruz Biotechnology Inc.) was employed and CD4, CD8, and CD16-positive T lymphocytes in the peripheral blood were counted by three-color flow cytometry using anti CD3-PE-Cy5.5, anti CD4-FITC, and anti CD8 and CD16-PE.

Statistical analysis

Significance of the difference in each parameter among groups was assessed by *t*-test and the Dunnett comparison test following analysis of variance. Values of $P<0.05$ were considered significant.

Results

Leukocyte counts

The number of blood leukocytes in normal mice is summarized in Figs.1. The number of leukocytes increased with time at least up to 7days to 30days after repeated dose of EF 2001 compared with 2Gy. Statistically significantly higher and increase of leukocyte counts in EF 2001+2Gy group were observed in comparison with2Gy group. In addition, at time of irradiation until day 12hp(hp;hours post), 3days to 30days, statistically significantly higher and more rapidly recovery of leukocyte counts in 2 Gy + EF 2001 group were observed in comparison with 2Gy group.

Lymphocyte counts

The number of blood lymphocytes in normal mice is summarized in Figs.2. The number of lymphocytes increased with time at least up to 7days to 30days after repeated dose of 2Gy + EF 2001 group compare with 2Gy group. Statistically significantly higher and increase of leukocyte counts in EF 2001+2Gy group were observed in comparison with2Gy group. In addition, at time of irradiation until day3hp and 12hp, 7days to 30days, statistically significantly higher and more rapidly recovery of lymphocyte counts in 2 Gy + EF 2001 group were observed in comparison with 2Gy group. Similar results were also in the Leukocyte counts Lymphocyte counts.

Analysis of T lymphocyte subsets in mouse peripheral blood.

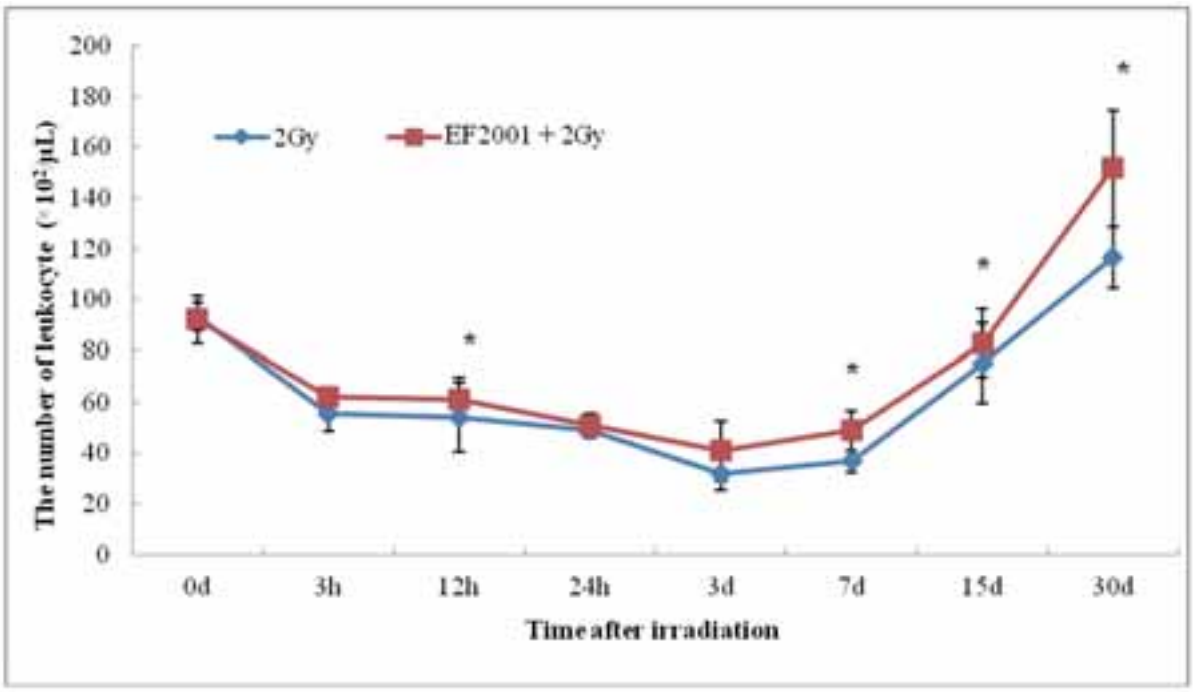

Fig.1. Leukocyte counts on different days after irradiation in mice of different groups. The number of leukocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. * Statistically significant ($p<0.05$) from the 2Gy group.

