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RESEARCH ARTICLE

The protective effects of apocynin on ionizing radiation-induced intestinal damage in rats

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Abstract

Background and aims: Radiation colitis typically emerges during radiotherapy of intra-abdominal malignancies. While the underlying mechanism remains unclear, it is considered that free oxygen radicals act like cellular mediators to cause colonic damage. Apocynin (APO) prevents oxidative stress and apoptotic cell death by inhibiting NADPH oxidase, and preventing the formation of free oxygen radicals. The aim of the present study was to investigate the protective effect of APO, a strong antioxidant and antiinflammatory agent, on radiation induced colonic oxidative damage in rats.

Materials and methods: Rats were randomly divided into four groups ($n = 8$/group). Group I (control group); Group II (Group RAD) received a single dose of 800 cGy ionizing radiation to the whole abdomen with a linear accelerator (LINAC); Group III (Group APO) received a single dose of 20 mg/kg of APO intraperitoneally for five days; Group IV (Group APO+RAD) received APO for five days before radiation exposure (similar to Group III), (similar to Group II).

Results: APO treatment prior to radiation led to protection in the biochemical and histopathological parameters.

Conclusions: Our study shows that APO treatment before radiation improves radiation induced colonic injury in rats, by decreasing oxidative stress and apoptosis.

Keywords

Apocynin, intestine damage, oxidative stress, radiation, rat

History

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Introduction

Radiotherapy is one of the key treatment options in cancer therapy. It is the basic treatment for abdominal and pelvic cancers. The intestines are highly sensitive to radiation, and dose-dependent toxicity occurs in the intestines[1]. Radiation colitis usually emerges during radiotherapy of intraabdominal malignancies[2]. This condition is a frequent and severe problem[3–5], considering the fact that approximately 50–70% of all cancer patients receive ionized radiation[6]. Radiation causes mucosal damage in activated inflammatory cells and the gastrointestinal (GI) epithelium. Different studies have shown that radiation causes destruction in crypt cells of GI epithelium, a decrease in size and number of villous structures, ulcers and necrosis[7–9]. Secondary toxicity to the lower GI tract can occur. These tissue injuries in the large intestine are most commonly localized in the rectum[10]. While the effect mechanism remains unclear, it is considered that free oxygen radicals act as cellular mediators to induce intestinal damage[11,12]. Following radiation exposure, elevated levels of free oxygen radicals in mitochondria cause DNA, protein and lipid damage[13]. As a result, it inhibits replication, transcription and protein synthesis. The intestines have a protective system that prevents oxidative stress or limits its effect, which is mediated by an enzymatic antioxidant system (superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px)) and a non-enzymatic antioxidant system (reduced glutathione (GSH), vitamin E and vitamin C)[13]. In light of these findings, several studies related to the use of antioxidants against oxidative damage have been conducted[14–17].

Apocynin (APO) (4-hydroxy-3-methoxyacetophenone) is an effective inhibitor of NADPH oxidase. NADPH oxidase is responsible for the production of reactive oxygen species (ROS). Elevated levels of ROS lead to apoptotic cell death, and several clinical disorders. Various experimental studies have demonstrated that the therapeutic effect of APO[18–20].

The aim of the present study was to investigate the protective effect of APO, a strong antioxidant and antiinflammatory agent, on radiation induced intestinal oxidative damage in rats.

Materials and methods

Animals

Thirty-two female Wistar-albino rats (mean weight: 180–220 g) were used in this study. Animals were kept in an environment

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with 12:12 dark:light cycle, 21 ± 2 °C room temperature, and 60 ± 5% humidity. Prior to the experiments, animals were fed with standard rat food and water. The study was approved by the institutional ethics committee (Reference Number: 2014/A-42). All experiments were carried out at Inonu University School of Medicine (IUSM) Experimental Animals Production Facility laboratories, according to the guidelines of World Health Organization (WHO) on animal research. Animals were purchased from IUSM Experimental Animals Production Facility.

Experimental design

Ionizing radiation was used to establish the intestinal damage model. Thirty two rats were randomly divided into four groups ($n = 8$/group).

Group I (Control, $n = 8$): Animals received saline solution intraperitoneally (ip) for five days.

Group II (Radiation, $n = 8$): A LINAC was used to administer a single-dose of 800 cGy radiation to the whole abdomen, under ketamine anesthesia (100 mg/kg, ip). Saline solution was administered ip for five days. The device was placed 100 cm away from the skin, and was capable of producing 6 mV laser.

Group III (APO, $n = 8$): A single dose of 20 mg/kg APO was administered ip for five days.

Group IV (APO+Radiation, $n = 8$): Prior to radiotherapy, APO treatment was performed similar to Group IV for five days. Twenty-four hours later, ionizing radiation was administered similarly to Group II.

The doses of radiation and APO were adjusted according to previous dose-response studies[12,21].

