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The Effects of Concomitant Giardia intestinalis Infection on Acute Gastrointestinal Toxicity in Rats Undergoing Pelvic Irradiation

Pelvik Radyoterapi Uygulanılan Sıçanlarda Akut Gastointestinal Toksisite Üzerine Giardi İntestinalis Enfeksiyonunun Etkileri

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ÖZET

GİRİŞ: Servikal, endometrial, mesane ve prostat gibi pelvik kanserin farklı türleri normalde ya sadece radikal radyoterapi ile yada cerrahi ve kemoterapi birlikte kombinasyon halinde tedavi edilebilir. Bu çalışmanın amacı, pelvik radyasyona maruz kalan sıçanlarda eşzamanlı *Giardia intestinalis* enfeksiyonunun akut gastrointestinal toksisite üzerine etkilerinin araştırılmasıdır.

METOD: Çalışma grubu, 250 g ağırlığa sahip kırk adet 6 aylık dişi Wistar sıçandan oluşmaktadır. Sıçanlar, herbir grupta 10 adet sıçan olacak şekilde dört gruba ayrıldı. Çalışma grupları; Grup 1, *Giardia intestinalis* ile enfekte olmayan ve radyoterapi almayan sıçanlar, Grup 2, radyoterapi almamış, ancak *Giardia intestinalis* ile enfekte olan sıçanlar, Grup 3, *Giardia intestinalis* ile enfekte olmamış fakat radyoterapi almış sıçanlar, Grup 4'te hem *Giardia intestinalis* ile enfekte olan hemde radyoterapi alan sıçanlardan oluşmaktadır. Radyasyon bittikten sonraki gün, hayvan vücut ağırlıkları kayıtedildi ve dışkılama sıklığı hesaplandı. Ratlar perfore edilerek sakrifiye edildi, ince bağırsak dokuları histolojik inceleme için alındı.

SONUÇ: Işık mikroskopik incelemesinin sonucu olarak, grup 3 ve 4 'te villus kısalması, yüzeyel epitelinde atrofi, kriptalarda kayıp gibi mukozal hasarlar ve goblet sayısında azalma tespit edildi.

TARTIŞMA: Bu çalışmanın bir sonucu olarak, *Giardia intestinalis* enfeksiyonu ile eşzamanlı olarak pelvik radyoterapi uygulanması sıçanlarda akut gastrointestinal toksisiteyi arttırmıştır.

Anahtar Kelimeler: Giardia intestinalis, Pelvik radyoterapi, Sıçan, İnce bağırsak toksisitesi

ABSTRACT

INTRODUCTION: Different types of pelvic cancer, such as cervical, endometrial, bladder and prostate, are normally treated by radical radiotherapy, which can be used both alone or in combination with surgery and/or chemotherapy. The aim of this study is to assess the effects of concomitant *Giardia intestinalis* infection on acute gastrointestinal toxicity in rats that have undergone pelvic irradiation.

METHODS: The study group consisted of forty female 6-month-old Wistar rats with the weight of 250 g. The rats were divided into four groups containing ten rats in each group. The study groups are as follows: Group 1 contained rats not infected with *Giardia intestinalis* and not irradiated, Group 2 contained rats infected with *Giardia intestinalis* but not irradiated, Group 3 contained rats not infected with *Giardia intestinalis* but irradiated, Group 4 contained rats infected with *Giardia intestinalis* and radiated. For the day after the end of radiation, the number of stool pellets was counted, and the operation of weighing rats was performed, and they were sacrificed the following day. The intestinal tissues were taken for histological evaluation.

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RESULTS: A mucosal damage, such as villus shortening, atrophy of surface epithelium, crypt loss, as well as a decrease in the number of goblet cells of the group 3 and 4, was detected as a result of the light microscopic examination.
CONCLUSION: As a result of the present study, the fact that concomitant *Giardia intestinalis* infection aggravates acute gastrointestinal toxicity in rats that have undergone pelvic irradiation has been verified.
Keywords: Giardia intestinalis, Pelvic irradiation, Rat, Small intestine Toxicity.

INTRODUCTION

Different types of pelvic cancer, such as cervical, endometrial, bladder and prostate, are normally treated by radical radiotherapy, which can be used both alone or in combination with surgery and/or chemotherapy (1). An increase in the number of people who recover from cancer has been observed in the last 30 years, and it increased 3-fold (2). Nevertheless, cancer treatment has a number of chronic physical consequences that have a negative impact on the life quality of 20-25% individuals who survive cancer (3). However, continuous enhancements have been recently observed in the outcomes from pelvic malignancy due to the presentation of new radiotherapy or combination of chemotherapy and radiation therapy (4).

