The Efficacy of Probiotic (*Lactobacillus rhamnosus* GG) and 5-ASA (Aminosalicylic Acid) in the Treatment of Experimental Radiation Proctitis in Rats

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Abstract The aims of the study are to demonstrate the effect of probiotic use on the healing of radiation proctitis (RP) and evaluate the efficiency of fecal biomarkers at follow-up of the treatment. Thirty-two male/female rats were randomly separated into four groups of eight rats. The first group (control) was not radiated. RP was created by 17.5 Gy single dose rectal irradiation. The second group (RP) was subjected to RP, but not treated. The third group (RP+ASA) was treated with 5-aminosalicylic acid (5-ASA) 250 mg/kg daily by gastric lavage for 14 days after the irradiation, and the forth group (RP+LGG) was treated with *Lactobacillus* GG (LGG) 25 × 100 million CFU daily. Feces

samples were taken at the 7th and 14th day of the treatment for fecal biomarkers. Rectums of the rats were resected at the 14th day by laparotomy. Samples were evaluated both macroscopically and microscopically. RP was achieved both macroscopically and microscopically. Weight loss of RP group is statistically significant ($p < 0.005$) than other groups. The healing ratio of RP+ASA and RP+LGG groups was significantly better than the RP group ($p < 0.005$) both macroscopically and microscopically. But there was no significant difference between ASA and LGG groups. Biochemically, fecal calprotectin was found to be more effective than fecal myeloperoxidase and fecal lactoferrin to show the efficacy of treatment of radiation proctitis. The results of our study demonstrate that probiotic is as effective as 5-aminosalicylic in the treatment of radiation proctitis, and fecal calprotectin is a useful biomarker in determining the response to the treatment.

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Introduction

The rectum is exposed during irradiation of pelvic tumors and tissue damage occurs as a result of tissue response to ionizing radiation [1]. Acute radiation proctitis (RP) occurs during or within 3 months of radiotherapy and chronic radiation proctitis either continues from the acute phase or begins after a latent period of at least 90 days [2]. About 75 % of subjects receiving pelvic radiotherapy experience rectal symptoms during treatment and almost 20 % continue with chronic proctitis [1, 3]. The incidence of both early and delayed intestinal radiation toxicity depends on radiation dose, frequency, and treatment time [2].

Although some histological features of acute colorectal radiation injury have been described, little is known about the development of individual changes during treatment and the association between histopathology and endoscopic and clinical signs of toxicity.

There are several therapy modalities used for the protection and treatment of acute radiation proctitis and consequent chronic sequelae, including diet, sucralfate, anti-inflammatory agents like mesalazine, corticosteroids, formalin, and misoprostol [4, 5]. 5-Aminosalicylic acid (5-ASA, mesalamine), one of the basic drugs in radiation proctitis, is a metabolite produced by intestinal bacteria from sulfasalazine [6].

Probiotics can be defined as live microbial food supplements which beneficially affect the health of the host by maintaining a balanced intestinal flora and stimulating its immune system [7–11]. Probiotics shows anti-inflammatory effect by several mechanisms [10] and are potential radioprotective agents with improved crypt survival and reduced epithelial apoptosis. *Lactobacillus* GG (LGG) or its conditioned medium reduce radiation-induced epithelial injury and improve crypt survival [12].

Fecal biomarkers are rapid and inexpensive noninvasive tests, which used diagnosis and follow-up of treatment of inflammatory bowel disease (IBD) and radiation proctitis [13]. Many studies have shown that neutrophil-derived proteins, myeloperoxidase, calprotectin, and lactoferrin are generally elevated in the feces of inflammatory bowel disease patients and also increased in the presence of malignancy. Their increased levels show a positive relationship with the severity of inflammation [14–17].

The aims of this study are to demonstrate the effect of probiotic use on the healing of RP and evaluate the efficiency of fecal biomarkers at follow-up of the treatment of RP.

