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INVESTIGATION OF ZONULA OCCLUDENS-1 PROTEIN LEVEL IN RECTUM TISSUE AFTER RADIOTHERAPY

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ABSTRACT

The transmembrane protein zonula occludens of rectal tissue has function to prevents the spread of bacterial toxins into the intestinal mucosa and to systemic circulation. But radiotherapy causes ablation of crypt cell proliferation, mitotic catastrophe, and apoptosis leading to gastrointestinal mucositis. We investigated the acute radiation effect on gastrointestinal mucosa of rectum tissue thickness with immunohistochemistry method for zonula occludens-1 (ZO-1) protein in animal model.

A total of 24 healthy Swiss Albino mice were divided into 4 groups, and except control group the groups of 1–3 was exposed to 500 cGy total body irradiation. All rectum tissue samples were taken from the groups of control, 24 h, 72 h, and 168 h after irradiation and stained with hematoxylin and eosin for histochemical examination, and for immunohistochemical staining with anti ZO-1 polyclonal antibody.

We observed edema especially in the groups 2 and 3 but not in group 1. Immunohistochemical examination of staining of rectum tissue samples for ZO-1 showed poor staining for control group (1.48 ± 0.06) and group 1 (1.38 ± 0.09) and group 2 (1.50 ± 0.01) but the group 3 (2.12 ± 0.04) samples showed moderate ZO-1 immunostaining. It was found that the amount and thickness of ZO-1 expression increased in the late period for more than 24 hours. The comparison of the values of ZO-1 between the group 3 which is the group in the late period after radiation exposure and control group or group 1 or group 2, showed statistically significant differences ($p < 0.001$).

It was concluded that ZO-1 protein may have a role in the side effects of radiation injury, and the understanding of cellular and molecular activity will help us to develop pharmacological modulators to mitigate or treat the injury.

1. INTRODUCTION

The radiotherapy (RT) is a widely used method in cancer treatment. The main mechanism of action of ionizing rays used in radiotherapy is to stop the proliferation of cells by causing DNA damage [1]. The normal tissues are effacted with the radiation used to destroy cancer treatment [2]. The gastrointestinal tract is one of the radiation-sensitive organs in the body and is characterized by gastrointestinal (GI) complications of radiation in the early stages such as nausea and diarrhea [3]. Endotoxemia and bacteremia may occur in septicemia at a later stage [4], [5], [6], [7], [8]. Intestinal injury occurs in patients receiving abdominal or pelvic radiotherapy and it is characterized by villous atrophy, mucosal edema, ulceration and increased mucosal permeability [9]. Enteral inflammation induced by radiotherapy increases bacterial translocation towards mesenteric lymph nodes [10].

Total body irradiation (TBI) causes ablation of crypt cell proliferation, mitotic catastrophe, and apoptosis leading to gastrointestinal mucositis [11]. TBI causes fatal gastrointestinal injury in high doses such as 14-18 Gy in 7–10 days in mice [12]. In the mouse model, low doses of TBI such as 3–7.5 Gy result in temporary injury to the tight junction between epithelial cells of the intestinal mucosal lining [13].

The function of the epithelial barrier is the first line of defense in the gastrointestinal tract that prevents the spread of bacterial toxins into the intestinal mucosa and into the systemic circulation. The tight junctions (TJ), the highly specialized intercellular junctions, confer epithelial barrier function in the gastrointestinal tract [14]. The TJ, which form most units of apical, define the boundary between apical and basolateral membranes, and are often the speed-limiting factor in the paracellular passage [15]. The TJ has multiprotein complexes made up of both transmembrane proteins such as occludin, tricellulin, different claudins and junctional adhesion molecules (JAMs), as well as peripheral membrane proteins such as zonula occludens (ZO)-1,-2,-3 and cingulin [16], [17].

We investigated the acute radiation effect on gastrointestinal mucosa of rectal tissue with immunohistochemistry method for ZO-1 protein in animal model.