The effects of radiotherapy on certain complex neurological, hormonal, muscular, immune, and enzyme functions of the human gastrointestinal tract have been examined by a number of researchers. However, definitive studies are quite limited. Attenuating chronic side effects were reported at the most in patients to which radiotherapy alone or in conjunction with other therapies was applied for the treatment of pelvic cancer (5). Gastrointestinal symptoms are regarded to be chronic physical side effects most frequently encountered, and their impact on daily activity is the greatest (6).

There is a risk of complications to normal tissues located around the tumour as a result of radical radiotherapy applied to pelvic cancers (7). Most of the information concerning the cellular and molecular reaction of the gastrointestinal tract to radiation therapy was obtained from experiments carried out on animals. A sequela of events, in which oedema develops into an inflammatory, generally mucosal, reaction that expands to the submucosa in the future, is indicated by the data that are available in the literature (5). Radiation damages the DNA content of the cells that are dividing in an active way, and therefore, causes its therapeutic effect. However, not only affected malignant cells but also adjacent normal tissues may be damaged as a result of radiation. In general, adverse effects develop secondarily to fibrosis and progressive endarteritis occurring in submucosal and muscular tissues that are poorly oxygenated, and this may cause further tissue scarring accordingly (8).

In case the adverse effects of radiation emerge within 90 days of treatment, they are defined as acute. There is a relation of acute effects with the upregulation of inflammatory mediators, fibrotic cytokines, coagulation cascade activation, and vascular damage (9). However, the late effects of radiation may develop months or even years after the completion of the treatment, and their symptoms may be mild or severe, selflimiting, or progressive, and their development may be gradual or sudden. There is a tendency of late effects to occur in tissues in which a slow turnover of cells is observed. Despite the diverse pathology of the lesions they contain fibrosis, necrosis, atrophy, and vascular injury (9). In the acute phase of radiation therapy, a small-bowel bacterial overgrowth is observed in 25% of patients. During the chronic setting, radiation therapy leads to motility changes which are the main reason for this overgrowth17—especially that of gram-negative bacilli, which may lead to different gastrointestinal symptoms observed in 4–45% of patients (10).

The potential risk of late intestinal damage increases to a significant extent during the application of radiotherapy in patients in which increased acute toxicity and diarrhea are observed (11). The most frequently encountered enteric protozoan pathogens that have an impact on humans play an important role in morbidity, particularly in developing countries. These pathogens lead to continuous diarrhea and enteritis and, therefore, damage the small intestine at first (12).

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The small intestine is infected by a flagellated unicellular parasite, known as *Giardia intestinalis*, which leads to watery diarrhea (13). Both humans and livestock are infected by this parasite. Furthermore, it is believed that some genetic variants of *Giardia intestinalis* have zoonotic potential (14). This parasite, which is considered to be the main reason for protozoan diarrhea, is encountered around the world. The range of clinical symptoms is quite rich, and it includes both nonsymptomatic infections and acute and chronic diarrheal diseases. The attachment of *Giardia* trophozoites to the epithelium of the upper part of the small intestine occurs at the acute phase of the infection, and a number of events, such as the disruption of epithelial barrier function, diffuse shortening of brush border microvilli, small intestinal malabsorption and maldigestion, chloride hypersecretion, and increased rates of small intestinal transit, and finally diarrhea, is induced (15,16).

The development of bacterial overgrowth in the small intestine has been caused by changed gastrointestinal motility (17). Bacterial overgrowth has a wide range of clinical symptoms, among which mild abdominal discomfort, bloating, diarrhea, as well as frank malabsorption with weight loss and nutritional deficiencies take place (18). In a study conducted by Roland et al. (2015), they examined and defined a relation between small intestinal transit time and increased bacterial colonization in the small intestine (19).

Transcriptional changes occurring under various stress conditions in *Giardia* have been examined in a number of studies conducted recently (20-25). Nevertheless, no study that evaluates Giardia infection following the application of UV radiation histologically has been encountered in the literature. Accordingly, in this study, it was aimed to assess the prevalence and violence of the effects of concomitant *Giardia intestinalis* infection on acute gastrointestinal toxicity in rats that have undergone pelvic irradiation.