Tissue samples

Ten days after irradiation (day 16), the skins of animals were shaved under high-dose anesthesia mixture [ketamine 100 mg/kg (Ketolar; Parke-Davis, Spain), xylazine 10 mg/kg (Alfazyne 2%; Alfasan, the Netherlands) ip], then sterilized with iodine, and laparotomy was performed afterward. Five milliliter of blood samples were collected from inferior vena cava, and rats were sacrificed afterward. Samples from distal colon were collected under sterile conditions. A part of the tissue specimens were stored in 10% formalin for histopathological examination. Another part of the specimens were frozen in liquid nitrogen, and stored at −35 °C until analysis. Oxidative stress markers malondialdehyde (MDA), total oxidant status (TOS), and oxidative stress index (OSI), and antioxidant system markers SOD, CAT, glutathione peroxidase (GPX), GSH, and total antioxidant capacity (TAC) levels were analyzed biochemically. Histopathological examination was performed under light microscopy.

Biochemical analysis

Two hundred milligrams of frozen colonic tissue was cut into pieces on dry ice and homogenized in 10 volumes of ice-cold Tris-HCl buffer with respect to tissue weight (50 mmol/L, pH 7.4) using a homogenizer (Ultra Turrax IKAT18 basic homogenization; Werke, Staufen, Germany) for 3 min at 6000 rpm. The supernatant solution was extracted with an equal volume of an ethanol/chloroform mixture (3/5, volume per volume [v/v]). After centrifugation at 3000*g* for 30 min, the upper layer was used in the analysis of total tissue protein levels.

Determination of MDA

The MDA contents of the homogenates were determined spectrophotometrically by measuring the presence of thiobarbituric acid reactive substances (TBARS)[22]. Three milliliters of 1% phosphoric acid and 1 ml 0.6% thiobarbituric acid solution were added to 0.5 ml of homogenate pipetted into a tube. The mixture was heated in boiling water for 45 min. After the mixture cooled, the colored part was extracted into 4 ml of *n*-butanol. The absorbance was measured by spectrophotometer (UV-1601; Shimadzu, Kyoto, Japan) at 532 and 520 nm. The amount of lipid peroxides was calculated as TBARS of lipid peroxidation. The results were given in nmol/g tissue, according to a prepared standard graph,which was prepared using the measurements of standard solutions (1,1,3,3-tetramethoxypropane).

Determination of protein content

Protein content of the samples was determined by the method of Lowry et al.[23] using bovine serum albumin as a standard.

Determination of SOD activity

Total SOD activity was determined based on the method of Sun et al.[24]. The principle of the method is the inhibition of nitrobluetetrazolium (NBT) reduction by the xanthine–xanthine oxidase system as a superoxide generator. One unit of SOD was defined as the enzyme amount causing 50% inhibition in the NBT reduction rate. SOD activity was calculated as units per milligram protein (U/mg protein).

Determination of GPX activity

Determination of GPX activity was measured by the method of Paglia and Valentine[25]. An enzymatic reaction in a tube containing NADPH, GSH, sodium azide and glutathione reductase was initiated by the addition of H_2O_2, and the change in absorbance at 340 nm was observed by a spectrophotometer. Activity was given in units per gram protein (U/g protein).

Determination of GSH content

The GSH concentration in homogenate was measured spectrophotometrically according to the method of Ellman. GSH content in the tissue as nonprotein sulfhydryls was analyzed with this described method[26]. Aliquots of tissue homogenate were mixed with distilled water and 50% trichloroacetic acid in glass tubes and centrifuged at 3000 rpm for 15 min. The supernatants were mixed with tris buffer (0.4 mol, pH 8.9) and 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 0.01 mol) was added. After shaking the reaction mixture, its absorbance was measured at 412 nm within 5 min of the addition of DTNB against blank with no homogenate. The absorbance values were extrapolated from a glutathione standard curve and expressed as GSH (micromol/g tissue).

Determination of TAC

TAC levels were determined using a novel automated colorimetric measurement method developed by Erel[27]. In this method, the hydroxyl radical, the most potent biological radical, is produced by the Fenton reaction and reacts with the colorless substrate O-dianisidine to produce the dianisyl radical, which is bright yellowish-brown in color. Upon the addition of sample, the oxidative reactions initiated by the hydroxyl radicals present in the reaction mix are suppressed by the antioxidant components of the sample, preventing the color change and thereby providing an effective measure of the total antioxidant capacity of the sample. The assay has excellent precision values, which are lower than 3%. The results were expressed as mmol Trolox equivalent/L.

Determination of TOS

TOS was determined using a novel automated measurement method, developed by Erel[27]. Oxidants present in the sample

oxidize the ferrous ion-O-dianisidine complex to ferric ion. The oxidation reaction is enhanced by glycerol molecules, which are abundantly present in the reaction medium. The ferric iron makes a colored complex with xylenol orange in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated with hydrogen peroxide and the results were expressed in terms of μmol H_2O_2 equivalent/L.