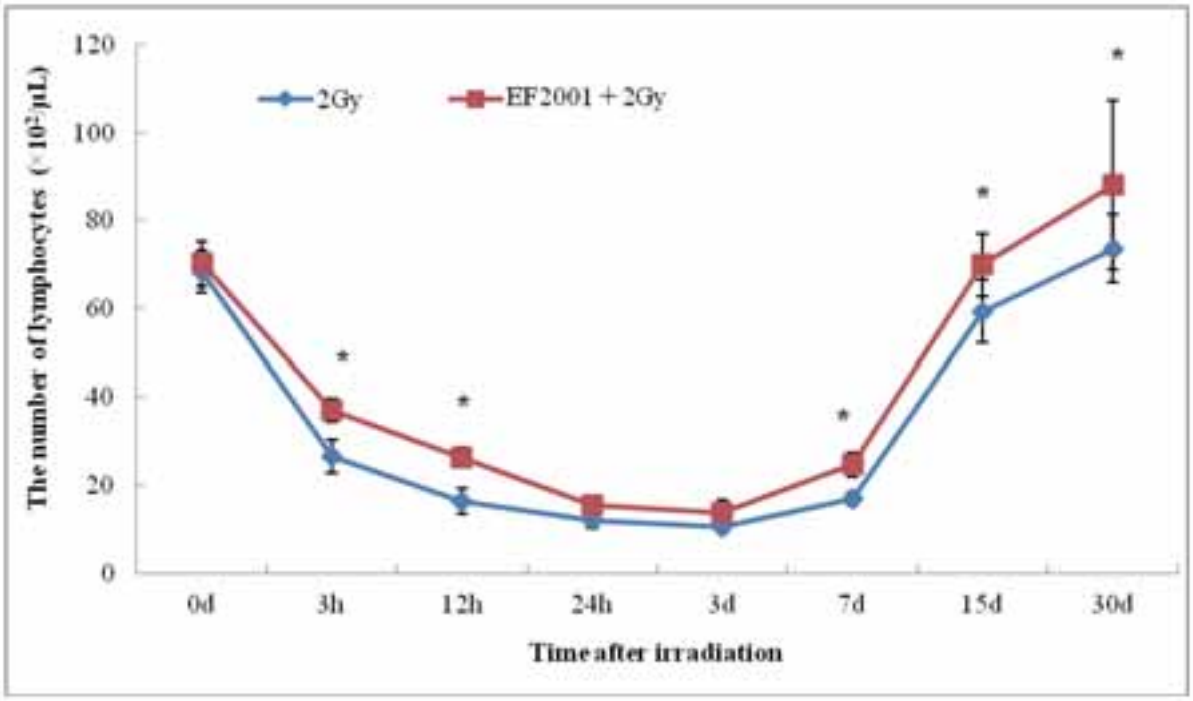

Fig.2. Lymphocyte counts on different days after irradiation in mice of different groups. The number of leukocyte was calculated from the pre-irradiation values taken as 100%. The bars represent standard deviation. * Statistically significant ($p<0.05$) from the 2Gy group.

On the cytogram, the lymphocyte fraction was gated (cells pass through the nozzle flow cytometry), so that CD4-positive (CD3+CD4+) and CD8-positive (CD3+CD8+) cells and CD16-positive (CD3+CD16+) cells were counted. First, CD4-positive cells were counted by flow cytometry for comparison. As shown in Fig.3, the number of CD4-positive cells was increased in only the β-D-glucan group. Likewise, the number CD4-positive cells increased by 11 times in the β-D-glucan with irradiation group compared with the 2Gy group. This indicates that EF 2001 administration increased CD4-postive cells, i.e., helper T cells in the peripheral blood.

