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New Screening for the Development of Radioprotectors: Radioprotection and Anti-Cancer Effect of β -Glucan (*Enterococcus faecalis*)

Yeun-Hwa Gu

Abstract

In this study, β -glucan was orally administered and irradiated with whole body 2 Gy. It was then confirmed that the mortality of mice and tumor growth of mice with tumors were significantly reduced. Since the number of leukocytes and lymphocytes increased with a single dose of β -glucan, the crystal was encountered where the radioprotective effect of β -glucan was probably increased by the hematopoietic action of irradiated mice. In previous studies, β -(1–3)-D-glucan extract has a radioprotective effect and an antitumor effect, and regarding the mechanism of action, the immune activity and antioxidant were elucidated. In this study, we investigated the antitumor effects of β -glucan on radiation, protection of immune disorders, and antioxidant effects. After intraperitoneal inoculation of about 2×10^6 sarcoma 180, ICR mice were administered 200 mg/kg β -glucan every other day every two weeks. We irradiated 2 Gy radiation 3 times and counted the number of white blood cells and lymphocytes. In addition, body weight and tumor size were measured 2 weeks after cancer cells were seeded. Antioxidant activity was measured using the AAPH (2,2-azobis (2-amidinopropane) dihydrochloride) method. There was a clear decrease in tumor size in the radiation and glucan groups compared to the group receiving only cancer cells that increased tumor size over time. Almost all mice inoculated with only cancer cells died two weeks after radiation, but two-thirds of radiation and the glucan group were alive. Regardless of radiation exposure, the number of leukocytes and lymphocytes increased when β -glucan was administered. Antioxidant activity has been demonstrated in both groups of glucans. These results may indicate that administration of β -glucan increases immune activity, prevents side effects during cancer radiotherapy, and provides a supplemental tool for the treatment of cancer.

Keywords: lymphocyte, CD4 + , CD8 + , β -(1–3)-D-glucan, radioprotection, radiotherapy

1. Introduction

Recent studies have demonstrated the immunomodulatory effects of heat-killed lactic acid bacteria. The aim of this study was to evaluate the protective effect of

heat-killed *Enterococcus faecalis* EF-2001 (EF-2001) on a radiation protective and immunopotentiating Effect.

Drugs that suppress the radiosensitizer that enhances the biological action of ionizing radiation are collectively called radioprotector. Drugs that show protective effects by radical scavenging or hypoxic action, such as WR-2721, need to be administered before exposure [1]. Interleukins and the like are also effective with prior administration. Drugs that enhance *in vivo* production of hematopoietic cell growth factors, such as hematopoietic cell growth factors such as G-CSF and immunostimulants such as 432OK, are effective even after administration [2]. Chelating agents and iodine agents that prevent radionuclides from being absorbed from the skin and gastrointestinal tract and deposited in tissues and promote excretion are broadly considered protective agents [3].

Protective agents for radical scavengers have been studied as an adjunct to cancer radiotherapy, and WR-2721 (S-2-(3-Aminopropylamino) ethylphosphorothioic acid) is the most promising among many compounds. It is thought to show a protective action by eliminating radicals caused by indirect action of radiation, hydrogenation, reduction of oxygen effect, etc. A drug that can reduce radiation damage when administered immediately before radiation exposure is called a radioprotective drug. Typical examples are aminothiols derivatives such as cysteamine (mercaptoethylamine) and WR-2721. At the time of tumor radiotherapy, clinical application has been considered for protecting normal tissues around the tumor, but it has not been put into practical use. In addition, there is a possibility that these radiation prevention drugs may be used to protect rescue workers and decontamination workers in the event of a nuclear facility accident, but all currently known drugs are stamina, judgment, agility. Side effects such as decreased sex and vomiting are strong and have not been put to practical use [4].

Mn, Zn, and Cd ions, as activators of biological defense mechanisms, induce protective effects by inducing biosynthesis of metallothionein with radical scavenging ability in cells. In addition, gelsolin, interleukin 1 (IL-1), lipopolysaccharide (lipopolysaccharide, LPS), muramyl dipeptide (MDP) derivatives, ginseng extract, etc. Effective when administered prior to exposure. Leucos and innocuities are nucleic acid precursors that are approved as drugs for leukopenia and have radiation protection but are not strong [5].

