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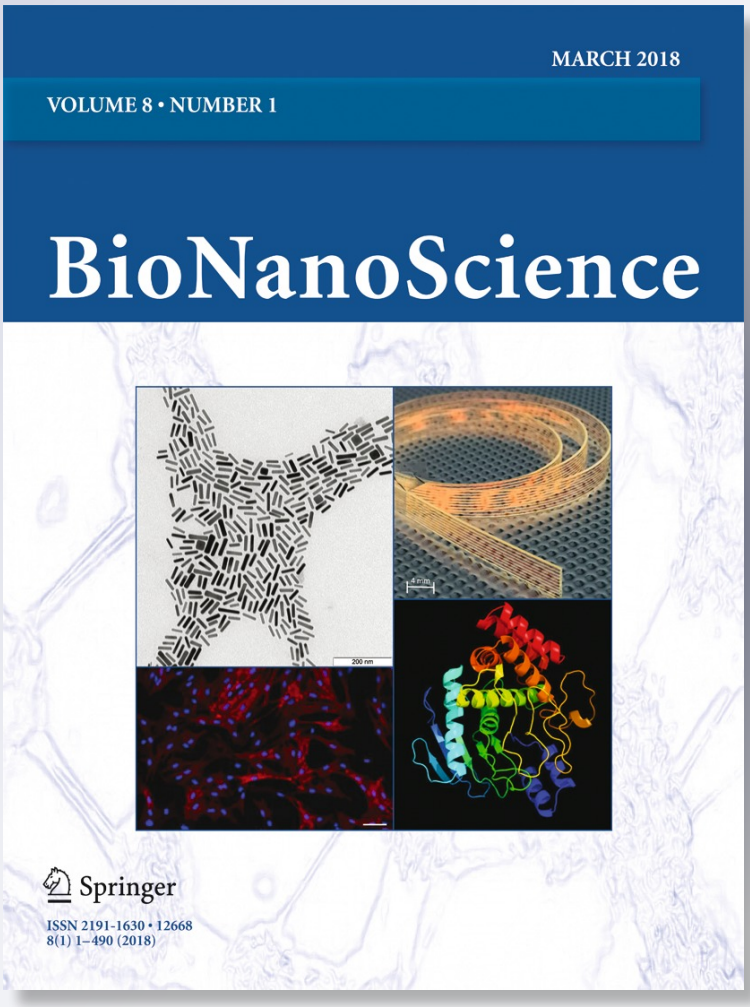

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Gamma-Irradiated Bifidobacteria Establish a Protective Effect on Mice to Experimental Radiation Exposure

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Abstract The purpose of this work is to assess the radioprotective effect exerted by the irradiated form of *Bifidobacterium bifidum* probiotic on experimentally irradiated mice. As a result of the research, we were able to determine experimentally the optimal dose of gamma rays (12–14 Gy) that ensures a switching of the metabolism of probiotic microorganisms towards the synthesis of superoxide dismutase (an antiradical enzyme) and activates production of interferon (a mediator of immunopoiesis) by immunocompetent cells. The mice were exposed to 8.0 Gy doses of ^{137}Cs gamma radiation at an exposure dose rate of 3.13×10^{-5} C/(kg s). Twenty four hours after the exposure, the animals were administered subcutaneously a single 0.2-ml dose (1×10^8 CFU) of either native bifidumbacterin or the radiation-modified form of this probiotic. The radioprotective effect was evaluated according to various parameters, such as the change in hematological parameters, the quantitative composition of the gut microbiome, and the ability of the drug to induce the release intercellular interaction mediators (interferons) by stimulated immune cells of the host. A single subcutaneous injection of 1×10^8 CFU of either native bifidumbacterin or its irradiated form, administered in the composition of the growth medium 24 h after the irradiation, protects 60 to 80% of lethally irradiated white mice. The radioprotective effect of the biopreparation is associated with a milder form of acute radiation syndrome, makes pancytopenia less severe (1.13–1.21 times against 2.7–4.9 times in the irradiated control group), and reduces the number of opportunistic enterobacteria (2.2 lg against 4.9 lg in the irradiated animals) in the intestine.