CD8-positive cells were also counted by flow cytometry. As shown in Fig.4, CD8-positive cells were increased by 11.6% in the EF2001 group compared with the control group. Compared with the irradiation alone group, CD8-positive cells were decreased by one of 20 in the EF2001 with irradiation group. This indicated that β-D-glucan administration increased CD8-positve cells, i.e., suppresser T cells and killer T cells in the peripheral blood. However, in the β-D-glucan with irradiation group, irradiation decreased the number of CD8-positive cells.

CD16-positive cells were also counted by flow cytometry. As shown in Fig.5, CD16-positive cells were increased by 1.9 times in the EF2001 group compared with the control group. Compared with the irradiation alone group, CD16-positive cells were decreased by 2.9 times in the EF2001 with irradiation group. This indicated that β-D-glucan administration increased CD16-positve cells, i.e., suppresser T cells and killer T cells in the peripheral blood. However, in the β-D-glucan with irradiation group, irradiation decreased the number of CD16-positive cells.

Discussion

EF2001 is well known to exert radioprotective effect and anti-tumor effect in vivo[12] and these effects were reproduced in this study. To confirm the elucidative mechanisms by which EF 2001 these effects, the number of leukocyte and lymphocyte was monitored as a hemopoietic action. Furthermore, T-cell activity was measured as immunological parameters. The results of these parameters demonstrated that the

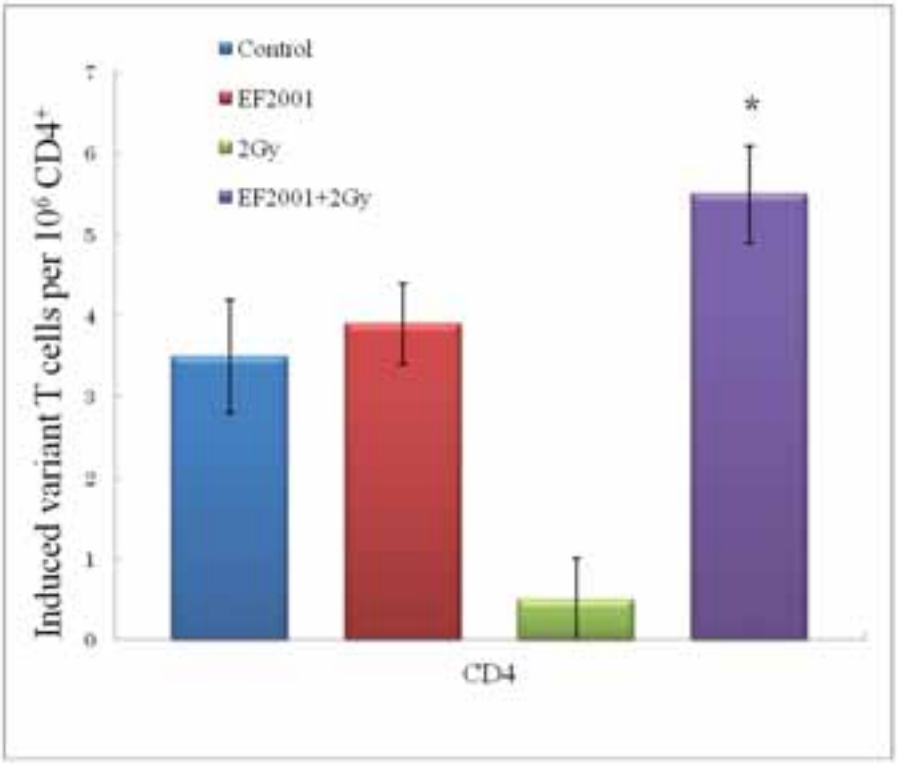

Fig.3. Comparison of the induced frequency of CD4+ in C3H/Hej mice. (7 days after irradiation)
CD4+ T cells from C3H/Hej mice were immunomagnetically purified and then cell were sorted for low CD3 expression. Purity was assessed by FACS analysis. Naive status of cells was confirmed by staining for CD4, CD3, Phenotype of CD4+ T cells after transfer.

