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ORIGINAL RESEARCH

Effects of β-Glucan on Colon Anastomotic Healing in Rats Given Preoperative Irradiation

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ABSTRACT

Background: Radiation therapy is an essential therapeutic modality in the management of a wide variety of tumors. We aimed to investigate the short-term effects of pelvic irradiation on the healing of colon anastomoses and to determine the potential protective effects of β-glucan in this situation. *Material and Methods*: Sixty Wistar albino rats were randomized into three experimental groups: a control group ($n = 20$), an irradiation (IR) group ($n = 20$), and an irradiation+β-glucan (IR+β-glucan) group ($n = 20$). Only segmental colonic resection and anastomosis were performed on the *control group*. The *IR group* underwent the same surgical procedure as the control group 5 days after pelvic irradiation. In the *IR+β-glucan group*, the same procedure was applied as in the IR group after β-glucan administration. The groups were subdivided into subgroups according to the date of euthanasia (third [$n = 10$] or seventh [$n = 10$] postoperative [PO] day), and anastomotic colonic segments were resected to evaluate bursting pressures and biochemical and histopathological parameters. *Results*: Bursting pressure values were significantly lower in the IR group ($p < .001$). Malondialdehyde (MDA) levels were significantly higher in the IR group, whereas β-glucan significantly decreased MDA levels on the third PO day ($p < .001$). Granulation tissue formation scores were significantly lower in the IR+β-glucan group compared with the control group and the IR group ($p < .001$). *Conclusions*: The results of this study indicate that irradiation has negative effects on the early healing of colon anastomoses. The administration of β-glucan ameliorates these unfavorable effects by altering bursting pressures and biochemical parameters.

Keywords: anastomotic healing; β-glucan; irradiation; relaparotomy; bursting pressure; protective effects

INTRODUCTION

Radiation therapy is an essential therapeutic modality in the management of a wide variety of tumors, but its immediate and delayed side effects in normal tissues limit the effectiveness of the therapy [1]. After the bone marrow, the gastrointestinal tract is the organ that is most sensitive to the effects of radiation. As a result of ionizing radiation, acute morphological changes in the intestine are observed within 24–48 hr. The detrimental effects of ionizing radiation on living systems are mainly due to its indirect effects. Upon exposure to radiation, free radicals are generated from the radiolysis of water molecules. These free radicals interact with various biomolecules and cause changes in their structure and function [2]. The local effects of irradiation on the bowel are believed to involve a two-stage process that includes both short- and long-term components [3]. The short-term changes are manifested histologically by a loss of the intestinal epithelial cell lining, disappearance of mitotic figures, and oedema of the submucosa and muscularis. There is usually marked

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improvement 7–9 days after treatment, with the bowel appearing histologically near-normal 22 days after treatment [4].

β-glucan is a glucose polymer derived from the cell walls of baker's yeast (*Saccharomyces cerevisiae*), fungi, and cereal plants [5, 6]. Several mechanisms have been proposed for the protective effects of β-glucan molecules. These mechanisms are antibacterial, antifungal [7], antitumoral [8], antilipid [9], antitoxic, and radioprotective [10]. Moreover, these polymers stimulate hematopoiesis and improvements in the wound healing process [7, 10, 11]. In addition to these important functions, β-glucan is an effective antioxidant and a free-radical scavenger.

Thus, in the present study, we aimed to investigate the short-term effects of pelvic irradiation on the healing of colon anastomoses and to determine the potential protective effects of β-glucan in this context. For this purpose, the effect of β-glucan on the irradiated anastomotic colon was assessed by mechanical, biochemical, and histologic analyses.

MATERIALS AND METHODS

Animal Models

The study was approved by the Ethics Committee on Animal Experimentation of the Faculty of Medicine of Ankara University (protocol no. 2011–105–386). Sixty male Wistar albino rats, weighing between 180 and 220 g, were used in this study. The animals were housed in stainless steel cages under controlled temperature (23°C) and humidity conditions and 12-hr cycles of darkness and light. All rats were clinically healthy and were fed with standard laboratory food and water.

