RESEARCH ARTICLE / Araştırma Makalesi

Amifostine Protects Small Bowel Against Radiation-induced Apopitosis by Reducing Caspase-3

Amifostin İnce Bağırsakları Kaspaz-3'ü Azaltarak Radyasyonun İndüklediği Apopitozise Karşı Korur

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Abstract

Aim The aim of the study was to investigate the protective effect of amifostine against radiotherapy-induced small bowel injury.
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Methods Forty rats were divided equally into four groups as control, irradiation (IR), IR + Amifostine, IR+N-acetyl cysteine (NAC) groups. Caspase-3 expression, villus lengths, and microscopic tissue injury were evaluated histopathologically. Superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), myeloperoxidase (MPO) and malondialdehyde (MDA) were measured biochemically in jejunum tissue. Pre- and post-radiation weights of the rats were recorded.

Results Caspase-3 was at the highest level in the IR group, at the lowest level in the control and weak in the Amifostine+IR group ($p<0.001$). The lowest microscopic score was determined in the IR group and the difference between the groups was statistically significant ($p<0.001$). There were significant differences in MPO, CAT and MDA concentrations between groups in the jejunum tissue ($p=0.001$, $p=0.034$, $p=0.032$, respectively). In Amifostine+IR group, the villus length was significantly longer than that of the IR group ($p=0.001$). Amifostine was also observed to protect against weight loss.

Conclusion Administration of Amifostine can substantially reduce apopitosis and support the repair of the structure and function of intestinal tissues which have been damaged by exposure to radiation. These results suggest that Amifostine may be a promising therapeutic agent against radiotherapy-induced small bowel injury.

Keywords Amifostine; Apoptosis; Caspase-3; Oxidative stress; Radiotherapy

Öz

Amaç Bu çalışmanın amacı, amifostinin radyoterapiye bağlı ince bağırsak hasarına karşı koruyucu etkisini araştırmaktır.
(Sakarya Tıp Dergisi 2018, 8(3):611-619).

Yöntem Kırk rat, kontrol, radyasyon (IR), IR + Amifostin, IR + N-asetil sistein (NAC) grupları olmak üzere dört gruba eşit olarak ayrıldı. Kaspaz-3 ekspresyonu, villus uzunlukları ve mikroskobik doku hasarı histopatolojik olarak değerlendirildi. Jejunum dokusunda süperoksit dismutaz (SOD), katalaz (CAT), glutatyon peroksidaz (GPx), miyeloperoksidaz (MPO) ve malondialdehid (MDA) biyokimyasal olarak ölçüldü. Ratların radyasyon öncesi ve sonrası ağırlıkları kaydedildi.

Bulgular Kaspaz-3, IR grubunda en yüksek düzeyde, kontrol grubunda en düşük düzeydeydi ve Amifostin + IR grubunda zayıftı ($p<0.001$). En düşük mikroskopik skor IR grubunda gözlendi ve gruplar arasındaki fark istatistiksel olarak anlamlıydı ($p<0.001$). Jejunum dokusunda MPO, CAT ve MDA düzeyleri arasında istatistiksel olarak anlamlı farklılık vardı (sırasıyla, $p=0.001$, $p=0.034$, $p=0.032$). Amifostin + IR grubunda, villus uzunluğu, IR grubundan anlamlı derecede daha uzundu ($p=0.001$). Amifostinin kilo kaybına karşı da koruduğu gözlendi.

Sonuç Amifostin uygulaması apopitozu büyük ölçüde azaltabilir ve radyasyona maruziyet sonrası zarar gören bağırsak dokularının yapısını ve işlevini tekrar kazandırabilir. Bu sonuçlar, Amifostinin radyoterapiye bağlı ince bağırsak hasarına karşı umut verici bir terapötik ajan olabileceğini düşündürmektedir.