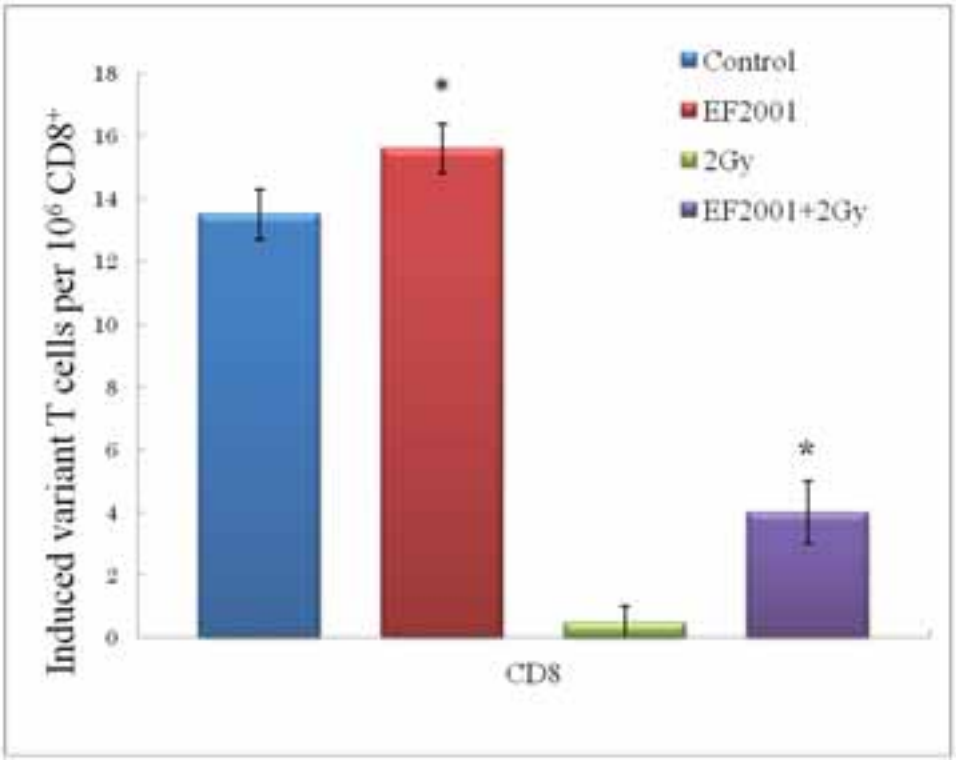

Fig.4. Comparison of the induced frequency of CD8+ in C3H/Hej mice. (7 days after irradiation)
CD8+ T cells from C3H/Hej mice were immunomagnetically purified and then cell were sorted for low CD3 expression. Purity was assessed by FACS analysis. Naive status of cells was confirmed by staining for CD8, CD3, Phenotype of CD8+ T cells after transfer.