Operative Procedure

The same operative procedure was performed on all groups by the same surgeon. All surgical procedures were performed under sterile conditions. After shaving the frontal abdominal wall, 2.5% povidone-iodine was used for skin disinfection, and the rats were anesthetized with ketamine hydrochloride (75–100 mg/kg) and xylazine (10 mg/kg) intramuscularly. The abdomen was entered through a 3 cm mid-line incision, and 0.5 cm of the colon, 3 cm proximal to the peritoneal reflection, was resected. An end-to-end anastomosis was then made using eight inverting interrupted sutures with 5/0 polypropylene (Ethicon). The muscles of the front abdominal wall and skin were closed by a continuous suture with 3/0 silk (Ethicon), and the rats were kept in their cages. During this period, all rats were housed in single cages in a room with controlled temperature (23°C) and humidity (50 ± 5%) and a 12-hr cycle of light and dark. The rats were fed with laboratory pellet chow, and water was given ad libitum. An evaluation of the general condition, food intake, and postoperative (PO) movements of the rats showed that there was no need to add analgesic or antibiotic administration.

Study Design

The rats were randomly allocated to one of three experimental groups: a control group ($n = 20$), an irradiation (IR) group ($n = 20$), and an irradiation+β-glucan (IR+β-glucan) group ($n = 20$). The groups were subdivided into subgroups according to the date of euthanasia (third [$n = 10$] or seventh [$n = 10$] PO days). The *control group* underwent surgery alone, and only a segmental colonic resection and an anastomosis were performed. The *IR group* underwent the same surgical procedure as did the control group 5 days after a single dose of 485 cGy pelvic irradiation. β-glucan was administered perorally to the rats in the *IR+β-glucan group*, with a daily dose of 100 mg/kg from the beginning of the study to the date of euthanasia. Two days after drug administration, a single dose of 485 cGy pelvic irradiation was applied to the group. As in the IR group, on the fifth day after irradiation, the surgical procedure was performed. On the third or seventh PO day, each subgroup was anesthetized with ketamine hydrochloride (75–100 mg/kg) and xylazine (10 mg/kg) intramuscularly and euthanized by intracardiac puncture. Following euthanasia, relaparotomy was performed for in vitro analytical procedures. A schematic diagram of the study is shown in Figure 1.

Measurement of Anastomotic Bursting Pressure

Anastomotic strength was measured in vitro by determining the bursting pressure. Segments of the colon, including the anastomotic area, were resected, and the bursting pressure was measured in vitro for every anastomotic segment. For this purpose, the segments were infused with 0.9% NaCl at 2 ml/min. The maximum pressure recorded immediately before a sudden loss of pressure was recorded as the bursting pressure. The site of the rupture (within or outside the anastomosis) was noted. After recording the bursting pressure, the anastomosis was opened longitudinally and divided into three equal longitudinal segments. One segment was placed in 10% formalin for histologic assessment. The second segment of the anastomosis site was frozen in liquid nitrogen and stored at –80°C until the anastomotic tissue's malondialdehyde (MDA) and advanced oxidation protein product (AOPP) levels were measured. The third segment of the anastomosis was prepared in the same manner for measurement of the anastomotic tissue's hydroxyproline (HP) content.