G-CSF (granulocyte colony-stimulating factor), a hematopoietic cell growth factor, is a growth factor for leukocyte progenitor cells such as neutrophils. Prevents infection and bleeding and provides lifesaving effect. GM-CSF (granulocyte/macrophage colony-stimulating factor) and interleukin 6 (IL-6) have similar effects, but G-CSF is most expected in terms of fewer side effects. These proteins are natural substances collectively called cytokines, but can be produced as recombinants by genetic engineering techniques. In addition, picibanil (OK-432), glucan, lactic acid bacteria preparations, etc. are said to enhance *in vivo* production of hematopoietic cell growth factor and are effective when administered after exposure. Hematopoietic hormones, such as GM-CSF, are actually post-accidents in Brazil and San Salvador used for exposed people [6].

New Protective Agents Stable nitroxide radicals have SOD activity and protect animal cells from oxidative stress caused by superoxide and hydrogen peroxide. Tempol, one of the stable nitroxide radicals, has been shown to exhibit radioprotective effects on C3H mice both *in vitro* and *in vivo*. It has also been shown to prevent radiation-induced bone marrow damage and appears promising as a protective agent in cancer radiotherapy [7].

Chelating agents and iodine agents that prevent radionuclides from being absorbed from the skin and gastrointestinal tract, or deposited in tissues, or

promote excretion as agents for preventing absorption and deposition of radionuclides in the body and promoting elimination these are protective agents in a broad sense. Prussian blue is used to prevent absorption of ^{137}Cs . To promote ^{90}Sr excretion, calcium citrate and sodium alginate are recommended. In addition, sodium citrate and a low phosphorus diet are effective. Germalmine (aluminum hydroxide gel) has the same effect as a low phosphorus diet because it inhibits the absorption of phosphoric acid. Zn-DTPA or Ca-DTPA is used to promote plutonium excretion. An iodine agent (potassium iodide agent) is effective for preventing radioactive iodine from collecting in the thyroid gland [8–10].

β -glucan is an edible mushroom, belonging to the β -glucan, and named β -glucan. It particularly resembles *L.shimeji* Hongo in its taste and touch. It is a stump mushroom, which grows in forest, having a grayish brown-shaped umbrella about 4–9 cm in diameter [11]. Glucan (β -glucan *decastes* Sing) was called the β -glucan aggregate and has highly been valued for a long time, and its artificial cultivation was difficult till now. Recently, there has been artificial cultivation using bacterial strains as seedlings [12]. Now you can see it with general partial exposure. The authors studied glucan for antitumor, angiotensin converting enzyme inhibition, and serum cholesterol-lowering activity [13]. Glucan was found to have higher activity than the same class of shiitake *Enterococcus Faecalis* in anti-tumor activity, β -1,6 D-glucan and β -1,3D-glucan, and their active β -1, 6 D-glucan contains β -1,3D-glucan [14]. Here, we report the effect of glucan extract on radiation therapy in mice with cancer.

2. Materials and methods

2.1 β -Glucan

EF-2001 is a commercially available probiotic that was originally isolated from healthy human infant feces. Nihon BRM Co. Ltd. (Tokyo, Japan) supplied it as a heat-killed, dried powder. One gram of dried EF-2001 is equivalent to over 7.5×10^{12} colony-forming units prior to being heat-killed. *Nihon BRM Co. Ltd. (Tokyo, Japan) supplied Enterococcus Faecalis 2001®, a glucan product, composed of yeast extract, dextrin and gelatin.*

Dry *Enterococcus faecalis* contains 35–45 g of β -glycan per 100 g. *Enterococcus Faecalis* was orally administered using a zoned for 2 weeks before the start of the experiment.

2.2 Animal

The experimental animals used in this study are ICR / Slc mice. ICR/Slc mice (5 weeks old, male) were obtained from SLC, Japan. Once received, the mice were pre-bred for a week and only healthy mice were used for testing. Mice were fed with commercial feed (CA-1, CLEA Japan) and water ad libitum, with a 12-hour light cycle (8:00 lights, 20:00 lights off). The room humidity and temperature were 60–65% and $22 \pm 2^\circ \text{C}$, respectively.

2.3 Experimental groups

The experimental groups were divided into control group, tumor seeding group, tumor seeding +2 Gy exposure group, β -glucan treatment group, β -glucan + tumor seeding group, β -glucan + tumor seeding +2 Gy exposure by 6 groups.

2.4 Irradiation device

The X-ray generator used was Philips MG226/4.5. For the pipe voltage, a dose of 200 kV was applied at a rate of 0.35 Gy/min. The total amount of X-rays exposed was 2 Gy. The exposed place of the mouse was fixed to the position of the front part of the left foot (place where cancer cells were seeded) with a holder, and the place not irradiated was covered with a lead container. X-rays were irradiated on the 1 and 3 days.