MATERIALS and METHODS

Experimental Design and Study Protocol

The study group consisted of forty 6-month-old Wistar rats, the average weight of which was 250g. The rats were divided into four groups containing 10 rats in each group as follows (n=10 in each group): Group 1 consisted of the rats not infected with *Giardia intestinalis* and not irradiated, Group 2 consisted of the rats infected with *Giardia intestinalis* but not irradiated, Group 3 consisted of the rats not infected with *Giardia intestinalis* but irradiated, Group 4 consisted of the rats infected with *Giardia intestinalis* and irradiated. Harvesting of *Giardia intestinalis* cysts was performed from stool samples for the rats in Group 2 and Group 4. Afterwards, they were processed with water and sucrose and administered via oral gavage. At the same time, the rats in Group 3 and Group 4 were given anesthesia and irradiated with a Cobalt-60 unit through parallel opposed pelvic portals with the use of five fractions of 5 Gy on five successive days, since the first week after oral gavage. For the day after the end of irradiation, stool pellets were counted, and the operation of weighing the rats was performed, and they were sacrificed the following day. For the purpose of histological assessment in regard to the overall intestinal damage score, the intestinal transit time was measured and intestinal tissue samples were obtained.

The rats were kept in cages separately from each other in a quarantine room, in which both light and temperature were controlled, and given standard chow and water ad libitum. The Experiment Animals Research and Application Center of Inönü University provided the rats that were used in this study (Malatya, Turkey), and the Animal Ethics Committee of Inönü University approved the study protocol on animals.

The formation technique of parasitic infection

The isolation of *Giardia intestinalis* cysts was performed with the technique developed by Buchel et al. (1991) (26). At first, stool samples containing a lot of *Giardia intestinalis* cysts were diluted in tap water at the rate of 1:10. Afterwards, they were homogenized and filtered. Following this, we added 0.75M sucrose solution into 3 ml of the diluted stool suspension and centrifuged the obtained mixture at 1500 rpm for 15 minutes. Then, a Pasteur pipette was used to collect cysts at the water/sucrose interphase. The cysts that had been collected at the previous stage were diluted in saline and, afterwards, centrifuged for the period 5 minutes. Following this, the

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obtained pellet was dispersed into 0.75 M sucrose, and the application of sucrose gradient was performed. The dilution operation of the cysts collected was repeated. After this, they were washed 3 times. The rats in groups 1 and 4 were given the obtained solution orally by gavage. Parasitic infections were observed in the period between 7-14 days. The assessment of the presence of parasitic infection in the stool specimen was performed using light microscopy.

The application method of radiation

In order to perform radiation therapy to the rats in groups 3 and 4, the front and back pelvic regions were used. For the purpose of external irradiation, 5 Gy (for each fraction) was applied as an average dose, and a Cobalt-60 teletherapy unit was used for the 5-day period. Therefore, the rats were given a 25 Gy dose of irradiation in total in 5 fractions. Before the application of every radiation fraction, the animals were given anesthesia with ketamine (80 mg/kg) and xylazine (5 mg/kg). Following this, the animals were fixed in the head and limbs, and clinical applications were considered on performing irradiation.

Light microscopic examination

We fixed the intestine tissue in 10% neutral buffered formalin and then put it in paraffin. Tissue sections were cut at 5 µm, mounted on slides, and then their staining with hematoxylin-eosin (H-E) and periodic acid Schiff (PAS) was performed. A Leica DFC280 light microscope was used to examine the sections.

Alterations in the epithelium, such as vacuolization, atrophy, and desquamation, crypt loss, decreased number of crypt cells, capillary hemorrhage and depletion of goblet cell are classified as an intestinal injury. The following scores were assigned for each criterium: 0 none, 1 mild, 2 moderate and 3 severe, and the highest score was found to be 15. In addition to the measurement of mucosal thickness performed from the base of the lamina muscularis mucosa to the villus tip, the measurements of villus height performed from the base to the tip of the villus and crypt depth were made with Leica Q Win and Image Analysis system (Leica Microsystem Imaging Solution Ltd, Cambridge, UK). The measurements of ten separate microscopic fields for all samples were performed using X20 objective per rats. Table 1 contains the findings obtained as a result of morphometric measurements and histological scores.