Measurement of OSI

The percentage ratio of the TOS to TAS yields the OSI, an indicator of the degree of oxidative stress[26]. OSI (arbitrary unit) = TOS/TAS. The OSI value of the distal colon samples was also calculated as an OSI (arbitrary unit).

Histological analyses

For light microscopic analysis, samples from distal colon were fixed in 10% formolin for 48 h dehydrated in ascending alcohol series, and embedded in parafin. Paraffin blocks were prepared for sectioning at 5 μm thickness by microtome. The sections obtained were stained with hematoxylin and eosin (H–E) for general morphology, periodic acid–Schiff (PAS) for secretion of goblet cell and 0.5% toluidine blue for mast cells.

Assessment of colonic injury was performed using microscopic damage scoring. The colonic damage was scored using the four criteria, including inflammatory cell infiltrations, loss or dilatation of crypts, hemorrhage and atrophy or desquamation of the lining mucosal epithelium. Each of criteria was graded on a scale from 0 to 3, depending upon the severity of changes (0: no change, 1: mild, 2: moderate and 3: severe). In addition to surface goblet cell, submucosal mast cell and mitotic figures in crypts were counted using Leica Q Win Image Analysis System (Leica Micros Imaging Solution Ltd., Cambridge, UK) in 20 areas under a 40 × objective. Microscopic scoring and cell counts were carried out blindly by two histologists.

For immunohistochemical analysis, thick sections were taken on to polylysine-coated slides. After rehydrating samples were transferred to citrate buffer (pH 7.6) and heated in a microwave oven for 20 min. After cooling for 20 min at room temperature, the sections were washed with phosphate buffer saline (PBS). Then sections were kept in 0.3% H_2O_2 for 7 min and afterward washed with PBS. Sections were incubated with primary rabbit-polyclonal cysteine aspartate specific proteinase (caspase-3) (Neomarkers, Fremont, CA) antibody for 30 min. And then rinsed in PBS and incubated with biotinylated goat anti-polyvalent for 10min and streptavidin peroxidase for 10 min at room temperature. Staining was completed with chromogen+subsrate for 15 min and slides were counter stained with Mayer's hematoxylin for 1 min, rinse in tap water and dehydrated. Caspase-3 kit was used according to the manufacturer's instructions except minor revision. The sections were examined by a Leica DFC 280 light microscope by a histopathologist unaware of the status of animals. Caspase-3 positive cell cytoplasm stained as brown color.

To calculate the apoptotic index in the superficial epithelium and crypts, we counted manually on digital images using point counting. We selected eight random fields and counted approximately 5000 cells for each samples using a 40 × objective. The apoptotic index was expressed as

$$\text{Apoptotic index} = \frac{\text{number of apoptotic epithelial cell} \times 100}{\text{total number of epithelial cells}}.$$

Statistical analysis

The data were expressed as either median (min–max) values or mean ± standard deviation (SD) depending upon overall variable distribution. Normality was assessed using a Shapiro–Wilk test. The normally distributed data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. The non-normally distributed data were compared by a Kruskal–Wallis *H*-test among the groups. When significant differences were determined, multiple comparions were carried out using a Mann–Whitney *U*-test with Bonfernoni correction. $p < 0.05$ values were considered as significant IBM SPSS statistics version 22.0 (IBM Corp, Armonk, NY) for Windows was used for statistical analyses.

Results

Body weights

None of the animals died during the experimental period. There was no difference between the body weights before and after the experiments among the groups (data not shown).

Biochemical assessment

The results of biochemical analysis of prooxidant and antioxidant parameters are shown in Table 1. Briefly, there was a significant difference in MDA levels between the radiation group and the control group.

There was a significant difference between Group RAD and Group APO + RAD ($p < 0.05$). GPX and GSH levels in Group RAD were significantly lower compared with the control group. These findings were statistically significant except for the SOD level ($p < 0.05$, $p < 0.01$). When we compared Group APO + RAD and Group RAD, these changes were statistically significant GPX, and GSH parameters levels ($p < 0.01$, $p < 0.01$) (Table 1). When we compared TOS and OSI levels between Group RAD and the control group, there were significant differences in these parameters ($p < 0.05$, $p < 0.01$). There were significant differences in OSI parameters between Group RAD and Group APO + ($p < 0.05$) (Table 1).

Histopathological results

The tissue sections of the rats in the control and APO groups showed normal intestinal structures. The colonic mucosa was observed as intact (Figure 1A and 1B). The PAS (+) reaction shows a magenta staining where goblet cells were present among surface epithelial cells. Numerous goblet cells were seen on the surface epithelium and crypt in the control and APO groups (Figure 2A and 2B). Mast cells stained with toluidine blue were distinguished by purple granules around the blood vessels in the submucosa (Figure 1E and 1F). Sections from RAD-treated rats showed slight focal inflammatory cell infiltration in the lamina propria as well as mild desquamation of the lining mucosal epithelium and loss of crypt in some areas (Figure 2A, Table 2). In addition to a decreased number of goblet cells was also recognized (Figure 2C). The decreased number of goblet cells was easily determined by the negative PAS reaction in these areas. Furthermore, decrease in number of mast cells in submucosa was marked in RAD group (Figure 2D, Table 3).