Keywords *Bifidobacterium bifidum* · Gamma radiation · Acute radiation syndrome · Interferon · Superoxide dismutase

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1 Introduction

The development of radiation sickness as a result of physiological changes is naturally accompanied by quantitative and qualitative alterations in the intestinal microflora. An analysis of the data reported in specialized literature give grounds to believe that the changes in the intestinal microflora in case of acute radiation syndrome may be attributed to the most severe form of dysbiosis, leading to a septic form of radiation-induced endogenous infection with lethal outcome [1]. Preparations containing microorganisms that are part of the normal intestinal microflora, such as Normoflora (Austria), Coniflora (Germany), Tetralactin (France), Lactobacterin, Colibacterin, Bifidumbacterin, Bifikol (Russia), are widely used for the prevention and treatment of various diseases associated with dysbiosis [2].

The efficacy of radiation methods to improve the action of preparations for various purposes has been proven earlier [3–5]. The sterilization of native solid and liquid nutrient media by gamma-ray doses ranging from 0.5 to 2.0×10^4 Gy was shown to be effective in [6]. Double exposure of cell cultures to low (0.05 Gy) and high (5.95 Gy) doses of radiation improves the adaptive response to ionizing radiation, simultaneously increasing radioresistance [7]. It has been proven that the strategy of constructing composite radioprotective preparations that exert a therapeutic and decorporation action, with the inclusion of immunotropic and biogenic stimulators and natural sorbents in their composition, is theoretically feasible [8].

It seems interesting that protecting an organism from the effects of radiation by exposing it to low doses of radiation goes together with an increase in the production of radioprotective (antioxidant) catalase enzyme, which leads to an increase in the radioresistance of the organism [9]. It is therefore quite likely that some membrane-bound enzyme complex released or activated by the radiation may play a certain role in the repair process of radiation-induced injures in the irradiated organism, stimulating at the same time an increase in the resistance of the organism when irradiated bacterial agents are used [10]. Substances of microbial origin have the property of stimulating the synthesis of cytokines, which have a pronounced therapeutic effect in cases of acute radiation injury [11, 12]. It has been demonstrated that the expression of the radioprotective Rad51 protein in the lungs, liver, and kidneys was significantly higher in mice that were exposed to radiation and were administered the *Deinococcus radiodurans pprI* plasmid gene [13]. The radioprotective efficacy of this protein was previously demonstrated in experiments with both human and mouse cell cultures [14]. A radioprotective effect is exhibited not only by proteins, but also by polysaccharides of some microorganisms [15], in particular by lipocalins produced by some plants and microorganisms [16]. Irradiated vaccines present more obvious immunogenic properties [17]. Consequently, the action of ionizing radiation on microorganisms may switch the metabolism of cells towards the synthesis of substances that can activate the production of antiradical enzymes and cytokines in the organism. Thus, it is possible to enhance the radioprotective effects exerted by bacterial preparations.

In connection with all the above stated, the purpose of this work is to investigate the radioprotective effect exerted by the irradiated form of *Bifidobacterium bifidum* probiotic on the organism of experimentally irradiated mice.

2 Materials and Methods

The study focused on the irradiated form of the probiotic "Bifidumbacterin" (JSC AO Partner, LLC AO Ecopolis, Russia), which is a powder containing 5×10^8 CFU/ml of viable bifidobacteria (*Bifidobacterium bifidum* strain 1). Before obtaining the irradiated form of the probiotic, the preparation was dissolved in water at a concentration of 1 ml per 1 dose. The optimal dose of irradiation was determined after irradiating the obtained suspension of bifidobacteria with ^{60}Co gamma rays in an Issledovatel IN-1 irradiator (Russia) with doses ranging from 1 to 20 Gy. Subsequently, to evaluate the effect of radiation on bifidobacteria, a special Blaurock medium was inoculated with the irradiated suspensions, then the cultures were incubated at 37°C for 48 h. Afterwards, we determined the concentration of superoxide dismutase (SOD) that had formed in the culture fluid. For this to be done, we dissolved 0.02 ml of the culture fluid in 3 ml of an incubation medium containing 0.41 mM nitrogen tetrazolium (NBT), 0.33 mM EDTA, and 0.01 mM N-methylphenazinium methosulfate. After that, we measured the optical density of the solution in a spectrophotometer at 540-nm wavelength, then added 0.1 ml of 0.6 mM NADH to the cuvette, stirred and left in the dark for 10 min. The reaction was evaluated by the difference between the first and second measurements in the spectrophotometer. As unit of activity, we took the amount of enzyme causing 50%-inhibition of NBT reduction. The activity of the enzyme was expressed in conventional units per 1 ml of culture fluid.