Body Weight and Gastrointestinal Assessment

At the end of the third day, the evaluation of the results obtained in this study was made following radiation therapy. After the completion of the experiment, tracking of the weight of the experimental animals was performed. The method developed by Berrak Yeğen (2005) was used for counting stool pellets in each group of animals (27). Furthermore, after the rats had been sacrificed, the method developed by Qin et al (2003) was used for the measurement of intestinal transit time with the Evans Blue (28). Also, the preparation of histological slides was performed, and the damage to the mucosa was assessed.

Statistical analysis

A computer program (SPSS 15.0) was used for statistical analysis. The measurement in each group was compared using one-way ANOVA test related to the normality of distribution of variables, post-hoc tests Tukey. Values of $p<0.05$ were considered significant. All results were expressed as means ±standart deviation (SD).

RESULTS

Intestinal measurement results

It was observed that significantly increased intestinal transit time in the group 4 compared with group1,2 and 3 ($p<0.001$). Additionally there was significantly difference between group 1 with other groups compared in terms of intestinal transit time ($p<0.001$). İntestinal transit time was increased in all groups compared to the control group. But there was no significantly difference between group 2 and 3 ($p>0.005$)(Figure 1A). On the other hand, there was highly significant increase compared with group 4 and other groups in terms of stool pellets. Amount of the stool pellets in group 4 was highly significant increased in comparison with the other groups ($p<0.001$). But there is no significantly difference between group 1 with 2 and group 1 with 3 and group 2 with 3 compared with each other, respectively ($p>0.005$)(Figure 1B). When the weight loss evaluated, there was significantly difference between group 1 with other groups ($p<0.001$). It was observed that significantly increased weight loss in the group

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2,3 and 4 compared with group1 ($p<0.001$). But no significant differences were observed between group 2 with 3 and group 3 with 4 ($p>0.005$) (Figure 1C).