Although in colonic tissues from rats treated with APO+RAD, slight inflammatory cell infiltration was still present in the lamina propria, hemorrhage in the lamina propria, desquamation of the lining epithelium or any damage for crypts were not observed (Figure 2B). Goblet cells in APO+RAD group were evident on the surface and crypt epithelium (Figure 2D). Additionally, the number of the mast cells was found to be significantly increased when compared with RAD group ($p < 0.01$) (Figure 2F).

Table 1. The effects of apocynin on tissue biochemical parameters in rats exposed to radiation.

Groups ($n = 8$)	MDA (nmol/g tissue) Median (min–max)	SOD (U/mg protein) Median (min–max)	GPX (U/g protein) Median (min–max)	GSH (micromol/g tissue) Median (min–max)	TOS (micromol/g tissue) Median (min–max)	TAC (mmol Trolox equivalent/L) Median (min–max)	OSI (arbitrary unit) Median (min–max)
Control	4.63 (3.86–6.44)	0.82 (0.56–1.10)	450.82 (359.95–516.82)	16.37 (9.46–19.03)	4.54 (2.39–8.58)	1.03 (0.58–1.26)	4.74 (3.38–6.87)
RAD	9.17 (5.56–14.32)*	0.66 (0.41–0.96)	199.76 (148.90–267.89)*	4.97 (3.74–7.95)*	7.16 (5.03–10.32)*	0.71 (0.63–1.13)	9.52 (6.99–11.27)*
APO	5.51 (4.10–6.70)	0.81 (0.63–1.01)	424.73 (361.30–494.19)**	14.10 (10.45–17.64)**	4.74 (3.09–5.78)	0.98 (0.78–1.32)	4.83 (2.99–6.21)**
APO+RAD	4.93 (2.93–7.83)**	2.11 (1.92–2.92)	456.03 (367.02–560.20)**	14.68 (11.93–17.68)**	5.16 (3.63–6.56)	0.99 (0.70–1.34)	5.29 (2.94–8.14)**
p Value	<0.015	<0.067	<0.002	<0.002	<0.028	<0.193	<0.004

RAD: Ionizing radiation; APO, apocynin. Results are expressed as median (min–max), $n = 8$.
*$p < 0.05$ versus group I, **$p < 0.05$ versus group II.

Caspase 3 immunostaining was seen only epithelial cells on the luminal surface in the control and APO groups (Figure 3A and 3B). On the other hand, caspase-3 positive cells as well as some crypt and surface epithelium were observed in the RAD group (Figure 3C).

Apoptotic index was found as significant increased in RAD group when compared with control group ($p < 0.01$) (Table 2). The APO administration decreased expression of apoptotic cells (Figure 3D). The apoptotic index was significantly decreased in the APO + RAD group when compared with RAD group ($p < 0.05$).

The results of semiquantitative histological grade, the number of goblet cells, mast cells and mitosis figures and the mean apoptotic index in all groups are shown in Tables 2 and 3.

Discussion

From all the GI organs, the rectum is most frequently acted by pelvic radiation[28]. Radiation-induced intestinal injury is a severe problem that results from radiotherapy to abdominopelvic tumors. This condition plays an important role in tumor spread to the neighboring and distant organs[29]. ROS are considered as the main cause underlying the pathogenesis of intestinal injury[30]. They are known to trigger oxidative stress and apoptosis[31]. Several studies have been carried out in light of these findings[32]. Previous studies have mostly focused on oxidative stress, which plays a dominant role in disease pathogenesis. Given the lack of studies on the use of APO for the treatment of radiation colitis, we tested the effects of APO, which has prominent antioxidant effects against oxidative stress[33].