The specific radiation irradiation method in each experimental group is shown below. For changes in blood cells, single irradiation with 2 Gy was performed. However, in the radiation tumor effect experiment, 2 Gy was divided into 3 divided doses.

2.5 Tumor inoculation

In order to obtain reproducible experimental data, mice were inoculated on the 15th day after breeding for more than 1 month. Approximately 2×10^6 sarcoma 180 cancer cells were inoculated into the muscle of the left foot of an ICR male mouse.

2.6 Administration method of β -glucan

Dissolve the extract in physiological saline, and for the control group, tumor-seeding group, β -glucan administration group, β -glucan + tumor seeding group, extract the water extract (200 mg/kg) every other day for several weeks. Administered. In the control group, tumor seeding +2 Gy-exposed group and β -glucan + tumor seeding +2 Gy-exposed group, only saline was injected. After 2 weeks of cancer cell seeding, the tumor size was measured weekly. We also measured body weight on exactly the same schedule as tumor size measurements.

Each group consisted of 10 mice. Tumor size was measured using.

Formula:

$$\text{Tumor size (cm}^3\text{)} = 3/4 \pi A^2 B / 2$$

A; Minor axis (cm);

B; Longer axis (cm)

At 14 (just before the first X-ray exposure), 1, 3, 5, 7, 9 and 11 days after tumor dissemination, each mouse is fixed with a holder and the caudal vein is covered with an extended female to get about 20 μ l of dripping blood. Blood was collected in a blood collection container (Dolamond Co. Ltd.) and diluted with diluent (Nihon Kohden Co. Ltd.). White blood cell counts, including lymphocyte counts and granulocyte counts, were measured using an automated blood cell analyzer (Nihon Kohden Co. Ltd., Celltac- α -MEK-6318). The results obtained were expressed as mean \pm standard deviation (S.D.). Student's t-test was applied and results above 0.05 were considered significant.

3. Results

3.1 Number of peripheral blood cells: leukocytes

Figure 1 shows the changes over time of leukocytes in the 4 groups.

Compared with the control group, the β -glucan group showed a significant increase in the number of white blood cells. The rate of significant increase throughout the treatment period was 5/8 (β -glucan group). These results suggest that β -glucan decisively increases white blood cell count.

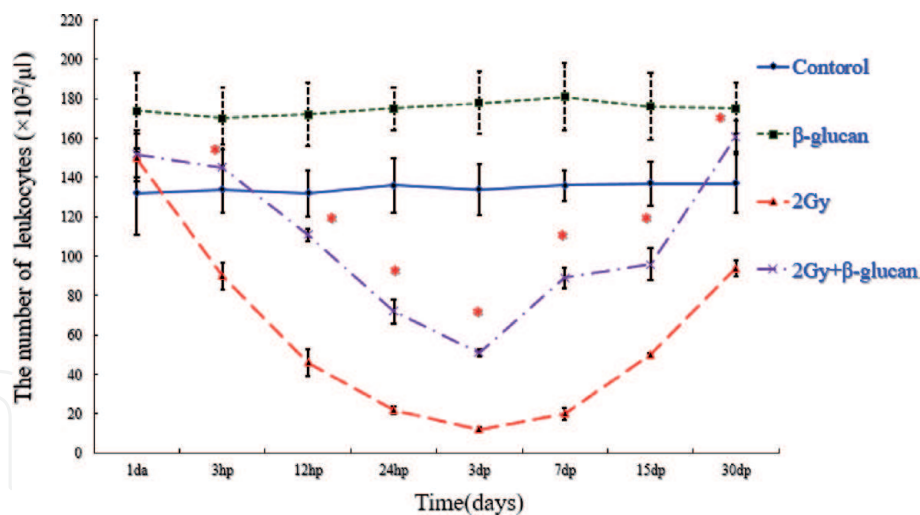


Figure 1.
β-glucan on blood leukocyte counts in mice. There were 10 animals in each experimental group. Data are mean ± standard deviation values. Statistically significantly different ($P < 0.05$) from the control group. Statistically significantly different (* $P < 0.05$) from the 2 Gy group. Administration of β-glucan was administered for 2 weeks and then treated with radiation. da; days after, hp.; hours post, dp; days post.*

In the irradiated group, white blood cell counts began to decrease in all experimental groups immediately after irradiation and tended to recover after 3 days. Compared to the 2 Gy group over time, the white blood cell count was always higher in the β-glucan + 2 Gy group. When observed in each period, the white blood cell count in the β-glucan + 2 Gy group was significantly higher than the 2 Gy single group the day before irradiation ($P < 0.05$). This suggests that an increase in white blood cell count is shown in the un-irradiated state. A significant ratio was observed in the β-glucan + 2 Gy group. These results suggest a decrease in 2 Gy-induced decrease in white blood cell count in the β-glucan + 2 Gy group.