Methods

Experimental protocol was designed according to the principles of National Institutes of Health Guide for The Care and Use of Laboratory Animals mentioned by the Declaration of Helsinki. The study was approved by the local Animal Ethical Committee of the Marmara University Medical School.

Animals Thirty-two Wistar–Albino conventional rats, including equal number of male and female, obtained from the University of Marmara Medical School Animal Research Laboratory, Istanbul, Turkey, weighing 180–220 g, were used. Rats were kept caged at 20 ± 2 °C constant temperature and a relative humidity of 55 ± 10 %, with a constant 12-h light/dark cycles, and fed with Standard Purina Rat Chow and tap water.

Experimental Protocol The male/female rats were allocated randomly into four groups of eight rats. The first group (control) was not irradiated. The other three groups subjected to RP. The second group (RP) was irradiated, but not treated. The third group (RP+ASA) was treated with 5-ASA (Pentasa® sachet 1 g prolonged release granules, Ferring BV, UK), 250 mg/kg daily by gastric lavage for 14 days after the irradiation. The fourth group (RP+LGG) was treated with *Lactobacillus rhamnosus* GG (Culturelle®, CAG Functional Foods, US), 25×100 million CFU daily by gastric lavage for 14 days after irradiation. After irradiation, subjects were weighed and observed daily for signs of proctitis such as diarrhea and rectal bleeding until the day of sacrifice. Feces samples were taken at the 7th and 14th day of the treatment for fecal biomarkers, such as calprotectin, lactoferrin, and myeloperoxidase. ELISA method was used for evaluation. On day 14 after irradiation, the rectum was removed from each animal and evaluated both macroscopically and microscopically.

Irradiation Rats were irradiated using a modification of a method previously developed by Hubmann [18]. Each rat was anesthetized using 5 mg/kg of xylazine (Rompun®, Bayer Türk Kimya Sanayi, İstanbul, Turkey) and 50 mg/kg of ketamine hydrochloride (Ketalar®, Pfizer İlaçları, İstanbul, Turkey) intraperitoneally prior to irradiation. Then, each rat was bridled and taped by the tail and legs on an acrylic plate at supine position. Except for a 3×4 -cm area of pelvis containing approximately 2 cm length of rectum in the middle of the field, to cover the rat, a lead shield was used. Irradiation was delivered on a cobalt-60 unit (Cirrus) with distance of 80 cm from the source to surface using a single exposure of 17.5 Gy (121.5 Gy/min) defined at a depth of 1 cm through an anterior portal.

Weight Change Evaluation After irradiation, subjects were weighed daily until the day of sacrifice. Weight differences between groups were evaluated.

Macroscopic Evaluation All animals were sacrificed under anesthesia by injecting thiopental sodium (Pentothal®, IE Ulagay, Istanbul, Turkey) 200 mg/kg, intraperitoneally, and the rectum approximately 1 cm proximal to the anus was removed. The removed rectums were grossly evaluated and scored as (0) normal mucosa; (1) edema, moderate hyperemia, and loss of visible vascularity; (2) diffuse hyperemia, multiple macular bleeding areas, or jointed bleeding areas; (3) erosions or visible hemorrhages; and (4) visible ulcers.

Histological Evaluation The rectums obtained at autopsy were immediately fixed in 10 % neutral buffered formalin solution for 24 h. After fixing, examples of 0.3 cm were cut from the fields created proctitis and the samples were placed

into two tapes for each subject. The samples were also embedded in paraffin and processed by routine techniques. Each specimen was stained with hematoxylin and eosin and examined by light microscopy (Nikon® E600-Japan) by a pathologist blinded to the study. A slightly modified scoring system reported by Hovdenak et al. [19] was used for microscopic evaluation. The abnormalities in the used parameters were assessed as (0) normal, (1) mildly abnormal, (2) moderately abnormal, (3) markedly abnormal, (4) severely abnormal.