2. MATERIALS AND METHODS

2.1. ANIMAL EXPERIMENTS

A total of 24 healthy, 6-8 weeks old, male, adult Swiss Albino mice, weighing 25-35 g, were obtained from the Ankara University Experimental Animal Laboratory and used as subjects. The subjects were isolated from stress and noise and fed with water and food ad libitum at 25°C in a cycle of 12 h/12 h dark/light conditions before being included in the study. Care of the animals was performed at the Ankara University Experimental Animal Laboratory throughout and the study was approved by the Animal Tests Local Ethics Committee of Ankara University (approval number 2017-21-166, approval date: 10/18/2017). The mice were divided into 4 groups, each containing 6 mice. Except the control group, the mice in the experimental Groups 1–3 were exposed to total body irradiation (TBI) with 6 MV photon using a Varian linear accelerator device present in the Department of Radiation Oncology of Ankara University School of Medicine, with a source-to-axis distance of 100 cm, from anterior (250 cGy) and posterior (250 cGy) fields, and a total dose of 500 cGy for mid-axis in a single fraction. All mice in the experimental group 1-3 were injected 45-50 mg/kg intramuscular Ketamine before TBI to provide sedation during irradiation. The unexposed mice in control group were subjected to euthanasia after 45-50 mg/kg intramuscular ketamine injection, while the mice in Groups 1-3 were sedated with 45-50 mg/kg intramuscular ketamine injection 24 h, 72 h, and 168 h after TBI, respectively, and then subjected to euthanasia. The euthanasia was performed by the method of cardiac perfusion. After euthanasia, the pelvic region was dissected and the rectum was completely removed. The tissue samples obtained were embedded into paraffin at 60°C following the routine light microscopy paraffin tissue method. All rectum tissue samples were first washed in a solution containing 10% formol and then placed in screw-cap sampling containers containing 10% formol, with separate boxes used for every animal. Two sets of serial sections (5-µm thick) were cut and prepared – the first set was stained with Hematoxylin and Eosin (HE) for histochemical examination, while the second set of sections were used for immunohistochemical staining as described below.

2.2. IMMUNOHISTOCHEMISTRY

Formalin-fixed, paraffin-embedded rectum sections were used for immunohistochemical staining. Tissue samples were stored at 60°C overnight and then de-waxed with xylene for 30 min. After dehydration of the sections with ethanol, they were washed with distilled water. Subsequently, the samples were treated with 2% trypsin (ab970, Abcam, Cambridge, UK) at 37°C for 15 min and incubated in 3% H_2O_2 solution for 15 min to inhibit endogenous peroxidase activity. Then, the sections were incubated with anti-ZO-1 polyclonal antibody (ab216880, Cambridge, UK) in a 1/100 dilution for 18 h at 4°C. They were then given an additional three 5-min washes in PBS, followed by incubation with biotinylated IgG and administration of streptavidin peroxidase (Histostain Plus kit Zymed 87-9999, Zymed, San Francisco, CA, USA). After washing the secondary antibody with PBS three times for 5 min, the sections were stained with DAB Substrate system containing diaminobenzidine (DAB, K007, DBS, Pleasanton, CA, USA) to detect the immunoreactivity, and then stained with Mayer's hematoxylin (72804E, Microm, Walldorf, Germany) for counterstaining. For positive and negative controls, a mouse rectum was subjected to the same procedure; however, normal IgG in place of primary antibody was used as a negative control. All samples were then covered with a mounting medium (01730 Surgipath, Cambridge, UK) and observed with light microscopy (Olympus BX-40, Tokyo, Japan).

Immunostaining for ZO-1 expression in the rectum samples were evaluated semi quantitatively using H-SCORE analysis [18]. The immunostaining intensities were scored as follows: 0 (no staining), 1 (weak, but detectable staining), 2 (moderate staining), and 3 (intense staining). A H-SCORE value was derived for each specimen by calculating the sum of the percentage of the rectum cells that stained at each intensity category multiplied by its respective score, using the formula H-SCORE = $\sum$Pi (i+1), where i is the intensity score, with a value of 1, 2, or 3 corresponding to weak, moderate or strong staining respectively, and Pi is the percentage of stained cells for each intensity, varying from 0 to 100%. For each slide, five different fields were evaluated microscopically at 200× magnification. H-SCORE evaluation was performed independently by at least two investigators (KO, SG) blinded to the source of the samples as well as to each other's results; the average score obtained by both was considered.

2.3. STATISTICAL ANALYSIS

All statistical analyses were performed using IBM, SPSS for Windows version 20.0 (IBM Corp, Armonk, NY, USA). Kolmogorov-Smirnov tests were used to test the normality of data distribution. Continuous variables were expressed as mean ± standard deviation, median (25^{th}-75^{th} percentiles), and categorical variables were expressed as counts (percentages). Comparisons of non-normally distributed continuous variables between the groups were performed using the Kruskal Wallis one-way analysis of variance and Dunn's Post Hoc test. A two-sided *p* value < 0.05 was considered statistically significant.