The absence of bacterial and fungal microflora in the irradiated bifidumbacterin was checked by inoculation of the preparation into nutrient media, namely beef-extract agar (BEAM) and beef-extract broth (BEBM), according to GOST 28085-89, and subsequent incubation [18]. The test for acute toxicity was carried out in laboratory white mice conform to GOST 12.1.007-76 [19]. The safety of the preparation was determined by administration of a single intraperitoneal infusion of the maximum permissible dose of the probiotic to laboratory animals (white mice) according to guidelines [20].

The radioprotective effects of both the native and the radiation-modified probiotic Bifidumbacterin were tested in white male mice weighing 18 to 20 g. Each animal was

administered 0.2 ml of the preparation subcutaneously. The dose was equal to 1×10^8 CFU.

The animals were irradiated in a gamma-ray irradiator (^{137}Cs) with 8.0 Gy doses (absolutely lethal doses for these animal species) [21] at an exposure dose rate of 3.13×10^{-5} C/(kg s). The radioprotective effect was evaluated according to various parameters, such as the change in hematological parameters, the quantitative composition of the gut microbiome, and the ability of the drug to induce the release of intercellular interaction mediators (interferons). Hematologic parameters were studied by staining blood smears with May–Grünwald and Romanovsky–Giemsa stains, and the subsequent registration of the leukogram and quantitative analysis of leukocytes and erythrocytes in a Goryaev chamber (hemocytometer) [21]. To study the composition of the intestinal microbiome, the feces were collected 3–5, 8–10, 15–17, and 20–25 days after administering the preparation [22]. The feces were diluted in 0.9% NaCl at a ratio of 1 : 10 000, and then, the solution was inoculated in Endo medium (in portions of 0.1 ml). The culture was incubated at 37°C for 24 h; finally, we counted the total number of formed colonies (CFU). The interferon activity was determined by titration [23]. This method relies on the fact that the treatment of incubated cells (T lymphocytes) in vitro with interferon increases their sensitivity to the toxic effect of the polyribonucleotide complex.

The efficacy of the preparation was determined according to the percentage of surviving animals on the 30th day after the irradiation.

The results were processed by biometric statistical methods using the Biostat software package.

3 Results

3.1 Radiation-Induced Changes in the Bifidobacterial Preparation.

At the first stage of the study, we determined the optimal conditions for the exposure of bifidobacteria to radiation with the aim of changing their metabolism by switching the enzymatic and cytokine activator pathways as a consequence of the radiation stress. In so doing, we applied various doses of gamma rays in the range from 1 to 20 Gy (1, 2, 4, 6, 8, 10, 14, 16, 18, 20 Gy) to stimulate the metabolism of microorganisms.

The choice of the optimal doses of gamma rays was based on data reported in specialized literature, according to which low doses of gamma rays (from 1 to 20 Gy) exert a stimulating effect on the metabolism of microorganisms, affecting their growth, development, and metabolic functions [24]. The radiation-induced changing effect of gamma rays on the microorganisms was assessed accordingly to the change in the production of the key antiradical enzyme SOD provoked by irradiation. From data contained in Table 1, it can be seen that the optimal doses of gamma rays at which the antiradical effect increases are those in the range 12 to 14 Gy. The inhibition of the synthesis of SOD enzyme may be achieved by increasing or decreasing the dose of gamma rays.

The radiation-modified bifidumbacterin, obtained by irradiation of probiotics, was tested in solid (BEAM) and liquid (BEBM) nutrient media prior to tests in animals. The results of dynamic microbiological studies showed that no extraneous microflora appeared within 7 days.

3.2 Assessment of the Toxicity of the Irradiated Preparation

We experimentally reproduced acute toxicity conditions in white mice by administering subcutaneous injections of 0.1 to 1.0 ml of irradiated bifidumbacterin, thereby proving the absence of toxicity. Also, we administered to each animal the maximum permissible dose of the probiotic (1.0 ml) once during the whole observation period (10 days) and detected neither changes in the general condition, nor behavioral reactions, nor appetite disorders. This indicates that the preparation is safe.

3.3 Assessment of the Radioprotective Effect

The preparation obtained by exposure of bifidobacteria to radiation according to the above-described technology was tested for radioprotective activity in white mice irradiated with an absolutely lethal dose (8.0 Gy, $LD_{100/30}$).