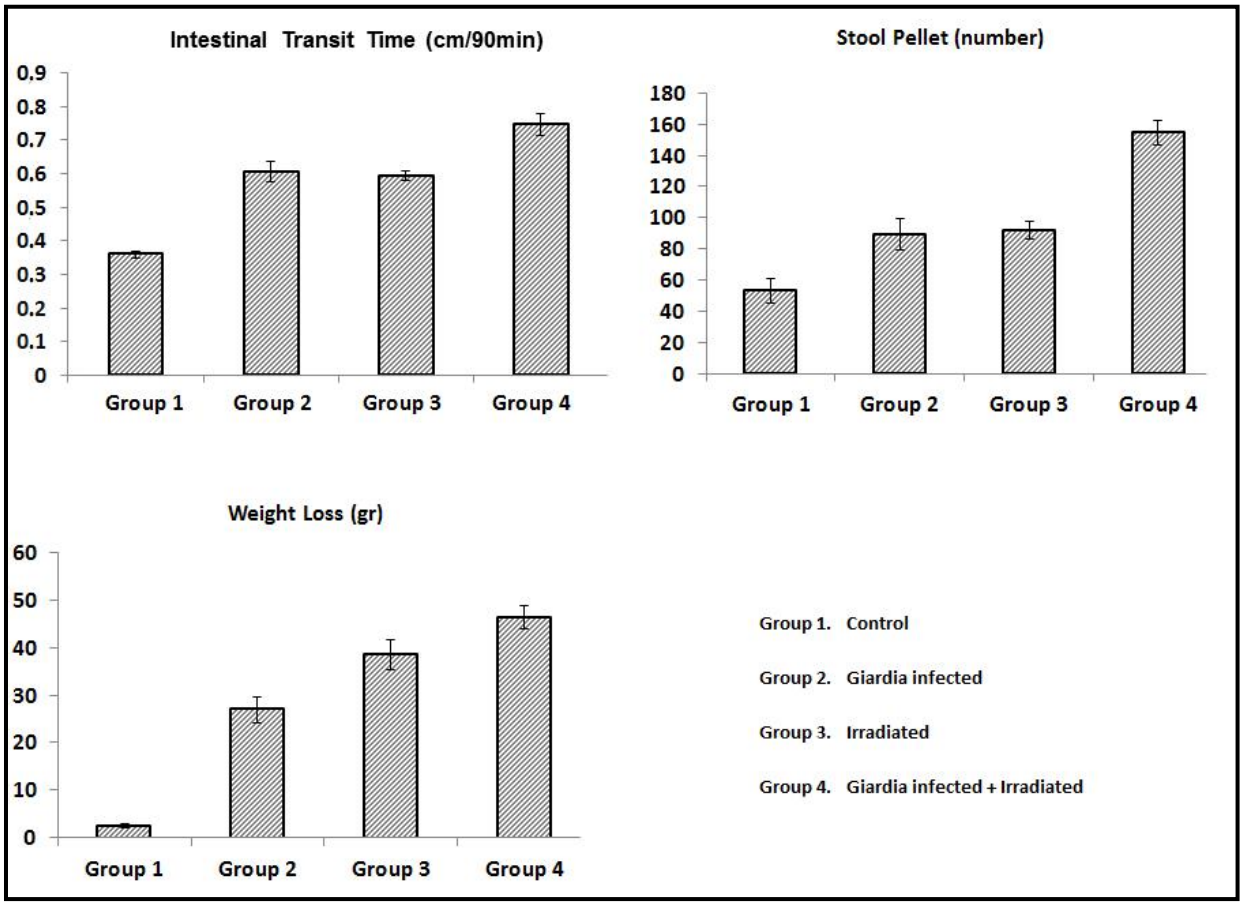

Figure 1 A, Transit time of intestine was no significant differences in between group 2, 3 and 4 ($p>0.005$). But transit time was significantly increase in group 2, 3 and 4 compared to group 1($p<0.001$). **B,** Amount of stool pellets were significantly increase group 2, 3 and 4 compared group1 ($p<0.001$). **C,** No significant difference in body weight of rats before operation was detected among group 1, 2, 3 and 4 ($p>0.005$). After operation weight loss in group 3 and 4 were significantly higher than the other groups ($p<0.001$).

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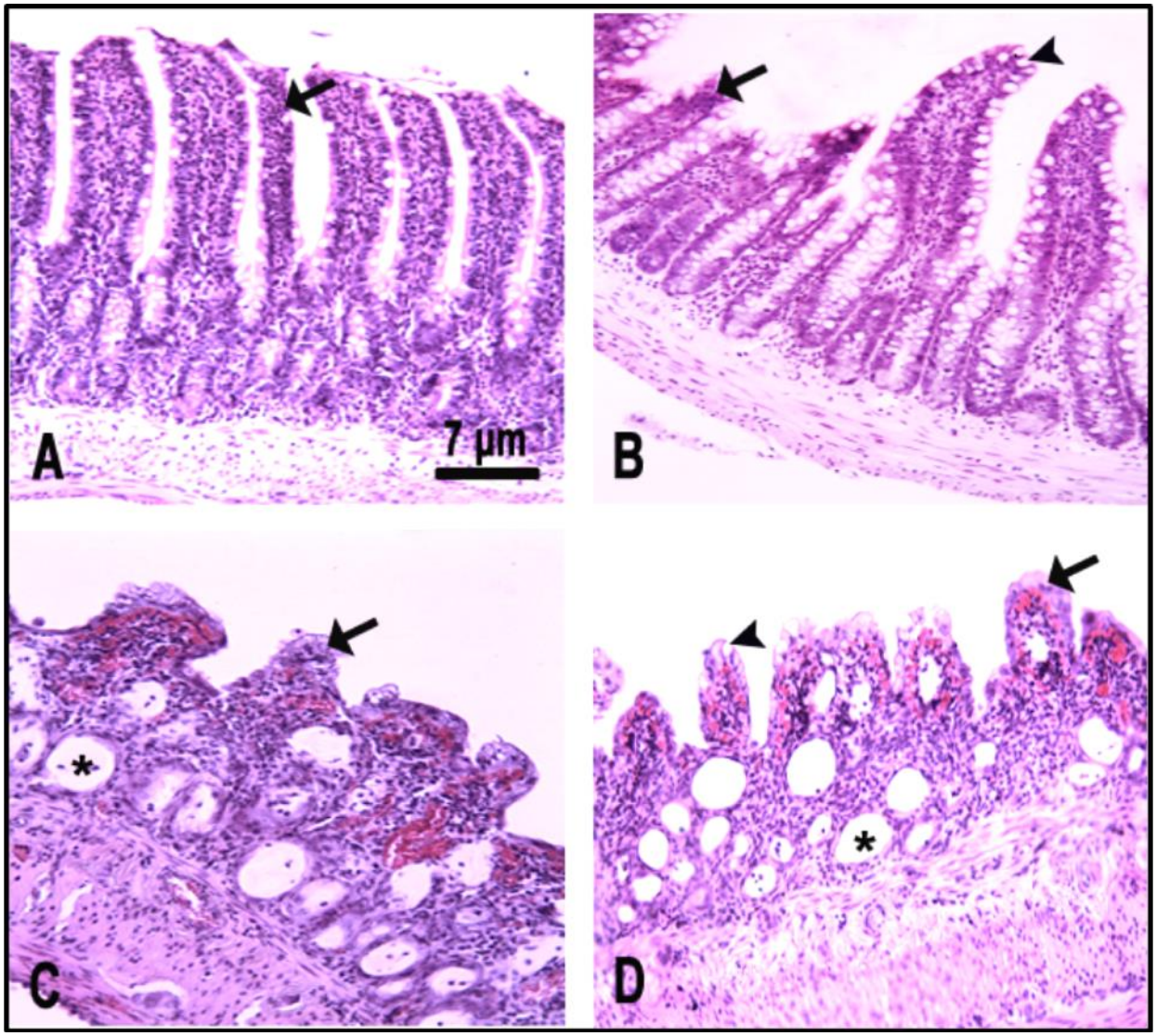