In the present study, we established an intestinal damage model by a single dose of radiation to whole abdomen. This condition was demonstrated by biochemical parameters and histopathological changes. Following APO treatment, we observed a decrease in oxidative stress, increase in antioxidant system and histopathological improvement. Experimental studies have shown that excess production of free oxygen radicals disrupts the equilibrium between the prooxidant and antioxidant systems in favor of the prooxidant system[34]. Similarly, another study demonstrated that vitamin E decreases oxidative stress through its antioxidant property[35]. In our present study, we used radiation to create intestinal damage, and observed that radiation caused significant tissue damage. When we compared Group RAD and the control group, there was a significant increase in MDA production in Group RAD. Thus, this finding confirms that radiation causes oxidative stress by increasing MDA levels in the intestinal tissue. MDA is a commonly used as a marker to indicate the level of lipid peroxidation in the tissue[36,37]. MDA is secreted as a result of ROS formation due to the oxidation of unsaturated fatty acids in the cell membrane. ROS targets especially membrane lipids, but also other lipids, proteins and DNA[38,39]. Previous studies have shown that MDA levels are elevated in radiation colitis[34]. The increased level of lipid peroxidation in radiation induced intestinal damage in previous studies is consistent with our current findings. The present study showed that there was a significant decrease in radiation induced oxidative stress in Group APO + RAD. This finding is consistent with the literature[34]. In literature, there is different time interval after radiation for model of radiation-induced injury in the experimental studies. This interval time varies from 3 days to 1 month[34,40–42]. As reported before, the initial phase of the effects of ionized radiation arise in the first 1–3 days. However, life threatening findings occur in the two weeks after ionized radiation exposure[43]. Therefore, in the current study, we chose the 10-day interval for formation of maximum intestinal damage[34].

Another important finding in the present study is the decrease in tissue SOD, CAT, GPX and GSH activities after

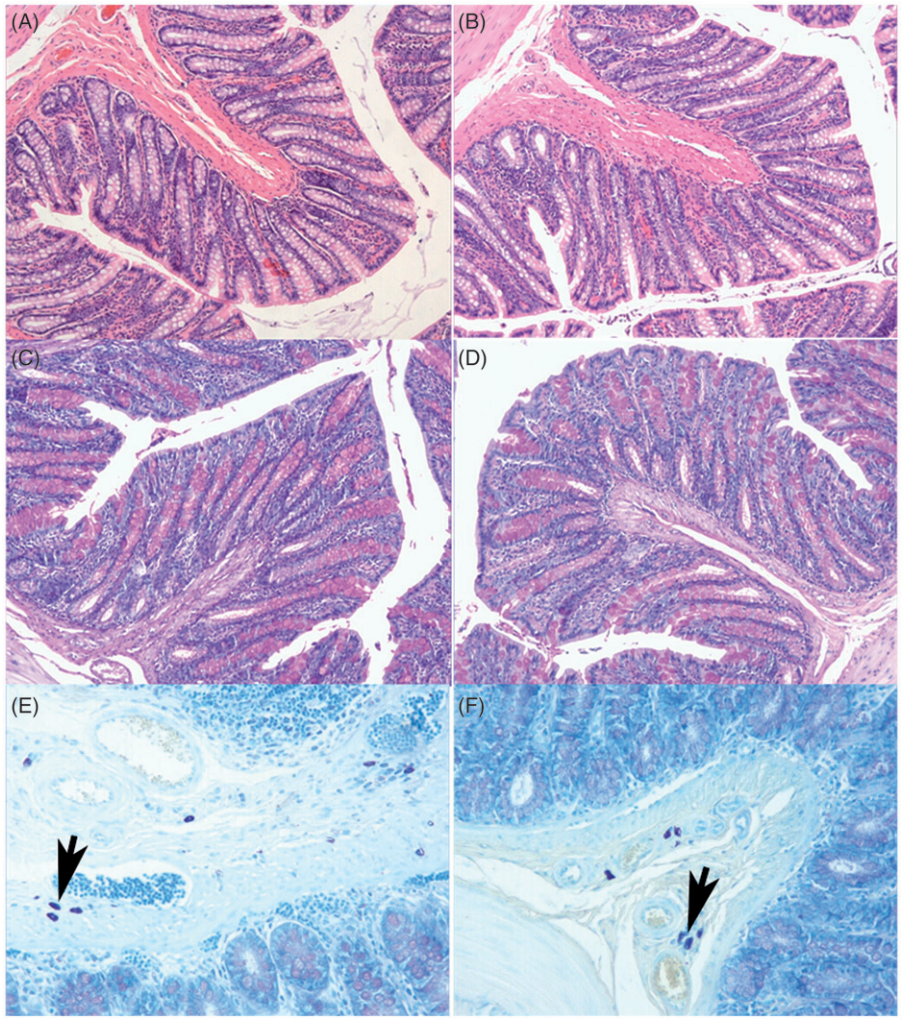

Figure 1. The appearance of regular epithelial lining and intact crypt (A and B) (H–E) and abundant goblet cells containing mucus (C and D) (PAS) × 33. Mast cells stained purple around the blood vessels in the submucosa (E and F) (Toluidin blue) × 132.