Stool Samples and Analyses The stool samples as 1 to 5 g were collected in a plastic container and stored at -20°C until the assays were performed. Calprotectin in fecal extracts was analyzed, using the calprotectin ELISA (Calprotectin ELISA Kit; Immundiagnostik AG, Bensheim, Germany). Lactoferrin in fecal extracts was analyzed, using the lactoferrin ELISA (HK329 Human Lactoferrin ELISA Kit; Hycult Biotech, Uden, Netherlands), and myeloperoxidase in fecal extracts was analyzed, using the myeloperoxidase ELISA (MPO ELISA Kit; Immundiagnostik AG, Bensheim, Germany). The techniques for measurement of three fecal markers followed the manufacturers' guidelines.

Statistics SPSS for Windows 16.0 program was used for statistical analysis. Results were presented as mean $\pm$ SD. Relationship between the groups was assessed using pair sample *T* test and one-way ANOVA. Nonparametric methods Kruskal–Wallis analysis and Mann Whitney *U* test were also used. *p* values less than 0.05 were considered statistically significant.

Results

The rats were apparently healthy until the day of sacrifice. There was no death during the study period and no subjects were disqualified. Radiation proctitis was achieved both macroscopically and microscopically (Fig. 1).

Weight Loss The average weight change values after treatment are $+19.9\pm 8.4$ in control group ($p=0.001$), -13.8 ± 8.2 in RT group ($p=0.002$), $+10.3\pm 6.7$ in RT+ASA group ($p=0.004$), and $+14.1\pm 5.3$ in RT+LGG group ($p=0.003$). While there was significant weight loss in RP group ($p=0.002$), the rats in other three groups gained weight significantly.

Macroscopic Results The macroscopic scores are shown in Table 1. The mean macroscopic score of RT group was significantly higher than those of others ($p=0.0001$). While the scores of RP+LGG and RT+ASA groups were significantly lower than those of RT group ($p=0.015$ and $p=0.038$,

Fig. 1 Histological changes in rectal mucosa with common inflamed gland and lamina propria after radiation (Hematoxylin–eosin (HE) $\times 200$)

respectively), there was no significant difference between each other ($p=0.574$).

Histopathological Results The mean histopathological scores of the specimens and comparisons between them are shown in Table 1. The RT group had the highest scores, followed by the RT+ASA and RP+LGG groups. There was significant difference between groups in all parameters (<0.05). When statistical comparisons were done, scores of treatment groups were significantly lower except a few parameters than those of RT group. On the other hand, there were no significant differences between the treatment groups (Figs. 2 and 3).

Biochemical Results Biochemical evaluation results are shown in Table 2. The mean fecal calprotectin results, both on the 7th and 14th days, were significantly higher in RT and treatment groups than those of control group. While the results for treatment groups were significantly lower than those of control, there were no significant differences between each other. The results on the 14th day were significantly higher than those of the 7th day in all groups but control.

The mean fecal lactoferrin results did not differ between groups, both on the 7th and 14th days. There were also no differences between the 7th and 14th days.

The mean fecal myeloperoxidase results, both on the 7th and 14th days were significantly higher in RT and treatment groups than those of control group. While the results for treatment groups were significantly lower than those of control, there were no significant differences between each other. There were also no differences between the 7th and 14th days in all groups.

Discussion

In the present study, radiation proctitis was achieved in each rat. The induced proctitis was treated by either 5-ASA or

Table 1 Histopathologic and macroscopic scores and statistical evaluation of the groups