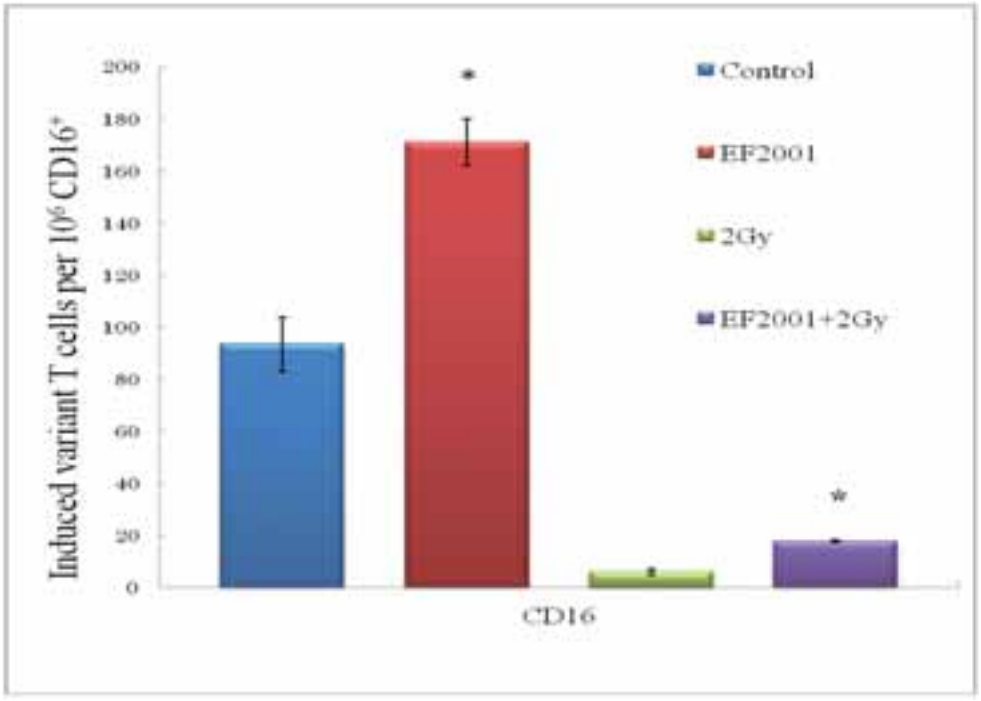

Fig.5. Comparison of the induced frequency of CD16+ in C3H/Hej mice. (7 days after irradiation)
CD16+ T cells from C3H/Hej mice were immunomagnetically purified and then cell were sorted for low CD3 expression. Purity was assessed by FACS analysis. Naive status of cells was confirmed by staining for CD16, CD3, Phenotype of CD16+ T cells after transfer.

radioprotective effect of EF 2001 is probably mediated at least in part by a hemopoietic action in irradiated mice since the leukocyte and lymphocyte number was increased by a single dose of EF 2001. Increased white leukocytes count of elapsed time after the experiment, may be the result of stimulation by blood sampling. In addition, augmented immunological activity as seen in increased T-cell activity by EF 2001 seems to play a role in preventing secondary infections associated with irradiation. Natural killer (NK) cells are well known to be associated with cytotoxic effect on various kinds of tumor cells[13]. Therefore, increased activity of NK by EF 2001 contributes probably to attenuated tumor growth in tumor-bearing mice. From these, EF 2001 is expected to be promising for the treatment of cancer patients receiving radiotherapy. Accordingly, we used peripheral blood cell counts as indicators of bone marrow function in order to assess the radioprotection of normal tissue, which is critical for survival in this study. From the previous study, the data from our experiments showed that prior oral administrations of EF 2001 to mice with 200 mg/kg/day for consecutive 7 days rendered 80% survival in irradiated mice and its survival was significantly higher than that of irradiated control group as well as that of EF 2001 administration[14]. Stimulating recovery of peripheral hematocytes were also observed in mice pre-treated with EF 2001, but protecting actions of EF 2001 on leukocytes and nucleated cells were much stronger than those of EF 2001, although its protection against the decrease of lymphocytes counts was lower than that of EF 2001. The active ingredient of the EF 2001 is due to radiation-induced radical scavenging and stimulation of hematopoietic stem cells as a reason.

We inferred that EF 2001, like EF 2001, was an effective radioprotector against radiation-induced death by stimulating the rehabilitation of hematopoiesis. These properties have been associated provisory with radioprotection[15].

In summary, the results of the current study demonstrated that mice pre-treated with EF 2001 have shown some effects of promoting survival and accelerating the rehabilitation of hematopoiesis by protecting bone marrow stem cells and peripheral

hematocytes against radiation-induced regression and stimulating proliferation and differentiation of hematopoietic cells. Although our preliminary investigations might EF 2001 information basis for the possibility of EF 2001 to be as a selective radioprotector of hematopoietic system, the evidence was not enough to apply yet and its active constitutions in radioprotection should be further examined individually.

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