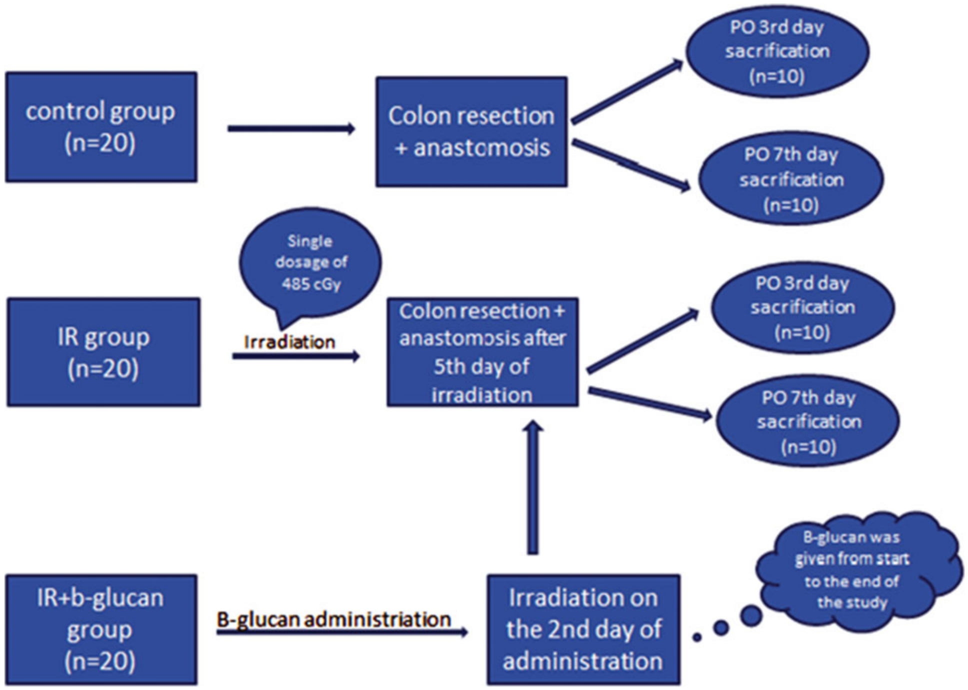

FIGURE 1 **S**chematic diagram of the study.

Biochemical Analyses

Hydroxyproline
The anastomotic HP content was measured using the method of Jamall et al. [12]. In this method, 25 μg of homogenate taken from hydrolyzation was lyophilized and dissolved in 1 ml of 50% (vol/vol) isopropyl alcohol. Ten minutes later, chloramine T was added to these samples. Next, the samples were incubated for 90 min at 50°C after adding 1 ml of Ehrlich's reagent. The absorbance was measured at 560 nm, and the amount of HP was calculated from a standard curve constructed using HP at concentrations between 0.25 and 40 μg/ml. The results were expressed as μg/mg.

Advanced Oxidation Protein Products

Spectrophotometric determination of the AOPP levels was performed using a modification of Witko's method [13]. The samples were prepared as follows. In total, 200 μl of supernatant was diluted 1:3 in phosphate-buffered saline (PBS), and 100 ml of 1.16 mol/l potassium iodide (KI; Sigma Chemicals, St Louis, MO, USA) was added to each tube, followed 2 min later by the addition of 200 ml of acetic acid. The absorbance of the reaction mixture was immediately read at 340 nm against a blank containing 1200 ml of PBS, 100 ml of KI, and 200 ml of acetic acid. Concentrations of AOPP were calculated using the extinction coefficient 26 $mM^{-1} \times 3\ cm^{-1}$.

Malondialdehyde

The levels of MDA in the homogenate from each group were measured using the method of Uchiyama et al. [14]. Determination of malondialdehyde precursor levels in tissues using a thiobarbituric acid test was performed as follows. Briefly, 250 μl of 10% tissue homogenate was added to 1.5 ml of 1% H_3PO_4 and 0.5 ml of 0.6% tert-butyl alcohol (aqueous solution). The mixture was then stirred and heated in a boiling water bath for 45 min. After cooling, 2 ml of n-butanol was added. The mixture was shaken, and the butanol layer was separated by centrifugation. The optical density of the butanol layer was determined to be 535 and 520 nm, and the optical density difference between the two determinations was calculated (corresponding to the tert-butyl alcohol value). The MDA concentrations were expressed as nanomoles per gram of tissue.

Histologic Evaluation

The perianastomotic colonic segments were sampled for examination by an expert pathologist who was blinded to the experimental groups. A segment of each anastomotic ring was removed for histologic examination and fixed in 10% formaldehyde. The samples for histology were dehydrated and embedded in paraffin. The paraffin blocks were cut into four μm-thick serial sections and stained with hematoxylin-eosin (H&E). Histologic changes in anastomotic wound

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TABLE 1 Parameters of histological changes of anastomotic wound healing, granulation tissue development, and local inflammatory response[15–17]

1. Mucosal anastomotic reepithelialization				
Grade 0	Absence of epithelialization on the anastomotic line			
Grade 1	Incomplete coating of the anastomotic wound with a single layer of cells			
Grade 2	Complete coating of the anastomotic wound with a single layer of cells			
Grade 3	Complete reepithelialization with glandular epithelium			
2. Inflammatory granuloma and granulation tissue formation				
	Inflammatory cell presence	*Neovascularization*	*Fibroblasts*	*Fibrosis formation*
Grade 1	Absence	Absence	Absence	Absence
Grade 2	Slight	Slight	Slight	Slight
Grade 3	Mild	Mild	Mild	Mild
Grade 4	Intense	Intense	Intense	Intense
3. Muscle layer destruction				
	Ischemic necrosis	*Muscle layer continuity*	*Inflammatory infiltration*	
Grade 1	Absence	Complete interruption	Absence	
Grade 2	Slight	Muscle synechia	Slight	
Grade 3	Mild	Complete restitution	Mild	
Grade 4	—	—	Intense	
4. Anastomotic wound inflammatory infiltration				
	Neutrophils	*Lymphocytes*	*Histiocytes*	*Giant cells*
Grade 1	Absence	Absence	Absence	Absence
Grade 2	Slight	Slight	Slight	Slight
Grade 3	Mild	Mild	Mild	Mild
Grade 4	Intense	Intense	Intense	Intense