3.2 Number of lymphocytes

Figure 2 shows the time course of lymphocyte counts by β-glucan administration in each non-irradiated and 2 Gy whole body irradiated groups. In the non-irradiated group, all treatment groups showed higher lymphocyte counts compared to the control group. Specifically, there was a significant ratio in the β-glucan

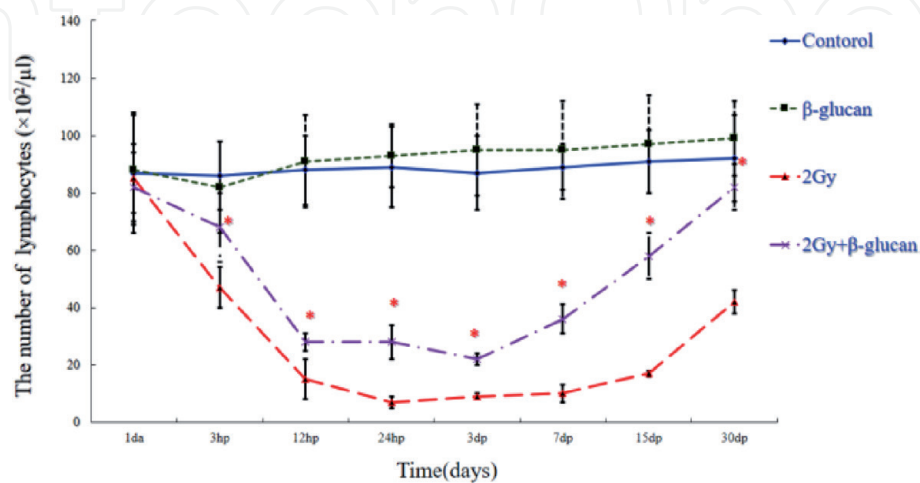


Figure 2.
β-Glucan on blood lymphocyte counts in mice. There were 10 animals in each experimental group. Data are mean ± standard deviation values. Statistically significantly different ($P < 0.05$) from the control group. Statistically significantly different (* $P < 0.05$) from the 2 Gy group. Administration of β-glucan was administered for 2 weeks and then treated with radiation. da; days after, hp.; hours post, dp; days post.*

group (4/8), and statistically significant differences were observed over many periods ($P < 0.05$). Overall, lymphocytes tended to increase compared to the control group.

This is the same trend seen in the non-irradiated group. Time-dependent changes after irradiation, all experimental groups showed a decrease, and subsequent recovery was observed 3 days after irradiation. The β -glucan +2 Gy group also showed higher lymphocyte counts than the X group. A significant ratio was observed in the β -glucan +2 Gy group ($P < 0.05$). These treatments have been shown to be particularly effective with a recovery period of 3 days after irradiation.

3.3 CD4 + and CD8 +

Figures 3 and 4 show the changes in CD4 + and CD8 + after β -glucan administration compared to the control group.

Figures 3 and 4 shows the increase frequency of CD4 + or CD8 + by β -glucan administration in each non-irradiated and 2 Gy whole body irradiation groups.

The β -glucan +2 Gy group showed a higher increase in CD4 + or CD8 + than the X group.