Groups		Control ^a	RT ^a	RT+ASA ^a	RP+LGG ^a	RT and RT+ASA (<i>p</i>)	RT+ASA and RT+LGG (<i>p</i>)	RT and RT+LGG (<i>p</i>)
Macroscopic evaluation		0.00±0.00	2.25±0.89	1.00±0.76	1.25±0.74	0.015	0.574	0.038
Surface epithelium	Loss of cellular height/ flattening of cells	0.00±0.00	2.50±0.75	0.75±0.46	1.25±0.46	0.0001	0.162	0.03
	Cellular inflammatory infiltrate	0.00±0.00	2.50±0.75	1.12±0.35	1.50±0.53	0.001	0.234	0.021
Glands (crypts)	Luminal migration of epithelial nuclei	0.00±0.00	2.38±0.52	1.00±0.00	1.50±0.53	0.0001	0.105	0.021
	Loss of goblet cells	0.00±0.00	2.38±0.74	1.13±0.35	1.62±0.51	0.005	0.105	0.065
	Mitotic activity	0.00±0.00	2.62±0.51	1.38±0.51	1.62±0.51	0.002	0.442	0.007
	Cryptitis	0.00±0.00	2.38±1.06	1.50±0.53	1.50±0.53	0.105	1	0.105
	Eosinophilic crypt abscess	0.00±0.00	2.12±1.24	1.00±0.53	0.88±0.35	0.05	0.721	0.028
	Loss of glands	0.00±0.00	2.00±0.75	1.50±0.53	1.13±0.35	0.234	0.234	0.028
	Atrophy of glands	0.00±0.00	2.00±0.75	1.50±0.53	1.25±0.46	0.234	0.442	0.065
	Distortion of glands	0.00±0.00	2.00±0.75	1.50±0.53	1.25±0.46	0.234	0.442	0.065
Lamina propria	Inflammation	0.00±0.00	2.62±1.06	1.38±0.51	1.13±0.35	0.002	0.442	0.01
	Edema	0.00±0.00	2.62±1.06	1.00±0.00	1.50±0.46	0.021	0.442	0.005
	Congestion of blood vessels	0.00±0.00	2.50±1.06	1.00±0.00	1.00±0.00	0.002	1	0.002

^a Values are presented as mean±standard deviation (SD), and $p<0.05$

probiotic effectively. The effectiveness of the treatment was shown macroscopically, microscopically, and biochemically. The fecal calprotectin as a biochemical agent reflected the proctitis and healing most accurately. Probiotics were found as effective as the 5-ASA in the treatment of radiation-induced proctitis.

Radiation proctitis has been reported to occur in 5 to 20 % of the patients during radiation therapy in the pelvic region [2, 3]. The ionizing radiation causes inhibition of epithelial cell mitosis deep within the intestinal mucosal crypts and transmural injury of the bowel wall, which may lead to a progressive obliterative vasculitis, thrombosis, varying degrees of ischemia and necrosis, and ultimately, fibrosis. Clinically, episodes of severe rectal bleeding due to rectal wall injury and obstructive features may be seen [20].

Animal models of radiation proctitis [21] showed that the radiation injury of the rectum in rat develops in dose-dependent manner. A 17.5 Gy in single fraction was suggested as the optimum dose to induce proctitis model and to evaluate the protective effect of various medications for radiation proctitis in face of the clinical situation. It was also shown that radiation caused dose-related clinical and histological changes peaking at 7 to 15 days post-exposure [22]. The present study was based on this proctitis model. The macroscopic and histological changes were also evaluated on the 14th day after irradiation in order to evaluate peaking histological changes. We also used some more parameters, such as weight changes and biochemical markers, for evaluation proctitis model and comparing the efficacy of the treatments. The proctitis model was achieved

Fig. 2 Slightly inflamed mucosa of the rectum in RP+ASA group (HE ×100)

Fig. 3 Moderately inflamed mucosa of the rectum in RP+LGG group (HE ×200)