healing, granulation tissue development, the local inflammatory response, and neovascularisation were determined according to the parameters of Houdart et al. and Hutschenreiter et al., as modified by Garcia et al. [15–17] (Table 1).

Drug Administration

A 100 mg/kg β-glucan capsule (Mustafa Nevzat Company, Istanbul, Turkey) was suspended in 2 ml of saline and administered by intragastric gavage to the IR+β-glucan group from the beginning of the study until the date of euthanasia. The selection of the dose range was based on previous experience with β-glucan in animal models [18] and in humans [19, 20].

Irradiation Technique

Before the intramuscular administration of ketamine HCl (10 mg/kg) (Ketalar) for anesthetization, the rats were grouped into subgroups each containing five rats. Each group underwent half-body irradiation via the source axial distance (SAD) technique under a simulator (Verasim II, GE Medical Systems, Waukesha, WI). All of the groups were exposed to a single dose of 485 cGy g-ray (Cobalt 60 Teletherapy Theratron 780 C, Atomic Energy of Canada Ltd., Kanata, Ontario, Canada) in the same position, with openings above and opened parts below (parallel). The rats were put into cages and started to feed after 4 hr.

Statistical Analysis

Data analysis was performed with SPSS for Windows, ver. 11.5. The Shapiro–Wilk test was used to determine whether the distributions of continuous variables were normal. The homogeneity of the variables was determined by the Levene test. The data were expressed as the mean $\pm$ standard deviation or the median (minimum-maximum), where applicable. The Bonferroni-adjusted Kruskal–Wallis test was applied to determine the differences among more than two dependent groups. When the p value from the one-way ANOVA or Kruskal–Wallis test indicated statistical significance, to determine which groups differed from the others, the post-hoc Tukey multiple comparison test was used. A p value of $<.025$ was considered statistically significant. However, for all possible multiple comparisons, the Bonferroni adjustment was applied to control for Type I errors.

RESULTS

One death caused by the aggressive behavior of a rat was registered in the control group on the second PO day. Postmortem exploration revealed no anastomotic leakage from the anastomotic site. The other rats survived until euthanization. No peritonitis, anastomotic leakage, or abscesses were observed in any of the three groups. Adherences of different intensities were found in all anastomoses. In particular, perianastomotic omental tissues were much more intensive in

TABLE 2 Bursting pressure (mmHg) on postoperative third and seventh day

Days	Control group	IR group	IR+β-glucan	p value[a]
D 3	70.1 ± 10.0^{b}	$55.9 \pm 6.2^{b,c}$	66.9 ± 8.4^{c}	0.006
D 7	$185.4 \pm 23.6^{b,d}$	$124.9 \pm 20.2^{b,c}$	$163.6 \pm 42.8^{c,d}$	0.002

a. The comparisons within days, Kruskal–Wallis test, according to the Bonferroni adjustment ($p < .025$), was considered statistically significant.
b. The difference between the control group and the IR group was found statistically significant ($p < .001$).
c. The difference between the IR group and the IR+β-glucan group was found statistically significant ($p < .001$).
d. The difference between the control group and the IR+β-glucan group was found statistically significant ($p = .019$).

the IR group; however, it was easy to dissect those tissues away using blunt dissection.

Bursting Pressures

Bursting pressures were measured in the anastomotic segments on the third and seventh PO days (Table 2). Bursting pressure values were significantly lower in the IR group than in the control group ($p < .001$) and the IR+β-glucan group ($p < .001$) on the third PO day. On the seventh PO day, bursting pressure values in the IR group were significantly lower compared with values in the control group ($p < .001$) and the IR+β-glucan group ($p < .001$).