Anahtar Kelimeler Amifostin; Apopitoz; Kaspaz-3; Oksidatif stress; Radyoterapi

Introduction

Radiation therapy (RT) uses ionizing radiation (IR), which is delivered to destroy tumor cells. The aim of radiation therapy is to provide effective treatment of cancer with as few side-effects as possible. IR can cause a wide of range DNA damage, radiolytic hydrolysis, oxidation of lipids and proteins, production of reactive oxygen species (ROS), and an alteration in the pro-oxidant/antioxidant balance.[1] It is also likely to induce various forms of cancer cell death through multiple molecular mechanisms, including apoptosis, necrosis, and autophagy. Apoptosis may be triggered by direct activation of caspases, which have a key role in all apoptotic pathways (extrinsic, intrinsic, perforin /granzyme pathways) and is also affected by ROS.[2]

Following radiation therapy, IR interacts with biological systems to stimulate ROS, which assault cellular constituents including DNA, proteins, and lipids. The ROS negatively impact the antioxidant defensive processes, reducing the level of glutathione (GSH) superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx).[3] Myeloperoxidase (MPO) is the most abundant pro-inflammatory enzyme which catalyzes the formation of hypochlorous acid from hydrogen peroxide (H2O2).[4] Another of the biomarkers of oxidative deterioration is the formation of malondialdehyde (MDA) for lipid peroxidation.[5]

The intestines are highly sensitive to RT and with dose-dependent toxicity; radiation colitis frequently develops in the intestines. Although RT is known to lead to mucosal destruction, the effect mechanism is not clear.[6] Besides dosimetric modulation of the small bowel volume, pharmacological intervention has also been taken into consideration for the mitigation of small bowel toxicities. Radio-protectors are chemical compounds which protect cells against radiation-induced toxicity. They include sulfhydryl compounds, antioxidants, immuno-modulators, and other components. Thiol forms the effective class of radioprotection compounds.[7] Amifostine [S-2-(3-Aminopropylamino) ethylphosphorothioic acid (WR2721)] is used for antioxidant scavenging oxygen-free radicals caused by radiation. However, there are limited studies examining the effect of amifostin radiation-induced intestinal injury.

The aim of the present study was to investigate the protective effect and mechanism of Amifostine against radiotherapy-induced apoptosis, lipid peroxidation and antioxidant status in intestinal injury in Wistar albino rats. The events occurring after radiation that are responsible for the injury to normal tissue are described and discussed in the evaluations of preventing or treating normal tissue injury.

Materials and Methods

Animals and Experimental Design

This experimental study was carried out between December 2017 and March 2018 at Kahramanmaraş Sutcu Imam University and approved by The Animal Experiments Local Ethics Committee. The experiments were carried out on 40 female Wistar albino rats, each weighing 250-300 g, aged 8-12 weeks old. All the animals were housed in an environmentally controlled room (23-24 0C temperature, 50% to 60% humidity) with a 12:12 hour light/dark daily cycle, and were fed on commercial rat chow and tap water ad libitum. The rats were randomly divided into 4 groups as Group 1 (control, n=10), Group 2 (IR, n=10), Group 3 (NAC+ IR, n=10) and Group 4 (Amifostine+ IR, n=10).

The rats were treated with Amifostine (Er-Kim Corporation, Turkey) at a dose of 200 mg/kg on the second day in group 4 via an intraperitoneal injection 30 min before IR. The rats were treated with NAC (Husnu Arsan Chemical Co. Turkey) at a dose of 1000 mg/kg every day for 5 days in group 3 via an intraperitoneal injection.

Irradiation of Animals

Prior to IR, the rats were anesthesised using ketamine at a dose of 50 mg/kg and xylazine at a dose of 5mg/kg via an intraperitoneal injection on the 2nd day. Total abdomen irradiation was applied with 6 MV X-ray beams using a standard linear accelerator (Trilogy, Varian Medical Systems, Palo Alto, CA). The rats were irradiated with a single fraction total 12 Gy on the second day. The radiation dose was delivered with the gantry set at 0° and with the gantry at 180°. The dose rate was 200 MU/min.

Immediately after IR, 2 rats from Group 3 and 1 rat from Group 4 died. The remaining rats were sacrificed on the 5th day. Blood samples of 5 ml were collected from intracardiac blood and jejunum samples were collected under sterile conditions.