Table 2 Biochemical results

Characteristics	Control ^a	RT ^a	RT+ASA ^a	RP+LGG ^a	<i>p</i> value
Calprotectin 7th day	3.79±0.9	6.02±2.0	1.73±0.8	1.68±0.3	0.041
Calprotectin 14th day	5.50±1.0	40.95±9.5	15.07±3.8	15.44±2.5	0.017
	<i>p</i> =0.136	<i>p</i> =0.031	<i>p</i> =0.016	<i>p</i> =0.036	
Lactoferrin 7th day	0.21±0.2	1.68±1.1	1.25±1.0	1.14±1.0	0.645
Lactoferrin 14th day	0.64±0.6	1.36±1.0	0.39±0.3	0.23±0.1	0.570
	<i>p</i> =0.285	<i>p</i> =0.496	<i>p</i> =0.245	<i>p</i> =0.406	
Myeloperoxidase 7th day	763±159	1,637±246	1,019±205	1,070±173	0.031
Myeloperoxidase 14th day	672±162	1,774±241	1,150±224	1,467±177	0.005
	<i>p</i> =0.400	<i>p</i> =0.300	<i>p</i> =0.489	<i>p</i> =0.102	

^aValues are presented as mean± standard error of mean (SEM)

by 17.5 Gy in single fraction and it was proven by both macroscopic and microscopic evaluation.

The mean weight changes between groups at the beginning and the end of the study were measured in order to evaluate clinical effects of the irradiation and the treatments. When compared to the before irradiation weights, a significant weight loss was detected after irradiation, while the control and the treatments groups significantly gained weight. The weight loss in irradiated rats is suggested to be related to the clinical effects of proctitis itself. Additionally, the stress due to daily feeding of under ether anesthesia with orogastric probe could be effective in weight loss. The weight gain in the control group subjects was related to the natural growth between 8th and 12th weeks of life and they were not subjected to the orogastric feeding. The treatment groups gained significant weight, although they were not as much as control. Despite the treatment groups possibly stayed under stress due to daily feeding of under ether anesthesia with orogastric probe, treatments were effective to prevent significant weight loss. In a placebo-controlled balsalazid study on radiation proctitis [23], also a weight loss was observed in the placebo group, while a weight gain was observed in the group that used balsalazid.

In the present study, macroscopic tissue damage was detected more common in irradiated group without treatment. Diminished tissue damage was more dominant and frequent in groups treated with either 5-ASA or LGG, suggesting that 5-ASA or LGG treatment prevented or decreased radiation damage macroscopically.

Histopathologically, tissue damage caused by radiation is evaluated on surface epithelium, glands, and lamina propria [5]. The preventive effects of both treatments were also true histopathologically without any significant difference between them. Although the treatment modalities were effective to diminish the damage, the healing process was not complete. This is because the damage may be repaired later on the glands or applied treatments may be less efficient on glands compared to the other structures. In addition, lack of treatment doses may be an effect of treatment failure on

gland healing. Similarly, in experimental study of Korkut et al., the group given 5-ASA has lower histopathological scores [5].

The probiotics, as living microorganisms, may compete with pathogens for nutrients and mucosal adherence, produce antimicrobial substances, and modulate mucosal immune functions [24]. Probiotic therapies have been clinically evaluated and used as treatment for clinical conditions, such as human inflammatory bowel diseases, pouchitis, and cancer prevention [12, 24]. For inflammatory bowel diseases, recent investigations in mice describe anti-apoptotic and anti-inflammatory mechanisms for the probiotic LGG. LGG or its conditioned medium reduce radiation-induced epithelial injury and improve crypt survival in mice [12]. We preferred to LGG as probiotics because it was shown to be effective especially on the crypts after radiation therapy in experimental studies. In the present study, LGG which has healing effects on intestinal mucosa and 5-ASA, which has therapeutic effects detected before on radiation proctitis, are used. Because of the presence of only oral forms of probiotics, oral form of 5-ASA was preferred to compare with.

Many studies have shown that fecal biomarkers such as calprotectin, lactoferrin, and myeloperoxidase vary in parallel with the severity of inflammation occurring in the bowel, where the sensitivity and specificity of the inflammatory response is higher [13].