Tissue Levels of HP, MDA, and AOPPs in Control Group, IR Group, and IR+β-glucan Group on Third PO Day

MDA levels were significantly higher in the IR group (216.6 ± 87.7 nmol/ml) than in the control group (121.2 $\pm$ 48.2 nmol/ml) and the IR+β-glucan group (98.9 $\pm$ 32.9 nmol/ml) ($p < .001$). There was no significant difference between the groups in terms of AOPP ($p = .804$) or HP ($p = .054$) levels.

Tissue Levels of HP, MDA, and AOPPs in Control Group, IR Group, and IR+β-glucan Group on Seventh PO Day

There was no significant difference between the control group, the IR group, and the IR+β-glucan group in terms of HP ($p = .124$), MDA ($p = .639$), or AOPP ($p = .116$) levels (Table 3).

TABLE 3 Biochemical parameters of each group on postoperative third and seventh day

	Control Group	IR group	IR+β-glucan	p
MDA				
D 3	121.2 ± 48.2^{b}	$216.6 \pm 87.7^{b,c}$	98.9 ± 32.9^{c}	<.001
D 7	267.4 ± 151.7	184.3 ± 112.5	197.0 ± 72.7	.639
AOPP				
D 3	6.1 ± 2.0	6.0 ± 3.0	6.7 ± 4.7	.804
D 7	9.5 ± 6.7	4.1 ± 2.1	7.2 ± 4.7	.116
HP				
D 3	0.34 ± 0.06	0.45 ± 0.26	0.25 ± 0.09	.054
D 7	0.37 ± 0.12	0.50 ± 0.21	0.33 ± 0.11	.124

a. The comparisons within days, Kruskal–Wallis test, according to the Bonferroni adjustment $p < .025$ was considered statistically significant, **b.** The difference between control group and IR group was found statistically significant ($p < .001$), **c.** The difference between IR groupand IR+β-glucan was found statistically significant ($p < .001$).

Histologic Evaluation of Anastomotic Layer on Third PO Day

There were no significant differences between the control group, the IR group, and the IR+β-glucan group in terms of mucosal anastomotic reepithelialisation ($p = .938$), anastomotic wound inflammatory infiltration ($p = .184$), muscle layer destruction ($p = .526$), or granulation tissue formation scores ($p = .042$).

Histologic Evaluation of Anastomotic Layer on Seventh PO Day

The granulation tissue formation score was significantly lower in the IR+β-glucan group than in the control group and the IR group ($p < .001$). There was no significant difference between the control group and the IR group in terms of mucosal anastomotic reepithelialization ($p = .802$), anastomotic wound inflammatory infiltration ($p = .567$), or muscle layer destruction scores ($p = .854$). The results of the histologic evaluation by H&E staining are shown in Table 4 and Figure 2.

DISCUSSION

Radiotherapy is considered to be one of the most important weapons in the fight against cancer, either alone or in combination other treatments, and relies on ionizing radiation-induced DNA damage to defeat malignant cells. However, this therapy's use is limited due to the lethal effects of radiation on normal tissues. Radiation-induced reactive oxygen/nitrogen species (ROS/RNS), such as nitric oxide and superoxide radicals, react to produce reactive peroxynitrite, which is known to induce cytotoxicity by interacting with biomolecules. It is currently believed that X-irradiation leads to the generation of free radicals from radiolysis,

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TABLE 4 The results of histopathological grading

Variables	Control group	IR group	IR+β-glucan	p value[a]
Mucosal anastomotic reepithelization				
Day 3	2 (1–3)	2,5 (1–3)	2 (1–3)	.938
Day 7	1 (1–3)	1 (0–3)	1 (0–3)	.802
Inflammatory granuloma and granulation tissue formation				
Day 3	2 (2–3)	3 (2–4)	2 (1–3)	.042
Day 7	2.5 (2–3)[b]	3(2–4)[b,c]	2 (2–3)[c]	.009
Muscle layer destruction				
Day 3	3 (2–3)	3 (2–3)	3 (2–3)	.526
Day 7	2 (1–3)	2 (1–3)	2 (1–3)	.854
Anastomotic wound inflammatory infiltration				
Day 3	2 (1–3)	3 (2–4)	2 (1–3)	.184
Day 7	3 (2–4)	3 (2–4)	3 (1–4)	.567

a. p value $< .025$ was considered statistically significant according to the Bonferroni adjusted Kruskal–Wallis test, **b.** The difference between the Control Group and IR Group was statistically significant ($p < .001$)**, c.** the difference between IR Group and IR+β-glucan Group was statistically significant ($p < .001$).