Histopathological Procedures

After surgery, the intestinal specimens were removed and placed in 10 % formalin for 24 hrs then dehydrated in ascending alcohol series and embedded in paraffin for light microscopic analysis. Sections of 3.5 microns thickness were stained with hematoxylin and eosin (HE) for double-blind light microscopy studies for general morphology. The intestinal damage was evaluated with a histopathological scoring system. The assessment was expressed as the sum of the individual score grades from 0 to 4, depending upon the severity of changes (0: Regular morphology, 1: Subepithelial congestion, slight cellular desquamation at villus tips, 2: Congestion in mucosa, loss of less than half of the villus, 3: Loss of more than a half of the villus, 4: Degeneration extending to submucosa). Villus lengths were measured and the mean score was calculated with NIS-Elements D 4.6 (Nikon Corporation, Japan) imaging software in 10 villi at x10 magnification. Slides were transferred to polylysine-coated slides for immunohistochemical analysis. Caspase-3 (Abcam Cambridge, USA) was evaluated according to the criteria of distribution and intensity which could range from 0 to 4. (0: negative, 1: <50% or/and weak immunostaining, 2: <50% and moderate/strong immunostaining, 3: >50% and weak immunostaining, 4: >50% and moderate/strong immunostaining)
Biochemical Procedures

SOD, GPx, MPO, CAT and MDA measurements For Jejunum Tissue

SOD activity was measured using the Fridovich method for protein peroxidation and expressed as units per milligram protein (U/mg).[8] GPx activity was measured spectrophotometrically with the Beutler method.[9] GPx outcomes were expressed in units per gram protein (U/mg).[10] MPO activity was measured with a modification of the O-dianisidine method. The activity of MPO was defined as that giving an increase in absorbance of 0.001per min and specific activity was stated as U/L.[11] CAT activity was determined by measuring the degradation rate of H2O2 using the Beutler method and the CAT result was expressed in units per milliliter (U/ml) protein.[9] The concentration of MDA was assayed based on the method of Ohkawa with minor modifications for lipid peroxidation. MDA results were expressed in nanomoles per milliliter (nmol/ml).[12]Tissue results were expressed in nanomoles per milligram protein (nmol/mg prot.). Tissue protein was measured according to

Lowry.[13]

Statistical analysis

Group comparisons of variables showing normal distribution were examined with the Shapiro-Wilk test and those not showing normal distribution with the Kruskal- Wallis H-test. Post-hoc paired comparisons were made with the Dunn test. Data with normal distribution were analyzed with One-way analysis of variance (ANOVA) followed by the Tukey post hoc test. Correlations between variables were examined with Spearman's correlation test. Fisher's exact test and the Wilcoxon test were used for the assessment of histopathological parameters. The data were analyzed using SPSS Software (SPSS, 22.0 version, Chicago, USA). The level of statistical significance was set at $p< 0.05$.

Results

Histopathological results

Caspase-3 immunostaining was observed on all jejunum tissue slides. According to these data, the immunohistochemical expression of caspase-3 was at the highest level in the IR group and at the lowest level in the control group. The immunohistochemical expression of caspase-3 was weak in the Amifostine+IR group compared to the IR group. The difference between the groups in respect of caspase-3 staining was statistically significant ($p<0.001$). (Table 1, Figure 1). The distribution of the groups according to the microscopic scores is also shown in Table 1. The lowest score was determined in the IR group and the difference between the groups was statistically significant ($p<0.001$).

Table 1. Caspase-3 and microscopic score distribution between control, irradiation, NAC+IR and Amifostine+IR groups.

		GROUPS								
		Control		IR		NAC+IR		Amifostine+IR		
		n	%	n	%	n	%	n	%	
Caspase-3[a]	1,00	10	100,0	0	0,0	0	0,0	4	44,4	p<0,001*
	2,00	0	0,0	0	0,0	4	50,0	4	44,4	
	3,00	0	0,0	5	50,0	3	37,5	1	11,1	
	4,00	0	0,0	5	50,0	1	12,5	0	0,0	
Microscopic score[a]	0,00	10	100,0	0	0,0	0	0,0	0	0,0	p<0,001*
	1,00	0	0,0	0	0,0	0	0,0	0	0,0	
	2,00	0	0,0	2	20,0	5	62,5	6	66,7	
	3,00	0	0,0	6	60,0	3	37,5	3	33,3	
	4,00	0	0,0	2	20,0	0	0,0	0	0,0	

[a]Fisher exact test; a<0,05; *Distribution of groups are statistically significant. IR:Irradiation, NAC: N-acetylcysteine