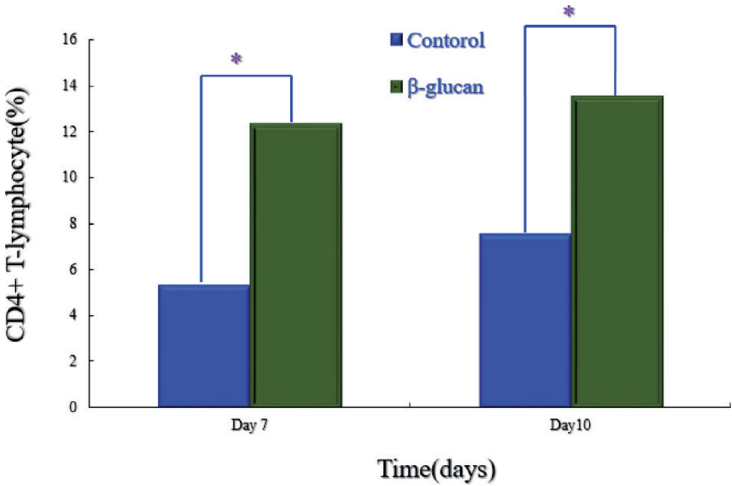


Figure 3. Lymphocytes were analyzed for CD4+ in C3H mic. The change of CD4+ cells after control and β -glucan administration group. The change of CD4+ cells control and β -glucan administration group. Statistically significantly different (* $P < 0.05$) from the control group.

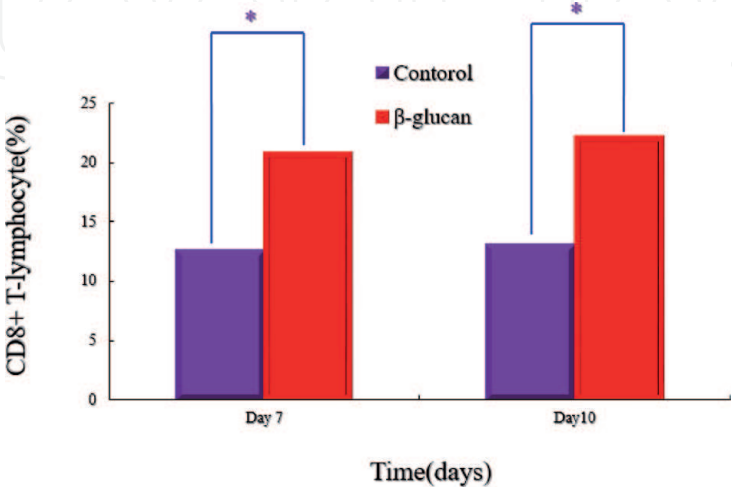


Figure 4. Lymphocytes were analyzed for CD8+ in C3H mic. The change of CD8+ cells after control and β -glucan administration group. The change of CD8+ cells control and β -glucan administration group. Statistically significantly different (* $P < 0.05$) from the control group.

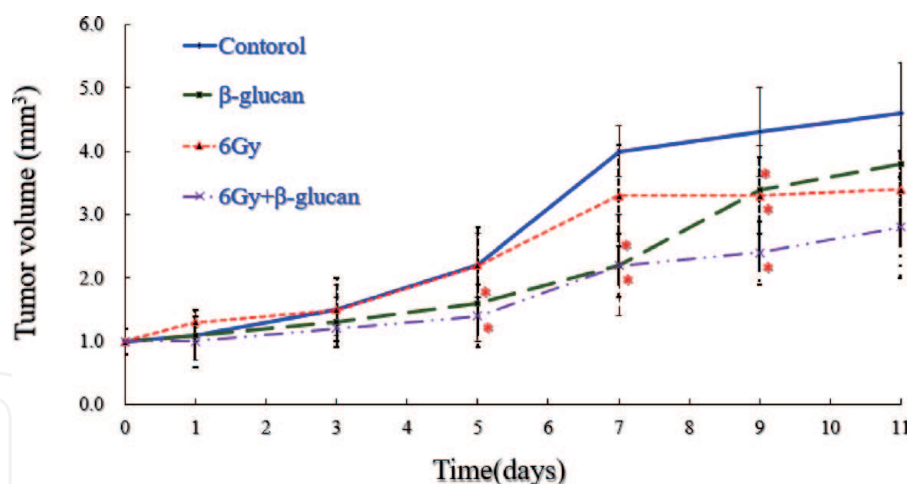


Figure 5.
Effect of β -glucan on the tumor growth in mice inoculated with 4 T1 (high grade) of mouse cancer cell line. Groups of ten mice each were subjected to each treatment. Results represent means \pm S.D. * Statistically significant ($P < 0.05$) from the control.

3.4 Effect of antioxidant activity (AOA_{AAPH})

Although an effect was observed in the β -glucan administration group, there was no statistically significant difference. The antioxidant activity against peroxy radicals after treatment was subtle.