The experiments were performed in 40 mice divided into four groups of 10 animals each. The animals of the first group were injected subcutaneously 0.2 ml bifidumbacterin (1×10^8 CFU) 24 h after having been exposed to radiation. The animals of the second group were administered irradiated bifidumbacterin under similar conditions. The animals of the third group were

Table 1 Gamma-ray doses provoking the production of the antioxidant protective enzyme SOD upon irradiation of bifidobacteria

Gamma-ray dose, Gy	SOD activity, units/ml
1	0.11 ± 0.05
2	0.13 ± 0.07
4	0.15 ± 0.09
6	0.17 ± 0.05
8	0.25 ± 0.03
10	0.33 ± 0.01
12	0.37 ± 0.03
14	0.41 ± 0.01
16	0.31 ± 0.05
18	0.29 ± 0.03
20	0.15 ± 0.05
Control (non-irradiated culture)	0.07 ± 0.01

administered a subcutaneous injection of 0.2 ml PBS (control group). The animals were irradiated in a gamma-ray irradiator (^{137}Cs) with doses of 8.0 Gy at an exposure dose rate of 3.13×10^{-5} C (kg s)$^{-1}$. The animals of the fourth group (irradiated control group) did not receive any treatment.

The results of hematological studies showed that pancitopenia was a typical disorder of the blood system in irradiated animals. This disorder was expressed in a decrease in the level of circulating leukocytes (5.3 times), lymphocytes (4.4 times), neutrophils (5.6 times), and platelets (3.2 times). The effect became apparent on the first day, gradually reached its maximum values by the third day, and kept at this level until the end of the experiments. During the research, these indices changed less rapidly in animals that were administered probiotics (either native or irradiated bifidumbacterin) than in those that were not (namely, leukocytes—2.1 and 1.6 times, lymphocytes —1.8 and 1.1, neutrophils—2.7 and 1.9, platelets—1.2 and 0.8, respectively). At the same time, it was noted that the prevention of radiation-induced pancytopenia in animals irradiated and receiving radiation-modified bifidumbacterin was more marked than in mice receiving native bifidumbacterin.

While studying the microbial colonization of the intestine, we observed a sudden increase in the number of colony-forming units in the feces of non-probiotic mice 5 days after having been irradiated (irradiated control group). During the main period of acute radiation syndrome in mice irradiated with an 8.0 Gy dose (a dose causing a severe degree of acute radiation syndrome), we detected in the intestinal discharge an increase in microbes by 4.9 lg; in contrast, in irradiated probiotic mice (receiving either native or irradiated bifidumbacterin), the amount of opportunistic microflora was two times less than in the previous group (2.2 lg). Consequently, the administration of probiotics to tested animals with a severe degree of damage led to the restriction of microbial multiplication in their organisms which was confirmed in the nature of the disease course and the development of endogenous infection. Most probably this is due to the development and activation of the normal microflora of the intestinal microbiome. Also, the interferon synthesis in irradiated probiotic mice (Table 2) was characterized by a positive dynamics, the most noticeable being in the group of animals that were administered the radiation-modified preparation.

All 10 animals in the irradiated control group died during the experiment. In the group of animals that were administered bifidumbacterin, six mice survived, while in the group treated with irradiated bifidumbacterin, eight mice survived. The more favorable course of the acute radiation syndrome associated with the use of probiotics raised the survival rate of lethally irradiated animals, amounting to 60% for native bifidumbacterin and 80% for irradiated bifidumbacterin.

4 Discussion

The development of dysbiosis in irradiated organisms creates a demand for drugs for its prevention and treatment. At present, there are some works indicating the potentiality of using bacterial preparations (probiotics) for the treatment of acute radiation syndrome [25]. Bifidumbacterin has already been used to reduce the severity of post-irradiation dysbiosis and treat radiation sickness [2]. In experiments with CBA mice and CBAxC57B1 hybrid mice exposed to an absolutely lethal dose of 8.0 Gy of ^{60}Co gamma rays (a dose causing 100% of mice to die within 30 days of the exposure, $LD_{100/30}$ mice), bifidobacterium strains 765, ABD, and 7541 were used as a therapeutic agent. Doses of 5×10^8 microbial cells in 0.25 ml of the preparation were administered orally on the 1st, 3rd, 5th, and 10th days after the exposure. It was established that oral administration of bifidobacteria increased the life span of animals from 5.0 ± 0.5 to 7.0 ± 0.6 days and raised the survival rate up to $40.7 \pm 3.7\%$.