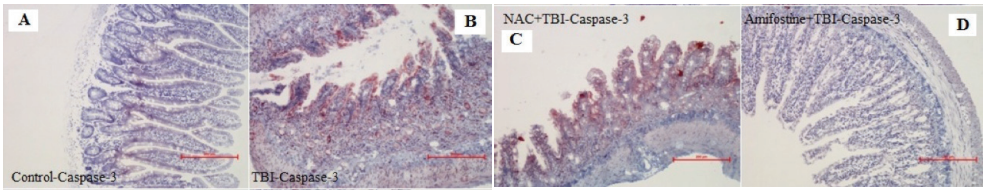

Figure 1: Caspase-3 stained section of rat intestine (Immunohistochemistry, x10 magnification) A: Control group, B: Irradiated with no treatment group, C: Irradiated and treated with NAC group, D: Irradiated and treated with Amifostine group

According to the HE stained slides, villus length was determined to be statistically significantly different (p=0.001). Under microscopic observation, normal jejunum structures and normal villus length were visible in the control group. The shortest villus lengths were seen on the IR groups. However, in the Amifostine+IR group, the villus length was significantly longer than that of the IR group (Table 2 and Figure 2). In accordance with these findings, Amifostine was observed to protect against weight loss (p<0.001) There was a significant difference between the groups in respect of bodyweight at the end of the experiment (p=0.002).

Table 2. Lose weight and villus length comparison between control, irradiation, NAC+IR and Amifostine+IR groups.

	Control	IR	NAC+IR	Amifostine+IR	p
Weight Before IR [e] Median (Min-Max)	270,00 (264,00-275,00)	267,50 (266,00-269,00)	270,50 (260,00-281,00)	270,00 (260,00-285,00)	0,467
Weight After IR[e] Median (Min-Max)	-	200 (185-207)[d]	206 (190-235)	230 (200-251)[b]	0,002*
Lose Weight[e] Median (Min-Max)	0,00 (0,00-0,00)[b,c]	68,00 (61,00-83,00)[a]	64,00 (31,00-84,00)[a]	30,00 (20,00-80,00)	p<0,001*
Villus Lengh[f] Mean ±SD	383,80±39,37[b,c,d]	166,80±29,40[a,d]	189,50±15,96[a]	227,44±27,25[a,b]	0,001*

eKruskal-Wallis H test; Post-Hoc: Dunn test; fOne-Way Anova; Post-Hoc: Tukey test : 0,05; *Difference is statistically significant; aDifference with Control Group is statistically significant; bDifference with IR Group is statistically significant; cDifference with NAC+IR Group is statistically significant; dDifference with Amifostine+ IR Group is statistically significant. IR:Irradiation, NAC: N-acetylcysteine.

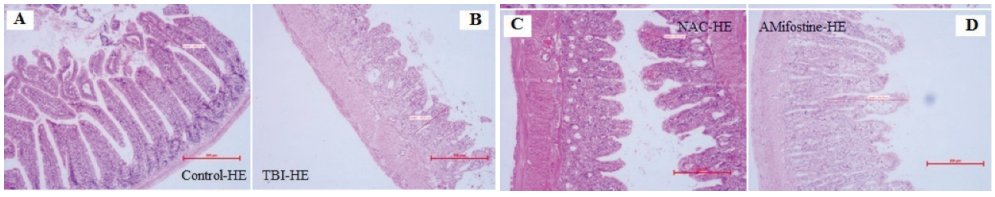

Figure 2: Intestinal cell morphology in irradiated rats. Representative images showing the villus length and tissue damage in the small intestine stained with Hematoxylin and eosin (x10 magnification).A: Control group, B: Irradiated with no treatment; severe tissue damage and villus length loss, C: Irradiated and treated with NAC; moderate tissue damage and villus length loss better than the group with no treatment, D: Irradiated and treated with Amifostine; tissue damage and villus length significantly improved compared to the other irradiated groups.

Biochemical results

The results of the biochemical analyses are shown in Table 3.

Table 3. Comparison of groups according to superoxide dismutase, glutathione peroxidase, myeloperoxidase, catalase, and malondialdehyde.