3.5 Anti-tumor effects

Figure 5 shows the measurement of tumor growth rate by β -glucan administration. The doubling time of the non-irradiated group was not different from the doubling time of the control group with respect to the number of days required to double the tumor size and the ratio of the control group to the 6 Gy group. In the local irradiation group (treatment group), the doubling time of the β -glucan +6 Gy group was 1.4 times longer than that of the control group. The doubling time of the β -glucan +6 Gy group did not exceed the doubling time of the 6 Gy group and tended to be slightly shorter. Compared to the 6 Gy group, it was confirmed that tumor growth was suppressed.

4. Discussion

White blood cells consisting of lymphocytes, granulocytes, and monocytes are deeply involved in immunity. Lymphocytes are roughly classified into T cells and B cells. T cells are associated with cellular immunity, while B cells are associated with humoral immunity through the production of antibodies. T cells are further classified into helper T cells and suppressor T cells, helper T cells play a role in directing and activating B cells, NK cells, killer T cells, cytotoxic T cells, and granulocytes are mediated by blood Phagocytoses vascular walls, bacteria and foreign bodies. Monocytes are transformed into macrophages through morphological changes after moving to tissues, and transmit antigen information to T lymphocytes. In addition, macrophages activate NK and LAK cells. Thus, in white blood cells, lymphocytes distinguish between self and non-self, give instructions, and play a central role in the immune response [15–19]. In this study, in the non-irradiated group, the β -glucan group showed an increase in the number of lymphocytes, suggesting an increase in the overall white blood cell count.

β -Glucan is a mushroom that contains abundant β -(1-3)-D-glucan and β -(1-6)-D-glucan. It has been reported that the number of granulocytes increases in response to Petrchenko et al. B-(1-3)-D-glucan. Treatment with β -(1-6)-D-glucan has also been reported to cause macrophage activation, increased T cells, and increased TNF- α and IL-release 8 macrophage activation [16–18]. However, intra-peritoneal administration of these polysaccharides has also been reported to induce inflammation in the peritoneal cavity due to difficulty in intestinal absorption [20]. Furthermore, β -(1-6)-D-glucan has been reported to accumulate in the liver and spleen after peritoneal and oral administration [21].

This study and previous reports strongly suggest the involvement of β -(1-3)-D-glucan and β -(1-6)-D-glucan. Polysaccharide polysaccharides such as β -(1-3)-D-glucan are difficult to absorb these substances in the peritoneal cavity and intestine and may stimulate intestinal immunity with a slight inflammatory condition. Intestinal mucosal epithelial T lymphocytes are located between intestinal mucosal epithelial cells, and Peyer's patch and lymphoid tissue are located around the digestive tract. The intestine is considered the largest immune system because 70–80% of B lymphocytes are present in the intestinal lymphoid tissue [22–24]. However, with the exception of β -(1-3)-D-glucan, there are few reports that food is directly related to the immune system, regardless of how difficult it is to absorb.

Therefore, this is thought to be due to slight differences in physicochemical structures such as the side chain of β -(1-3)-D-glucan. In addition, β -(1-6)-D-glucan is degraded by beneficial intestinal bacteria and is relatively well absorbed, so it is necessary to consider the relationship with intestinal bacteria [25]. It is therefore speculated that macrophages are activated by the intestinal immune system. IL-8 and TNF- α are released from macrophages, activate helper T cells, and activate the systemic immune system consisting of macrophages, cytotoxic T cells, killer T cells, NK cells, and B cells.

However, there is no good basis for connecting these series of mechanisms and further experimentation is required. Cell damage is the most important side effect of radiation, and lymphocytes are the most sensitive cells [22]. Radiation causes interphase death in the short term [23]. Therefore, the control of radiation effects on lymphocytes essential to the immune system is very important, and the effects on lymphocytes can be viewed as an indicator of radiation-induced cell damage. When the body is exposed to radiation, free radicals such as H \bullet , OH \bullet , and O $_2^{\bullet -}$ (superoxide anion) are generated by the decomposition of water molecules by radiation. DNA free radical damage is called an indirect effect. Administration of the redox agent reduced the cytotoxicity induced by O $_2^{\bullet -}$ produced by ionizing radiation [25].

It has been suggested that the administration of α -glucan, which has a radio-protective effect, does not inhibit the suppression of tumor growth by radiation, but inhibits tumor growth independently [26]. This may be due to low blood flow in hypoxic cells such as tumor cells and low radical scavenger factors in plasma. Therefore, the antitumor components and tumor suppressors of these substances act by activating immune cells [27–30].

In this study, we examined tumor growth of sarcoma 180 alone, but in the future, we will need to explore different types of tumors and adopt more accurate experimental systems.

Figure 6 shows mechanisms of cell repair in radiation protection.