such as hydroxyl radicals, which are largely responsible for significant tissue damage [21, 22]. The quest for an effective, nontoxic compound with optimal radioprotective capabilities addresses an immediate need, and this search has shifted research interests toward naturally occurring dietary antioxidants. A number of dietary antioxidants and medicinal plant extracts and their isolated constituents have been reported to have hepatoprotective, neuroprotective, anti-inflammatory, antioxidant, and radical-scavenging properties [23–25]. Although several studies have shown no significant rates of morbidity or mortality, there is controversy about the side effects of preoperative irradiation, such as impaired healing [26–28].

In their experimental study on dogs, Blake et al. concluded that low anterior resection with either an EEA-stapled or a hand-sewn anastomosis could not be performed safely after 6000 rad of preoperative irradiation [29]. Stumpf et al. investigated the PO courses of 400 consecutive patients who underwent colonic or rectal resection. The researchers concluded that the most important clinical risk factors for leakage were radiotherapy and blood transfusion [30].

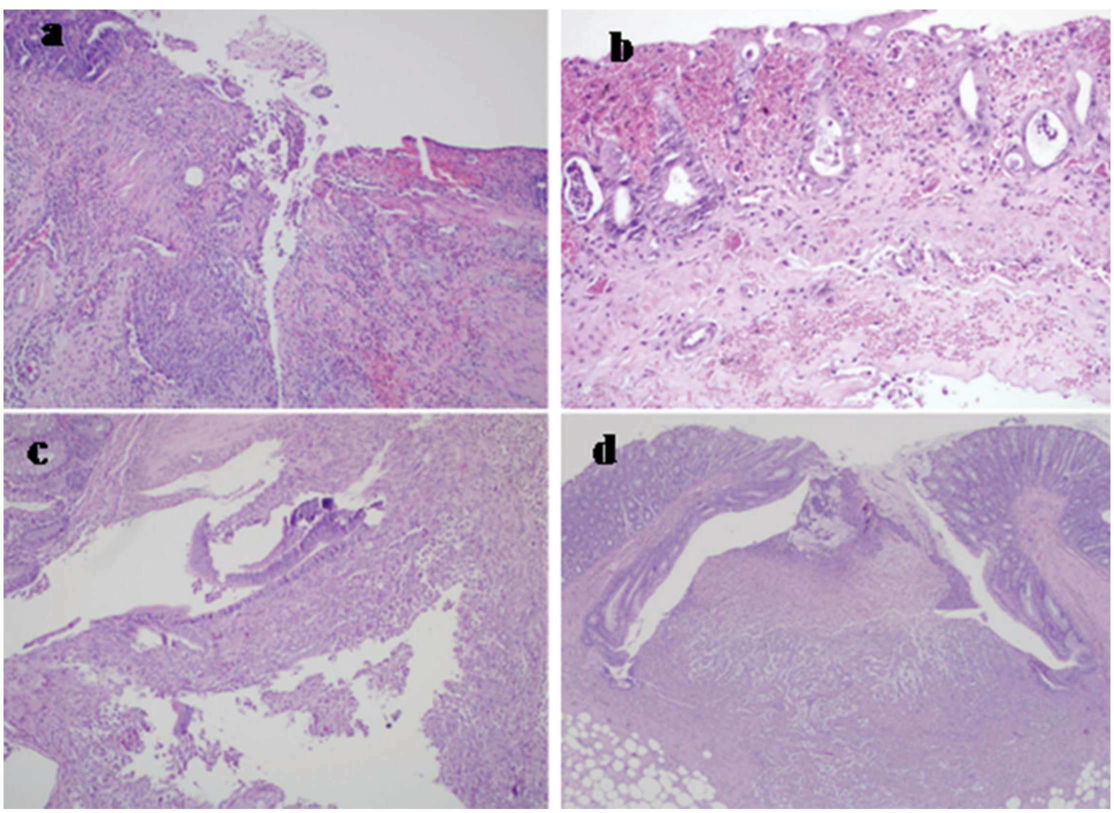

FIGURE 2 Anastomotic histology. H&E-stained sections of colon anastomoses collected on the third and seventh PO days. **a.** Granulation tissue formation, slight/mild inflammatory infiltration, and a narrow ulceration line in the middle, with renewed crypt formation on both sides, in the IR+β-glucan group on the third PO day (H&E, $\times$ 100). **b.** Incomplete epithelization on the surface, characterized by marked ischemic changes and regenerated crypt formation areas in the lamina propria in the IR group on the third PO day (H&E, $\times$ 200). **c.** Ulceration with partially epithelized areas in the IR+β-glucan group on the seventh PO day (H&E, $\times$ 100). **d.** Deep ulceration with complete interruption of the muscle layer, intense inflammation, and active granulation tissue in the IR group on the seventh PO day (H&E, $\times$ 40).

In the present study, we evaluated the short-term effects of irradiation on colon anastomosis and the possible ameliorative effects of β-glucan. The applied dosage of irradiation should have been enough to achieve mucosal damage, but not mortality, in rats. A single dose of radiotherapy (485 cGy) of less than 675 cGy, which is near the 50% lethal dose for gastrointestinal damage in humans, was preferred in our study. In the literature, several irradiation models using a variety of dosages have been used to investigate the effects of radiation on tissue healing. Fang et al. used a single dose of 500 cGy and claimed that this dose was well tolerated by all animals [31]. Ozdemir et al. used a single dose of 800 rad of abdominopelvic irradiation on the fifth preoperative day [32]. E. O. Demir et al. administered a single abdominal dose of 10 Gy to rats 5 days before operation and performed left colon anastomosis [33].