	Control	IR	NAC+IR	Amifostine+IR	p
SOD[e] Median (Min-Max)	791,10 (239,01-16501,35)	720,83 (421,06-999,02)	603,67 (309,40-1198,07)	671,79 (520,20-993,98)	0,441
GPx[e] Median (Min-Max)	49,95 (3,12-1464,22)	59,49 (0,00-201,74)	126,51 (9,50-549,58)	23,12 (0,00-1800,64)	0,288
MPO[e] Median (Min-Max)	1337,69 (415,94-34811,20)[b,d]	224,83 (13,49-453,71)[a]	706,78 (214,78-1579,56)	495,33 (168,14-1149,75)[a]	0,001*
CAT[e] Median (Min-Max)	275,30 (23,89-5770,49)[b]	91,25 (0,00-224,21)[a]	125,41 (17,49-377,16)	164,51 (40,64-556,80)	0,034*
MDA[e] Median (Min-Max)	204,76 (86,65-5947,75)	261,03 (225,47-352,26)[c]	173,52 (126,52-313,15)[b]	194,36 (111,40-369,55)	0,032*

[e]Kruskal-Wallis H test; Post-Hoc: Dunn test : 0,05; *Difference is statistically significant; [a]Difference with Control Group is statistically significant; [b]Difference with IR Group is statistically significant; [c]Difference with NAC+ IR Group is statistically significant; [d]Difference with Amifostine+ IR Group is statistically significant. SOD: Superoxide dismutase, GPx: Glutathione peroxidase, MPO: Myeloperoxidase CAT: Catalase, MAD: Malondialdehyde IR:Irradiation, NAC: N-acetylcysteine.

There were significant differences in MPO, CAT and MDA concentrations in the jejunum tissue. According to these parameters, Amifostine+IR ve IR grup had a lower level of MPO compared to the control group (p=0.001). After irradiation the level of catalase decreased. However, Amifostine and NAC prevented radiation-induced catalase decline (p=0.034). Treatment with NAC reduced the MDA level, which was increased due to radiation (p=0.032). No relationship was determined between the other tissue biochemical parameters and weight loss or villus length (Table 4).

Table 4. Correlation test for villus length and lose weight in groups relationship intestinal tissue SOD, GPx, MPO, CAT, and MDA levels.

		Control		IR		NAC+ IR		Amifostine+ IR	
		Villus length	Lose Weight	Villus length	Lose Weight	Villus length	Lose Weight	Villus length	Lose Weight
SOD	r	-,442	.	,091	,107	,108	-,262	,183	,653
	p	,200	.	,803	,769	,799	,531	,637	,057
GPx	r	,224	.	-,455	-,132	-,323	-,643	-,184	,429
	p	,533	.	,187	,717	,435	,086	,635	,250
MPO	r	-,188	.	-,212	-,496	-,144	-,071	-,217	,201
	p	,603	.	,556	,145	,734	,867	,576	,604
CAT	r	,200	.	,091	-,031	-,108	,167	-,450	-,552
	p	,580	.	,803	,931	,799	,693	,224	,123
MDA	r	-,224	.	-,212	,144	-,443	-,500	,450	,510
		,533	.	,556	,691	,272	,207	,224	,160

Spearman Correlation test; a:0,05; * Correlation is statistically significant; r:Correlation coefficient. SOD: Superoxide dismutase, GPx: Glutathione peroxidase, MPO: Myeloperoxidase, CAT: Catalase, MAD: malondialdehyde, IR: Irradiation, NAC: N-acetylcysteine.

Discussion

RT is a type of cancer therapy used clinically in which the processes use ionizing radiation leading to cell death through activation of apoptosis. The aim of cancer therapy is to destroy cancerous cells while preventing damage to healthy cells. However, IR can lead to DNA damage, radiolytic hydrolysis, oxidation of lipids and proteins, production of reactive oxygen species (ROS), and alterations in the pro-oxidant/antioxidant balance.[1] ROS are known to trigger oxidative stress and apoptosis is thought to be the major underlying factor in the pathogenesis of radiation injury.

Amifostine is the only radioprotective agent that is approved by the Food and Drug Administration (FDA).[14] However, there are a few studies investigating radioprotective effect of Amifostine on small intestine. Small bowel toxicities are often encountered during radiotherapy in patients who have pelvic malignancies, such as those in the uterus, rectum, bladder, or prostate. Moreover, some clinical findings suggest that the late effects of irradiation may be exacerbated due to acute effects.[15] This implies that preventing small bowel toxicities during pelvic radiotherapy is of great importance. In the current study we investigated the effect of Amifostine against radiotherapy-induced intestinal injury. This study was a quite comprehensive study including caspase-3, lipid peroxidation, protein oxidation, microscopic score, and length and body weight to show the effects of Amifostine on radiation-induced intestinal injury.