Calprotectin is a calcium- and zinc-binding protein, which accounts for 40 to 60 % of neutrophils' cytosol. It is resistant to enzymatic degradation and can be easily measured in feces. The presence of calprotectin in feces is directly proportional to neutrophil migration towards the intestinal tract [13, 25]. The main diseases that cause an increased excretion of fecal calprotectin are Crohn's disease, ulcerative colitis, and malign neoplasm [26]. Many studies showed that fecal calprotectin has higher specificity and sensitivity to follow the course of the disease and detection of recurrence in patients with bowel diseases associated with inflammation, when compared to other fecal markers [17, 27, 28].

Lactoferrin (LF) is a multifunctional protein of the transferrin family that is widely represented in various secretory fluids, such as milk, saliva, tears, and nasal secretions [29]. In intestinal inflammation, leukocyte infiltration of the mucosa causes a rise in lactoferrin concentration in the feces. Lactoferrin is resistant to proteolysis in the feces and can be detected after 4 days due to its excellent stability. It was found that fecal lactoferrin was a sensitive and specific marker in measuring the activity of IBD and a valid method for discriminating between inflammatory and non-inflammatory bowel diseases [13]. Sipponen et al. showed that in ileocolonic and colonic disease, endoscopic score for Crohn's disease and histological findings correlated significantly with fecal calprotectin and lactoferrin. Also, a normal fecal marker concentration was a reliable surrogate marker for endoscopically and histologically inactive Crohn's disease [28].

Myeloperoxidase, an enzyme that functions in the oxygen-dependent killing of microorganisms, is released from the primary granules of neutrophils during acute inflammation. The concentration of myeloperoxidase is also proportional to the number of neutrophils within that region [13]. In a study, it was reported that in follow-up of ulcerative colitis and Crohn's disease treatment, fecal myeloperoxidase was a biomarker as effective as fecal calprotectin [17].

In the present study, fecal calprotectin, lactoferrin, and myeloperoxidase were used as biochemical markers to show the presence of proctitis and the effectiveness of therapeutic agents. While many studies were performed related to fecal inflammatory markers such as fecal calprotectin, lactoferrin, and myeloperoxidase in remission, treatment, and monitoring of inflammatory bowel diseases, a limited number of publications were observed on radiation proctitis [18].

Determination of the measured 7th and 14th day's values of fecal calprotectin and myeloperoxidase high only in the group receiving radiotherapy shows the intensity of the inflammation and an increasing inflammation caused by radiotherapy. Both in radiotherapy and treatment groups, detection of the 14th day calprotectin results higher than the 7th day results suggest that the inflammation may be maximal in the 10–14th days. Myeloperoxidase levels in treated groups compared to untreated groups were lower in both the 7th and 14th day. In radiation damage created and untreated subjects, no significant difference was observed between the 7th day and 14th day. Also, no difference was found between groups and between the 7th and 14th days. Lactoferrin results evaluated no significant finding observed in diagnosis and monitoring treatment of radiation proctitis in early period. There was no significant increase in fecal lactoferrin levels. We suggest that lactoferrin would be useful in the late period of these diseases. These results suggest that fecal myeloperoxidase and calprotectin are more effective than fecal lactoferrin for monitoring treatment of radiation proctitis in early period.

Fecal biomarkers are inexpensive, easy to use, and non-invasive method for diagnosis and monitoring treatment. Fecal calprotectin could be a safe and simple biomarker at the determination of the response to the treatment of radiation proctitis in the early period. In order to determine exactly the effectiveness of fecal biomarkers in diagnosis and monitoring treatment of radiation proctitis, detailed and long-term experimental and clinical trials are needed by using various experimental models.

The drawbacks of the study are absence of pre-irradiation fecal biochemical results of the subjects, not having a comparison between biochemical and histopathological results on day 7, and absence of combined treatment of 5-ASA and probiotic.

In conclusion, LGG is as effective as 5-ASA in the treatment of experimental radiation-induced proctitis both clinically and histopathologically. Fecal calprotectin could be a safe and simple biomarker at the determination of the response to the treatment. More detailed experimental and clinical studies are necessary for determination of efficiencies of probiotics, their dosages, treatment durations, types of use, and also combinations with the other probiotics or drugs.

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