Anastomotic bursting pressure is a reliable and objective parameter for assessing colonic tissue healing [34]. There was no leakage of the colon anastomoses in each group, although the bursting pressures were significantly lower in the IR group compared with the control group and the IR+β-glucan group on the third and seventh PO days. It is believed that gains in breaking strength and bursting pressure are due to the healing process, and mainly collagen synthesis. Measuring HP levels is an indirect way to evaluate collagen content and is a useful method for assessing the biochemical tissue healing process. In our results, we did not find any significant difference among groups in terms of HP levels on the third or seventh PO day. According to these results, HP levels did not correlate with the assessed anastomotic bursting pressure levels. Several results have been reported that are similar to or different from the findings of our study. Dominguez et al. investigated anastomotic wound healing at varying times after irradiation. The researchers reported that increased HP content was observed after irradiation, but this event did not imply increased wound strength [35]. Ceelen et al. studied the effect of a fractionated, clinically relevant scheme of high-dose preoperative radiotherapy on colon anastomotic healing in rats and did not find any significant differences in terms of anastomotic bursting pressure or HP content [36]. Winsey et al. observed a significant decline in bursting pressure and HP levels on the seventh PO day in irradiated rats [37]. U. Ozel Turkcu et al. measured HP levels in an experimental animal study and showed that anastomotic HP levels were changed in the irradiation plus standard anastomosis group compared with the standard anastomosis group [38].

Several experimental studies in the literature have highlighted the antioxidant, radical-scavenging effects of β-glucan [39–42]. Patchen et al. suggested that early after irradiation, β-glucan might mediate its radioprotection by enhancing resistance to microbial invasion via mechanisms not necessarily predicated on hemopoietic recovery and implicated the radical-scavenging property of β-glucan [43]. However, in our literature search, we did not find any report on the effect of β-glucan on the irradiated anastomotic colon. The present study revealed that administration of β-glucan significantly decreased MDA levels on the third PO day compared with levels in the IR group. This finding suggests that the ameliorative effect of β-glucan on anastomotic bursting pressure levels can be attributed to the potent antioxidant effect of the drug.

The histopathological examination revealed a significant decrease in granulation tissue formation on the seventh PO day in the IR+β-glucan group compared with the IR group and the control group. There was no reference to the effect of β-glucan on granulation tissue formation when we reviewed the literature. In any case, this effect of the drug did not have a negative impact on bursting pressures or anastomotic healing. Certain other factors may have played a role in this situation.

In conclusion, our present study showed that irradiation has negative effects on the early healing of colon anastomoses. We suggest that administration of β-glucan ameliorates these unfavorable effects in terms of bursting pressures and biochemical parameters, and particularly MDA levels. Further studies are needed to reach a definitive conclusion.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the article.

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