radiation exposure. These findings demonstrate the hazardous effect of radiation on the antioxidant system. It is known that equilibrium exists between the prooxidant system and the antioxidant system. The disequilibrium between these systems forms the basis of various diseases. The antioxidant system consists of enzymatic and nonenzymatic systems[44]. SOD, CAT and GPX are endogenous enzymatic antioxidants, whereas GSH is a nonenzymatic antioxidant. Together, they protect the organism and cells from the harmful effects of ROS. In this present study for SOD activity was used total form of SOD. As you know, SOD is a class of antioxidant enzymes that is a component of the defense mechanism against cellular oxidative damage caused by the most common reactive oxygen species in the body called superoxide[21]. The level of this enzyme within host increase to protect the tissues during oxidative damage. Apocynin, a significant inhibitor of NOX and uses its pathway to suppress oxidative stress. It is shown, its effect by increasing this enzyme level in this study. However, one of the explanations of this status, probably it could not show this effect by the NOX pathway[21]. Superoxide formation does not occur only through NOX pathways. Li et al. shown that superoxide formation also occur with other pathway such as xantine oksidase, mitochondria, cytochrome P450, uncoupled endothelial nitric oxide synthase (eNOS)[45]. According to this knowledge, there are some other pathways (xantine oksidase, GSH) for this effect. Probably, other pathways may also cause to increase of SOD activity[45]. Apocynin may also have influenced these other pathways. SOD converts oxygen radical into hydrogen peroxide. Then, H_2O_2 is detoxified by the activities of CAT and GPX, and cleaved into H_2O and O_2[46]. Karbownik and Reiter[47] demonstrated that oxidative damage occurs in radiation colitis as a result of increased levels of prooxidants, and decreased level of the antioxidant system. Similarly, Kaya et al. demonstrated that MDA levels were elevated, whereas SOD and GPX levels are decreased in radiation induced tissue injury[48]. Our findings confirm the existing hypothesis in ROS associated intestinal damage mechanism, and are consistent with the literature. Furthermore, our results confirm that APO exerts its antioxidant effect by increasing SOD and GPX levels[49].

GSH is the most important intracellular antioxidant defense system that plays a role in cellular defense against oxidative stress[50]. Several antioxidant enzymes such as GPX use GSH as a substrate for their activity. The activity of these enzymes is required for detoxification[44]. Previous studies have shown that: (i) GSH, and other associated antioxidant systems are decreased in radiation induced intestinal injury and (ii) APO treatment increases GSH and GPX levels[47,49]. Similarly, we showed that RAD decreased GSH and GPX levels; whereas APO treatment increased the level of these parameters.

In the present study, Group RAD had higher TOS and OSI levels, and lower TAC levels, compared with the control group. On the other hand, Group APO + RAD had lower TOS and OSI levels compared with Group RAD; however, the increase in the TAC level was not significant. These findings suggest that radiation mainly effects oxidative stress, and probably has a weaker effect on the antioxidant system.