Figure 2. A; Histological appearance was normal in group 1 (control) (villus and crypts are normal), **B;** Intestinal damage was mild in group 2 (giardia infected), **C;** Histopathologic changes were evident in group 3 (irradiated group). Villus shortening, vacuolization and atrophy of surface epithelium, crypt loss, capillary hemorrhage and decrease number of goblet cells were observed, **D;** In group 4 (giardia infected+ irradiated) a high histologic damage score was determined in the histological sections (13±0.7). Villus shortening, atrophy, vacoulization and desquamation of surface epithelium, crypt loss, structural changes of the crypts and capillary hemorrhage and a decreased number of goblet cells were more pronounced compared to those in the group 3. (**arrow;** villus, **arrow head;** goblet cell, **asterisk;** vacuolization, **H&E** stain, **X60** magnification).

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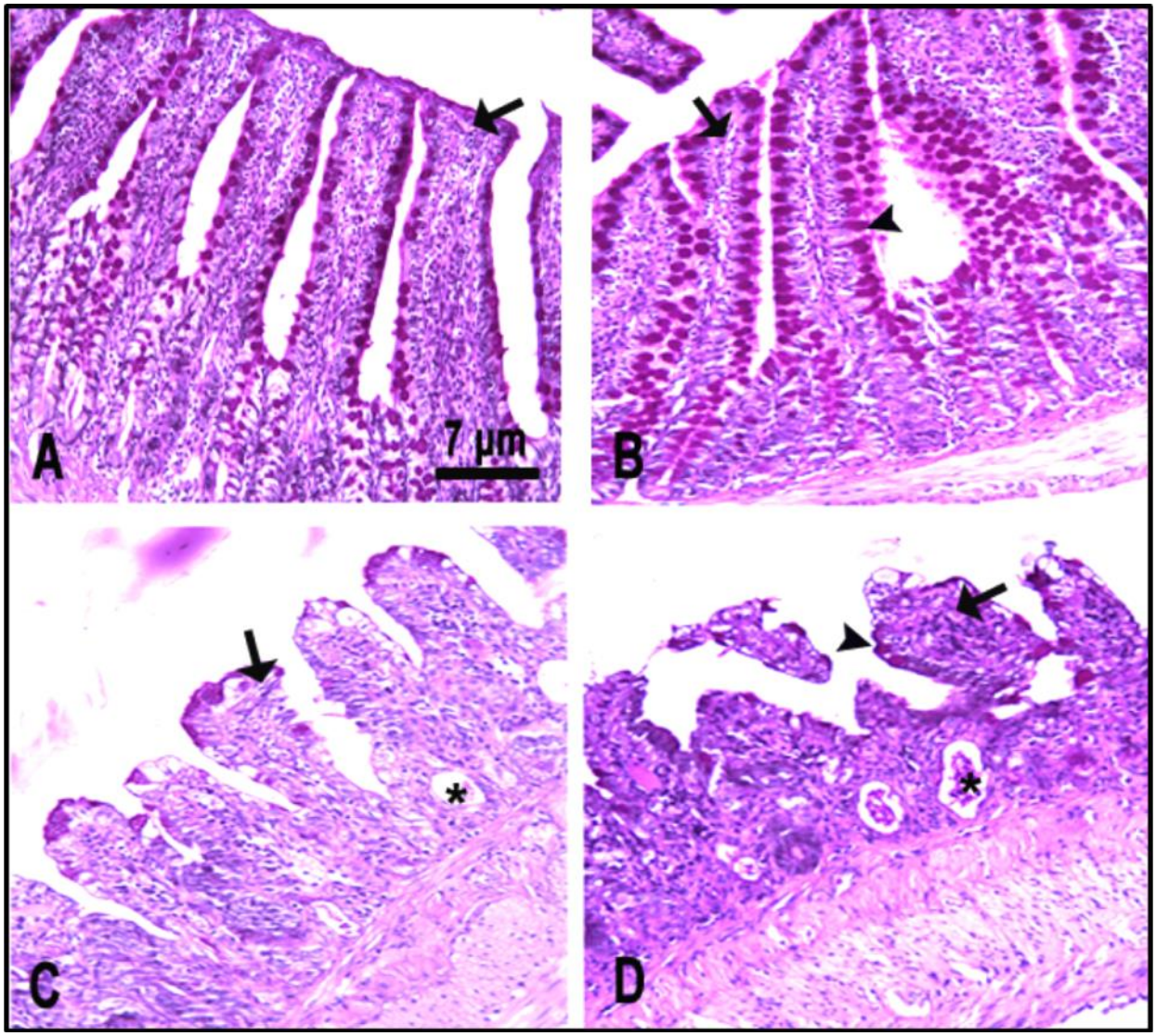