"Bifikol" (JSC AO I.I.Mechnikov Biomed, Russia), a probiotic obtained by the joint cultivation of aerobic microorganisms (*Escherichia coli* strain M-17) and anaerobic bifidobacteria (*B. bifidum* strain 1), each dose containing 10^8 CFU/ml of viable bifidobacteria and 10^7 CFU of *E. coli*, has been used for the treatment of sheep experimentally irradiated with 4.0 Gy, a dose causing 60–80% of these animals to die (LD_{60-80}) [21]. Bifikol was injected subcutaneously to the animals 24–26 h after the exposure to radiation. It was found that the use of Bifikol exerts a radioprotective effect, reflected

Table 2 Dynamics of interferon synthesis by blood cells in irradiated animals treated with irradiated bifidumbacterin

Groups of animals	Interferon concentration (lg/ml) at day after irradiation			
	7	14	21	28
Irradiated with 8.0 Gy + 1 subcutaneous injection of irradiated bifidumbacterin	17.3 ± 5.2	16.7 ± 5.5	16.9 ± 4.7	19.8 ± 5.3
Irradiated with 8.0 Gy + 1 subcutaneous injection of native bifidumbacterin	12.3 ± 1.1	10.1 ± 1.3	6.3 ± 1.9	6.5 ± 2.1
Control group (non-irradiated animals)	20.8 ± 5.6	21.0 ± 5.9	20.6 ± 6.2	21.1 ± 4.7
Irradiated control group: irradiated with 8.0 Gy and no probiotics	7.2 ± 2.4	5.8 ± 1.9	4.5 ± 1.1	3.3 ± 0.6

by more moderate clinical manifestations, a smaller decrease in the number of leukocytes, a faster recovery, and an increase in the survival rate of sheep by 50% compared with the control group. The author explains the protective effect of the drug by its immune corrective action, namely the restoration of T and B lymphocytes, T helper cells and, as a consequence, normal immunoglobulins of the M and G classes.

As can be seen from a brief analysis of materials regarding the issue we have considered here, the tested probiotics (Bifidumbacterin and Bifikol) have a certain radioprotective effect but it was relatively low, since the survival rate of animals irradiated with absolutely non-lethal doses ($LD_{50-60/30}$) did not exceed 50–60%.

This created a need to improve the technology of producing radioprotective preparations based on probiotics. In our research, we proceeded from the premise that, depending on the dose, radiation may have either stimulating, mutagenic, or lethal effect on microorganisms. The object of this study was a radiation-modified form of Bifidumbacterin, a probiotic containing 5×10^8 CFU/ml of viable bifidobacteria (*B. bifidum* strain 1). A suspension of bifidobacteria was irradiated with gamma-ray doses of 1 to 20 Gy. The optimal dose of gamma rays was determined to be 12–14 Gy, at which an increase in antiradical effect is observed. The radiation-modified preparation thus obtained was tested in mice to assess its radioprotective properties. These studies proved the efficacy of probiotics, both native and irradiated, which was reflected by a less pronounced pancytopenia, a decrease in the number of opportunistic enterobacteria, and an increase in the survival rate of lethally irradiated animals compared with the control group.

5 Conclusion

As a result of the study, we could experimentally determine the optimal dose of gamma rays (12–14 Gy) that ensures a switching of the metabolism of probiotic microorganisms towards the synthesis of superoxide dismutase (an antiradical enzyme) and activates production of interferon (a mediator of immunopoiesis) by immunocompetent cells.

According to the results of the study, a single subcutaneous injection of 1×10^8 CFU of either native bifidumbacterin or its irradiated form, administered in the composition of the growth medium 24 h after the irradiation, protects 60 to 80% of lethally irradiated white mice. The radioprotective effect of the biopreparation is associated with a milder form of acute radiation syndrome, makes pancytopenia less severe (1.13–1.21 times against 2.7–4.9 times in the irradiated control group), and reduces the number of opportunistic enterobacteria (2.2 lg against 4.9 lg in the irradiated animals) in the intestine.

Compliance with Ethical Standards The experiments in animal models were approved by the Ethical Committee of the Federal Center for Toxicological, Radiation and Biological Safety (Kazan, Russia).

Conflict of Interest The authors declare that they have no conflict of interest.

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