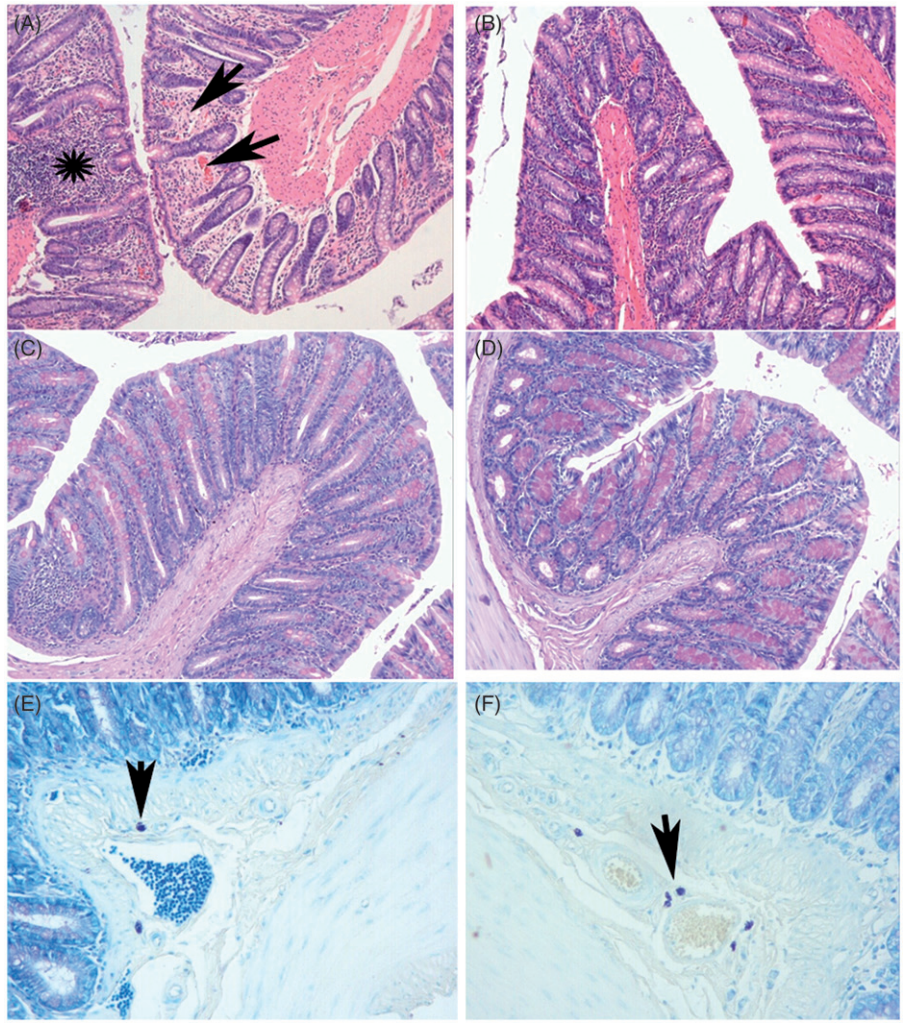

Figure 2. Partial loss of tubular glands (arrows) and inflammatory cell infiltration at lamina propria (star) are observed (A). The histological appearance is similar to control group except slight inflammatory cell infiltration in the around the glands (B) (H–E). Notice the number of goblet cells decreased compare with control group (C). Preservation of goblet cells are seen among surface epithelial cell according to RAD group (D) (PAS) × 33. Reduction in the number of mast cells is evident in the submucosa than control group (arrow) (E). Mast cells monitored around the blood vessels (arrow). The number of mast cells is higher according to RAD group (F) (Toluidin blue) × 132.

Table 2. The variance of histological damage score.

Groups	Hemorrhage	Inflammatory cell infiltration	Loss or dilatation of crypts	Epithelial alterations	Apoptotic index
Control	0.29 ± 0.49 0.0 (0.0 – 1.0)	0.57 ± 0.54 1.0 (0.0 – 1.0)	0.0 ± 0.0 0.0 (0.0 – 0.0)	0.29 ± 0.49 0.0 (0.0 – 1.0)	18 (17 – 18)
APO	0.0 ± 0.0 0.0 (0.0 – 0.0)	0.0 ± 0.0 0.0 (0.0 – 0.0)	0.14 ± 0.38 0.0 (0.0 – 1.0)	0.0 ± 0.0 0.0 (0.0 – 0.0)	12 (12 – 15)
RAD	0.14 ± 0.18 0.0 (0.0 – 1.0)	1.0 ± 0.58 1.0 (0.0 – 0.2)	0.29 ± 0.49 0.0 (0.0 – 1.0)	0.57 ± 0.54 1.0 (0.0 – 1.0)	24 (19 – 24)
APO + RAD	0.0 ± 0.0 0.0 (0.0 – 0.0)	0.29 ± 0.49 0.0 (0.0 – 1.0)	0.0 ± 0.0 0.0 (0.0 – 0.0)	0.0 ± 0.0 0.0 (0.0 – 0.0)	18 (18 – 19)
p Values Mann–Whitney *U*-test	C – RAD: 0.71 APO – RAD:0.71 RAD – APO + RAD: 0.71	C – RAD: 0.26 APO – RAD: 0.004 RAD – APO + RAD: 0.05	C – RAD: 0.38 APO – RAD: 0.71 RAD – APO + RAD: 0.38	C – RAD: 0.38 APO – RAD: 0.07 RAD – APO + RAD: 0.07	C-RAD: 0.007 C-APO+RAD: 0.058 RAD-APO+ RAD: 0.02

RAD, Ionizing radiation; APO, apocynin; C, control.

Macroscopic and histopathological examinations are the gold standards to evaluate inflammatory injury in the colon. The pathological changes in radiation induced intestinal damage are nonspecific, and pathognomonic findings do not exist[51]. Radiation causes submucosal edema in activated inflammatory cells, hyperemia and infiltration in lamina propria, thus leading to inflammation in the intestinal tissue[52]. In our study, the histopathological examination of four independent parameters (hemorrhage, inflammatory cell infiltration, crypt dilation or loss and epithelial changes) showed significant changes between Group RAD and Group APO + RAD, which proved antiinflammatory and protective effects of APO on the mucosa. This finding is consistent with the anti-inflammatory effect of APO, which is exerted by the inhibition of NADPH reductase by activated neutrophilic cells[49].

In the present study, the number of goblet cells and mast cells was decreased in Group RAD, compared with the control group. Moreover, the mitotic index was also lower in Group RAD. Thus,

Table 3. The number of goblet cell, submucosal mast cell and mitosis figures.