Figure 3.A; Goblet cells were detected as violet in PAS reaction in group 1 (control), **B;** The most prominent finding was intensity of the goblet cells in group 2 (giardia infected), **C;** Goblet cells aren't visible except of villus tips in group 3 (irradiated), **D;** Goblet cell depletion is seen. The secretion of goblet cells and degenerated crypt cell are visible in the lumens of crypt in group 4 (parasite infected+ irradiated). Due to the application of radiation, intestinal damage was more pronounced in group 3 and 4 compared to other groups. (**arrow;** villus, **arrow head;** goblet cell, **asterisk;** vacuolization, **PAS** stain, **X60** magnification).

Histopathological results

Figure 2 and 3 present a summary of histopathological results for each group. In order to examine the histoarchitecture of the intestinal mucosa, the observation of the intestinal sections stained with haematoxylin and eosin (H&E) was performed using a light microscope at 10x magnifications. Moreover, the evaluation of goblet cells population was made using periodic acid shift (PAS) staining. Normal histoarchitecture was observed in the sections of the control group. Staining mucus and the characteristic violet of PAS-stained mucus in obvious goblet cells were observed in the histological slides. In the control group, goblet cell density was observed to be normal in comparison to other experimental groups. Furthermore, the rats in the control group have the normal crypt and a regular villous architecture (Fig 2A-3A). Group 2 infected with *Giardia intestinalis* had a higher goblet cells density compared to the other groups.

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Moreover, mild intestinal injury caused by parasite infection was observed in group 2 (Fig 2B-3B). Regarding histopathologic structure, significant differences were observed in group 3 in comparison to groups 1 and 2. In the group 3, a mucosal damage, such as villus shortening, atrophy of surface epithelium, crypt loss, as well as a decrease in the number of goblet cells was detected as a result of the light microscopic examination (Fig 2C-3C). A high intestinal injury was observed in group 4 in comparison to group 3 (Fig 2D-3D).

DISCUSSION

A number of pelvic malignancies are generally treated with the use of radiotherapy (RT). Radiation therapy has a lot of benefits, however, it has a number of adverse effects, such as toxicity, observed in many patients and caused by the impact of the radiation on the small and large intestine (29). To date, a lot of effort was put in trying to develop enhanced methods of radiation. Nevertheless, in the studies performed recently, severe late effects have been confirmed in patients undergoing pelvic radiation therapy. The gastrointestinal problem is among them (30,31).

Acute small intestine damage, the characteristics of which are the increased apoptosis of crypt epithelial cells and lymphocyte infiltration of the underlying tissue, occurred as a result of radiation (32). The acute diarrhea was caused by inflammatory alterations by various mechanisms, among which the increased gastrointestinal motility, reduced bile acid reabsorption, and the impaired maturation and depletion of villi with denudation of the mucosa took place (33). In the comparison of the groups, a significant negative impact that radiation therapy has on the histologic structure of the intestine was demonstrated as a result of this study. In the assessment of histologic tissues, no negative effect was detected in the control group, however, severe negative effects were observed in the groups to which radiation was applied. Villus shortening, atrophy of surface epithelium, crypt loss, and a decrease in the number of goblet cells are among these adverse effects.