Only at radiation doses less than 15 Gy are the radioprotective drugs effective.[16,17] Therefore, similar to the other studies investigating the effect of radioprotectors on the small intestine, we

determined the dose to be 12 Gy to the total abdomen.[18] Considering its reported role as an antioxidant and a strong scavenger of hydroxyl radicals, we chose NAC in order to keep out the effect of general free radical scavenging.[19]

RT induces apoptosis which may be initiated by direct activation of caspases, which have a key role in all apoptotic pathways.[2] Milas et al. noted that Amifostine could histologically protect the jejunum, testes and hair loss against radiation, whereas it augmented radiation-induced enhancement of metastasis formation in the lungs.[20] A previous study by Oshima et al. showed that immunostaining of activated caspase-3 was mostly undetected in all tissues. Their data showed that Amifostine could trigger apoptosis as a result of increasing caspase-3 level in colonic cells.[21] Rozalski et al. reported that Amifostine had a protective effect in healthy cells against caspase-3 activation and apoptosis after chemotherapy compared to human acute promyelocytic leukemia cells.[22] A study by Segreto et al also demonstrated that Amifostine protects normal cells against caspase-3 activation and apoptosis after RT compared to cancerous cells from bone marrow granulocyte.[23] The results of the current study were consistent with those of Milas, Rozalski, and Segreto but not Oshima.

A study by Karbownik and Reiter demonstrated that in radiation colitis, oxidative damage occurs as a result of the increased pro-oxidant concentrations, and the decreased antioxidant concentrations.[24] The endogenous enzymatic antioxidants include SOD, GPx and CAT enzymes. It has been shown that SOD and GPx levels are decreased in radiation damage.[25] However, our data indicated that SOD levels decreased in IR group but not statistically significant, GPx levels are not also different between groups. Previous studies showed that the CAT concentration was elevated in spleen tissue of irradiated mice treated with Amifostine.[14] Our results demonstrated that CAT decreases after IR. However, Amifostine and NAC prevented radiation-induced catalase decline.

MDA is an important biomarker for lipid peroxidation in the tissue and MDA levels are elevated in radiation colitis.[26] Neal et al. suggested that NAC can protect cell membranes against lipid peroxidation.[27] Similarly, MDA level decreases in renal tissue with Amifostine treatment when compared to an RT group.[28] We found that MDA levels are increased after radiation. NAC administration decreased the MDA in intestinal tissue compared to IR groups. There were no differences between amifostine and other groups in regard to MDA levels.

MPO has a bactericidal effect and neutrophils have a microbial defence system as an important agent against microorganisms.[4] Demirel et al. showed that the MPO level of lung tissue is not significantly reduced when compared to those of RT rats administered with Amifostine.[29] Research by Kilciksiz et al. showed a significant decrease in MPO level in serum and liver tissue when compared to RT and RT+Amifostine groups.30 In the current study, MPO levels significantly reduced IR and IR+Amifostine group compared to the control group.

Loss of duodenal villi by irradiation leads to diarrhea, nausea and decreases digestion efficiency and even causes duodenal ulceration since intestinal villi are in charge of the absorption of food from alimentary canal. Results can be considerably reduced the patient's quality of life. For that reason, it is of great importance to protect intestines against radiation injuries. In their study, Das et al. observed a swift decrease in crypt survival which also caused the massive structural

alteration of the villi. An increase in the irradiation dose (2.5, 5 and 10 Gy) gradually decreased the length of the villi and crypts. After 2 d of 10-Gy irradiation, a severe loss of normal crypt architecture and cellularity, along with inflammatory cell infiltration was observed in the rats exposed to 10 Gy dose of irradiation.[31] In another study, the structure of the rat ileum was disordered on day 3 after irradiation; a large number of intestinal epithelial cells were necrotic; structures like 'pseudomembranous' were formed; a vast number of inflammatory cells were infiltrated and the number of villi and glands were reduced to a great extent. The reduced nutrient uptake occurs 3 days after abdominal irradiation as a result of villus shortening and dysfunction.[32] On day 7, the rats lost weight to reach the lowest body weight.[33] It has been shown that weight loss during RT affects survival in some abdominal cancers. Therefore, prevention of weight loss and malnutrition in cancer patients is clinically very important.

In conclusion, administration of amifostine with IR prevented CAT reduction in the small intestine and stopped apoptosis by decreasing increased caspase 3 levels. Therefore, it prevented weight loss by reducing villus loss and tissue damage. For this reason, amifostine can be used as a radioprotector in patients who are administered RT in the abdominal region.

The authors report no conflicts of interest.

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