Groups	Goblet cell	Mast cell (submucosa)	Mitosis figure
Control	50.6 ± 11.1 [51.0 (22.0 – 92.0)]	3.0 ± 2.6 [2.5 (0.0 – 13.0)]	4.2 ± 2.8 [4.0 (0.0 – 14.0)]
APO	44.5 ± 11.1 [44.5 (18.0 – 71.0)]	2.4 ± 2.3 [2.0 (0.0 – 11.0)]	4.0 ± 2.2 [4.0 (0.0 – 13.0)]
RAD	33.5 ± 12.1 [34.0 (8.0 – 63.0)]	1.2 ± 1.4 [1.0 (0.0 – 6.0)]	3.7 ± 2.5 [3.0 (0.0 – 13.0)]
APO + RAD	40.9 ± 11.1 [41.0 (12.0 – 73.0)]	1.9 ± 1.9 [1.0 (0.0 – 10.0)]	4.1 ± 2.4 [4.0 (0.0 – 11.0)]
p Values	One-way ANOVA	Mann–Whitney *U*-test	Mann–Whitney *U*-test
	Control – RAD: $p = 0.000$	Control – RAD: $p = 0.000$	Control – RAD: $p > 0.005$
	Control – APO+RAD: $p = 0.000$	Control – APO+RAD: $p = 0.000$	Control – APO+RAD: $p > 0.05$
	RAD – APO + RAD: P = 0.000	RAD – APO + RAD: $p = 0.002$	RAD – APO + RAD: $p > 0.05$

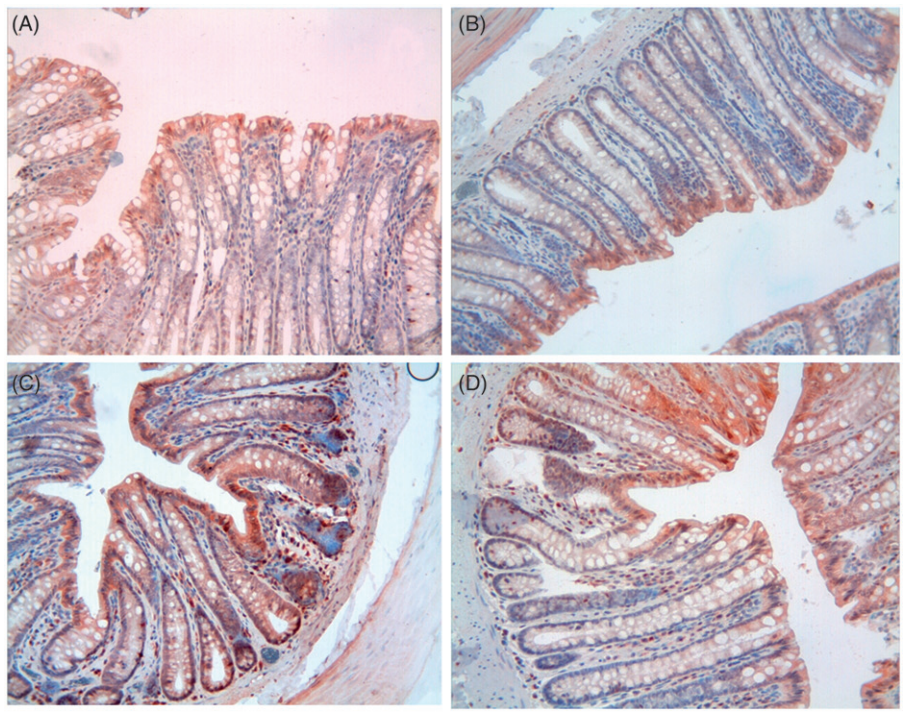

Figure 3. Control and APO groups, view of caspase-3 staining (A and B); RAD group, marked caspase-3 staining is seen in surface epithelium and crypt epithelium (C); RAD + APO group, there is a prominent reduction caspase-3 staining cells according to RAD group (D); X66.

considering the elevated oxidative stress in Group RAD, these findings supported the fact that radiation causes intestinal injury. The mucosal healing in Group APO + RAD is consistent with the improvements in tissue biochemical parameters.

When we compared the apoptotic index in Group RAD and the control group, we found a significant increase in the apoptotic index in Group RAD. This finding was also consistent with elevated biochemical parameters, and further indicated intestinal damage. In this regard, our findings are consistent with the study by Moon et al. APO treatment significantly decreases the apoptotic index in Group APO + RAD, compared with Group RAD, and reduced the level of intestinal damage[31]. Thus, our findings showed the protective effect of APO.

Different studies by Karbownik et al. and Kaya et al. have shown that oxidative stress parameters are increased, and antioxidant parameters are decreased in the radiation induced cell injury[47,48]. Thus, their results are consistent with our findings.

Conclusion

In the present study, biochemical and histopathological examinations showed that oxidative stress is elevated in radiation-induced intestinal injury pathogenesis. Furthermore, APO treatment before radiation exerted antioxidant effects by decreasing oxidative stress. Thus, these findings demonstrate the importance of oxidative stress in radiation colitis pathogenesis. Furthermore, our findings can indicate that future studies on radiation colitis might aim to identify agents that will reduce the effects of ROS. Given the positive effects of APO on lipid peroxidation and the antioxidant system in the intestinal tissue, our findings suggest that it can be a treatment option to stop the spread of injury. However, this warrants further studies.

Declaration of interest

The authors have no disclosures relevant to this publication.

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