3. RESULTS

Examination of the HE stained preparations of rectum samples revealed that; rectum is a hollow tube composed of four distinctive layers: mucosa, submucosa, muscularis externa, and serosa. The mucosa of the rectum has, simple columnar epithelium having straight, tubular intestinal glands with many goblet cells. In the submucosa, we observed light edema in experimental groups, especially in Groups 2 and 3 but not Group 1 (Figure 1)

Immunohistochemical examination of rectum tissue samples for ZO-1 showed different staining intensities in the rectum mucosa of four different groups (Figure 2). For each sample, the percentage of rectum cells stained in each density category was calculated by multiplying with the corresponding density to obtain an H-SCORE value. The values for each group are given in Table 1. We observed poor staining for control group (1.48 ± 0.06), and group 1 (1.38 ± 0.09). The group 2 (1.50 ± 0.01) and group 3 (2.12 ± 0.04) samples showed moderate ZO-1 immunostaining

We noticed that, 24 hours after radiotherapy the staining amount and thickness of ZO-1 decreased from 1.44 to 1.38 because of the destructive effect of radiotherapy. Then, because of the repair and protective effect on rectum tissue we noticed increase to 1.5 at 72 hours and then to 2.12 at 168 hours after radiotherapy, respectively.

The comparison of the H-SCORE values of ZO-1 positive cells between the control group and the late exposure to radiation groups was statistically significant (control group versus group 3; $p<0.001$). The expression of ZO-1 in the rectum could not be demonstrated in relation to early exposure to radiation. On the other hand, it was observed

that the amount of expression increased in the late period for more than 72 hours (Group 3 versus groups 1-2; $p < 0.001$). No significant difference was observed between the control group versus 24 hours group 1, and between the control group versus 72 hours group 2, also between group 1 versus group 2. For ZO-1 positive cells, the main statistically significant difference was seen between 168 hours group 3 versus other groups ($p < 0.001$).

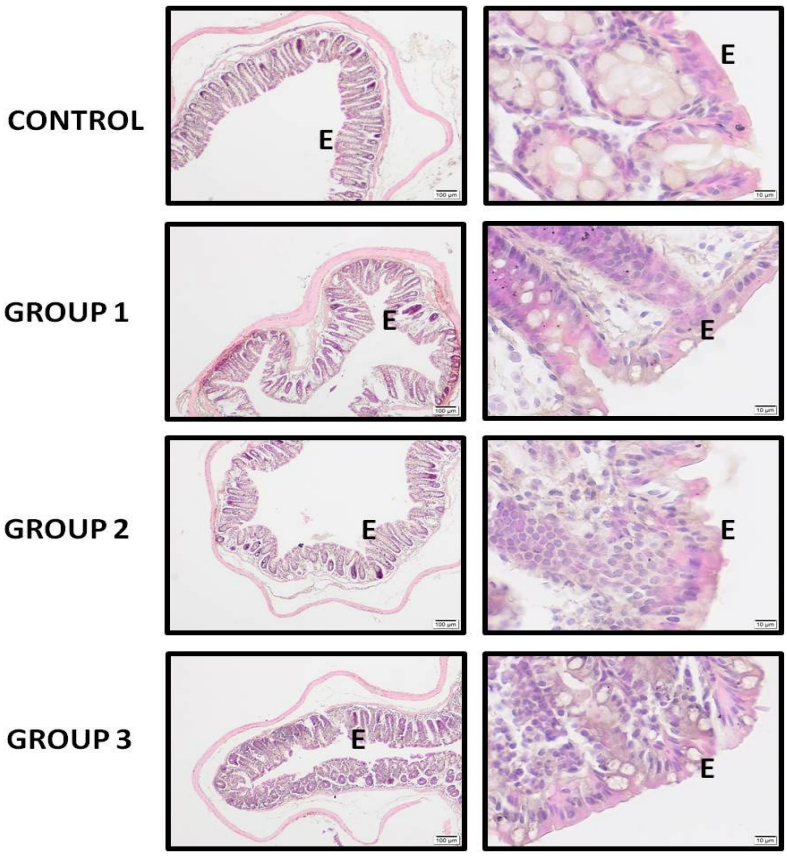

Figure 1: Hematoxylin-Eosin staining properties of the rectum were seen. The simple columnar epithelium (E) is lined the rectum surface. Light edema were seen especially in the group 2 and 3