Figure 7 shows radiation protection and radical scavenger processes.

Figure 8 shows the free radical removal mechanism of β -glucan by radiation.

Enterococcus faecalis is a gram-positive bacterium that belongs to the LAB family. Its cell walls are reported to induce B-cell activation along with stimulation of IgA

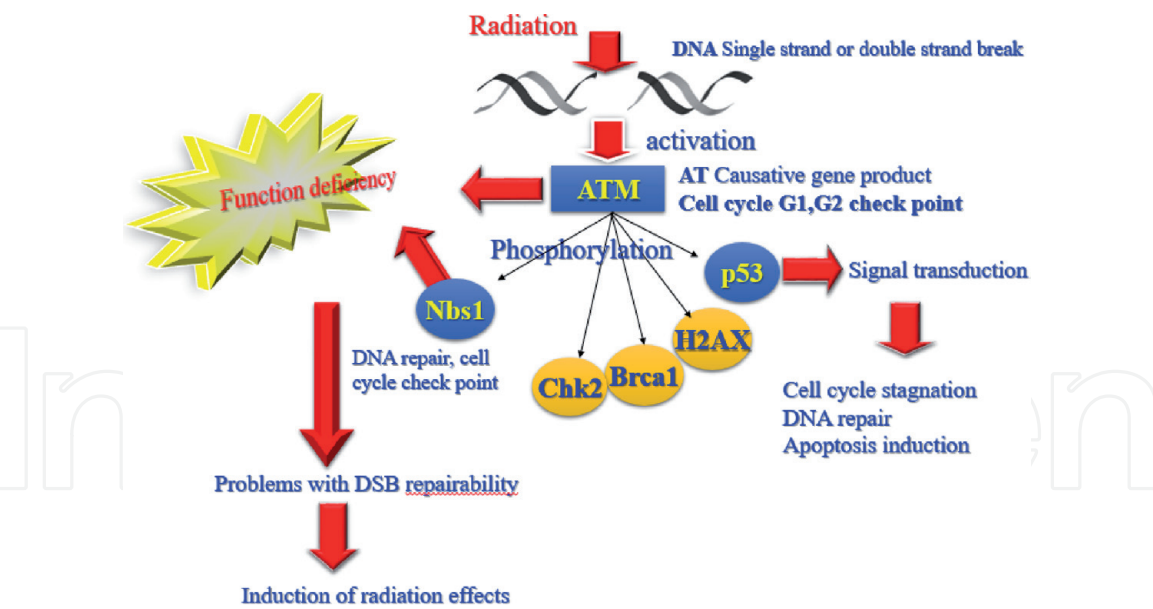


Figure 6.
Mechanisms of cell repair in radiation protection.