The enhancement of patient response and survival was focused on in the oncology studies performed in the last few decades. As a result, the effects of cancer treatment in the long term have not been considered enough. It is irrational to anticipate that oncologists will have the skill to deal with all adverse effects that cancer treatment has. Nevertheless, since the efficiency of cancer treatment is gradually improved and survival of many cancer patients in the long term is ensured nowadays, oncologists can determine problems caused by cancer treatment in the long term and, therefore, improve the life quality of patients (5).

The reaction to the radiation of ultraviolet light (UV) is considered to be a characteristic stressor to the intestinal protozoan parasite *Giardia intestinalis* (14). Reactive oxygen species (ROS), which may alter the structure of membrane and proteins, can be generated as a result of UV radiation of cells (34). Alterations in goblet cell response and mucin generation occur in various intestinal infections, the causes of which are parasites, bacteria, and viruses. In some infections caused by parasites, hyperplasia of mucin-secreting goblet cells has been detected (35). Moreover, some intestinal protozoan parasites can generate a number of enzymes that degrade mucin and may take part in degrading host mucins and, therefore, help to penetrate the host mucus barrier (36). As a result of the histologic analysis conducted in this study, an increase in the density of the goblet cells in intestinal mucosa was detected in group infected with Giardia intestinalis. Differently from the group infected with Giardia intestinalis, a decrease in the number of goblet cells was detected in the group to which radiation was administered. Furthermore, a score of histologic injury, such as villus shortening, atrophy, vacuolization and desquamation of surface epithelium, crypt loss, alterations in the structure of the crypts and capillary hemorrhage, was found to be high in the histological sections of this group in comparison to other groups in this study. That the application of radiation increases adverse effects on the intestinal tissue, particularly, in the presence of giardia intestinalis, has been confirmed by histologic analysis conducted in detail in the present study.

In a study conducted by Deselliers et al. (1997), they defined an increase in the rate

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of gastrointestinal transit time caused by *Giardia intestinalis* infection (37). Similar findings were obtained in this study as well. The transit time results obtained in this study demonstrated a significant increase in groups 2, 3 and 4 in comparison to the rats in the control group, i.e. group 1. An increase in transit time was found to be similar in a number of other models of intestinal parasitism, for instance, the Trichinella spiralis and Nippostron gylus brasiliensis infections, and the Yersinia enterocolitica model of bacterial enteritis (37). Furthermore, in this study, an increase in the weight loss was observed in the rats in the experimental group in comparison to the rats in the control group. In addition to this, the number of stool pellets was found to be significantly higher in groups 2,3 and 4 than in the control group in the present study which was justified bu the histological images.

The results of this study demonstrate that *Giardia intestinalis* and radiation cause significant changes in the gastrointestinal system. In this study, an increase in the intestinal tissue damage has been observed, particularly in group 4. According to these results, the damage was also observed in the intestinal transit system, weight and stool pellets. Moreover, infection was determined to be the reason for softness and unpleasant smell in the stool specimen.

Gastrointestinal manifestations with a negative impact on the life quality may be encountered more frequently at least 1 year following pelvic radiation therapy, than is widely known (7). Hidden diseases and chemotherapy are among the causes of c*ancer patients' being under risk, and therefore, such patients are significantly affected by various bacterial and fungal infections. At the same time, it should be taken into account that all cancer patients that have infections need special attention. Because of chemotherapy regimes developed recently, the algorithms that are created by experts and societies in this area have to be combined with the local institutional patterns, as well as the pattern of the immunosuppression.* It is believed that, depending on these results, an assessment of medications may convincingly evaluate its capacity to diminish gastrointestinal complication of radiation therapy. According to the opinion of patients, advice given by doctors and alternative practitioners was equitably useful.

CONCLUSION

Many studies have investigated the effects of irridation, and researchers have explored potential associations between the effect of exposure to irridation and the functioning of the digestive system. Irradation emitted from cell cause biological damage in a number of organs, including intestine. Our findings indicate a significant damage in intestinal mucosa. A solution must now be found to the deleterious effects of irradiation. Animal studies should also focus on the potential effects of irradiation exposure.

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Declaration of interest
The authors declare that they have no conflict of interest related to the content of this manuscript, including employment or personal financial interests.

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