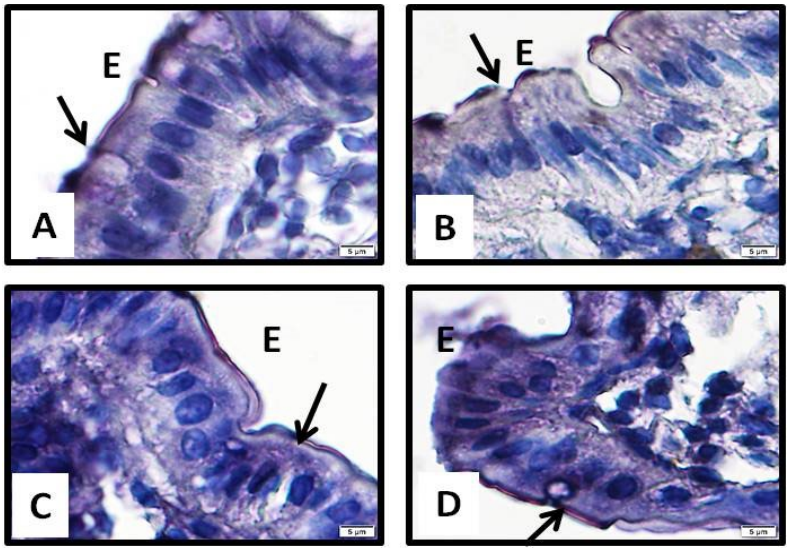

Figure 2: ZO-1 thickness of the rectum with ZO-1 antibody in Control (A), Group 1 (B), Group 2 (C), and Group 3 (D). There were no differences between control and both group1 and 2. However the thickness of ZO-1 was increased in Group 3 compared the control group. E: epithelium.

Table 1: The thickness of ZO-1 in Groups and statistical analyses.

	CONTROL	GROUP 1	GROUP 2	GROUP 3
	1,6	1,4	1	1,6
	1,4	1,4	1,6	2
	1,8	1,4	1,4	2,4
	1,4	1,8	1,2	2,2
	1,4	1,6	1,6	2,4
	1,6	1	1,4	2,2
	1,2	0,8	1	2,2
	1,4	1,6	2	2
	1,8	1,4	1,8	2
	1,2	1,4	2	2,2
1,6 mean±SD *P<0.001	1,48±0.06	1,38±0.09	1,5±00,1	2,12±0,04*

4. DISCUSSION

The function of the epithelial barrier is important because it is the first line of defense in the GI channel that prevents the spreading of bacterial toxins from the intestinal mucosa into the systemic circulation [9], [10]. ZO-1 is one of the important peripheral components of the TJ complex [16], [17].

The side effects of GI secondary to radiotherapy appear in a wide spectrum from mild symptoms to mortal effects. Total body irradiation (TBI), crypt cell proliferation ablation, mitotic catastrophe and; GI leads to mucositis [16]. In the mouse model, high doses of TBI (14 - 18 Gy) cause fatal gastrointestinal damage [17]; low TBI doses (3 - 7.5 Gy) have been observed to cause temporary injuries in tight junctions (TJ) between intestinal mucosal epithelial cells [11]. In our study; 24 hours after radiotherapy the amount and thickness of ZO-1 decreased from 1.44 to 1.38 because of the destructive effect of radiotherapy, but increased to 1.5 at 72 hours and then to 2.12 at 168 hours after radiotherapy because of the repair and protective effect on rectum tissue.

Abdominal RT applications are known to affect intestinal epithelial cells and cause death, hypoplasia and ulcerative lesions by pouring cells. The mechanism of GI side effects observed in the RT result is not fully known. However, it can be suggested that epithelial barrier disorder may play an important role in the formation of these side effects. Only one study has been found in the literature. In this study; In mice treated with ionizing radiotherapy, damage was observed in ZO-1 at 2 and 24 hours after radiotherapy [18]. In our study, ZO-1 staining measured in the apical region of the rectum epithelial cells after RT was observed to be the same as the control group after 24 hours. This result was evaluated as an indication that the effect of RT on TJ complexes was observed after 24 hours. It was thought that ZO-1 staining increased on 3rd and 7th days after RT application and it could be associated with proliferation. In the previous study, it has been reported that the increase in TJ complex's proteins has emerged as a response to barrier destruction [18].

The ZO-1 and occludin levels in mouse sperm cells were examined after 100 cGy radiotherapy that a significant decrease and irregular immunolocalization were observed at 3 and 6 months [19].

In the murine model assay, the relationship of other proteins constituting the TJ complex with radiotherapy was increased. On the 4th and 12th day after the exposure to radiation, the occludin level increased. An increase was observed in JAM-1 on day 7. Exposure to radiation caused a reduction in the amount of E-cadherin, but did not significantly affect the amount of fragmented caspase-3 [20].

5. CONCLUSION

It was observed that RT started to affect ZO-1 expression in the rectum epithelial cells after 24 hours, and the effect was observed on day 3, and its effect continued in 7 days. It was concluded that ZO-1 protein may have a role in the side effects of RT on the intestine. Unraveling the cellular and molecular activity in response to intestinal radiation injury will help understand the mechanisms involved and develop pharmacological modulators to mitigate or treat the injury.

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CONFLICT OF INTEREST

The author have declared that no competing interests exist.

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