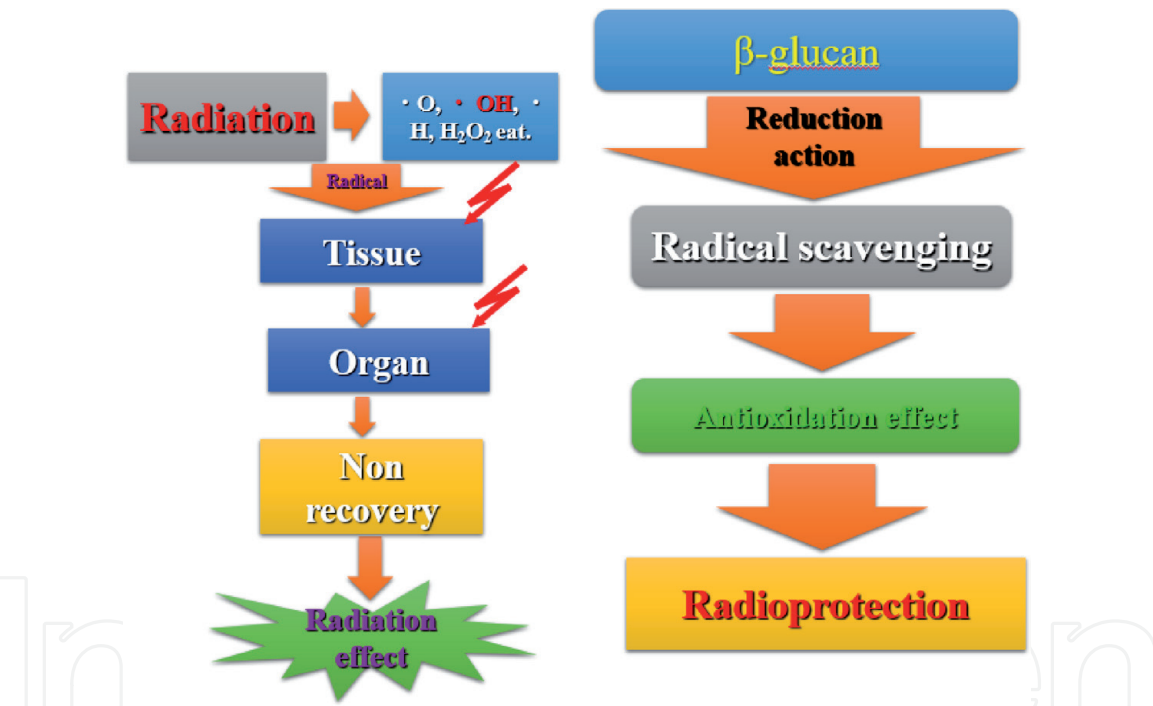


Figure 7.
Radiation protection and radical scavenger processes.

secretion in the intestine [31], which could remove pathogens from the intestine [32]. To date, several functions of EC-12 have been reported [33, 34]. However, the preventive effects of heat-killed EC-12 on intestinal carcinogenesis have not yet been elucidated. In this study, we demonstrated that administration of heat-killed EC-12 weakly decreased intestinal tumorigenesis in Min mice, Apc-mutant mice that develop many intestinal polyps through activation of β -catenin signaling. Moreover, we revealed that heat-killed EC-12 possesses suppressive function of β -catenin signaling in vitro by measuring T-cell factor/lymphoid enhancer factor (TCF/LEF) transcriptional activity.

High IFN- γ production as part of the Th1 immune response has been associated with colitis in mice [33]. Furthermore, Ito et al. reported that IFN- γ plays a

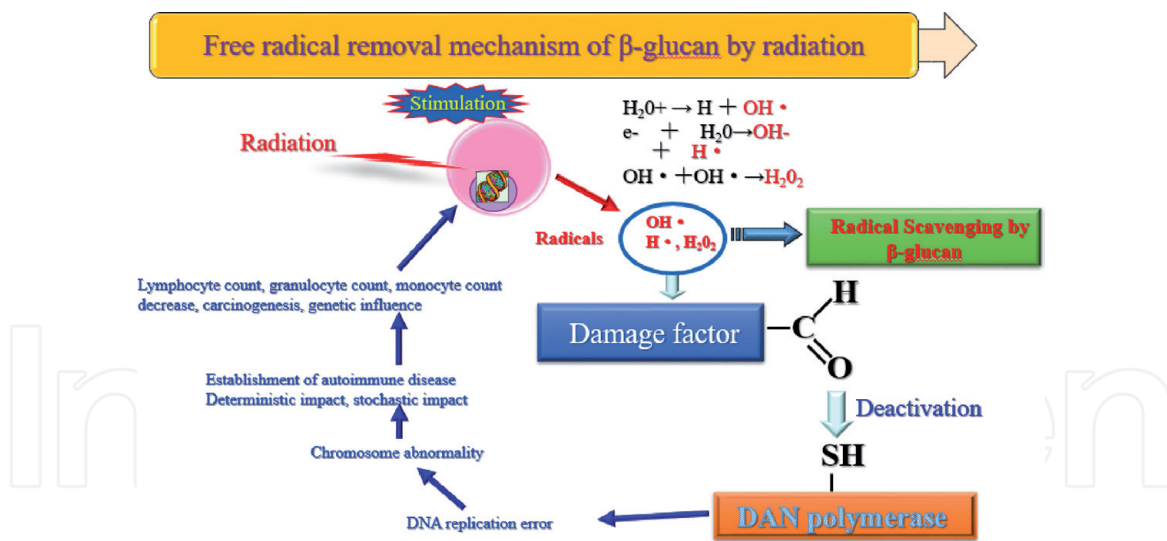


Figure 8.
The free radical removal mechanism of β -glucan by radiation.

fundamental role in the development of colitis in mice [34]. In addition, IFN- γ activates downstream effector cells to produce inflammatory cytokines such as IL-1 β . Therefore, suppression of IFN- γ and IL-1 β induction may explain the anti-inflammatory properties observed with EF-2001. In addition, Th1 or humoral responses are important for resistance to extracellular pathogens and these cells produce certain IL family cytokines, including IL-1 β , IL-6, and IL-10 [35–37].

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