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Punica Granatum Peel Extract Protects Against Ionizing Radiation-Induced Enteritis And Leukocyte Apoptosis In Rats

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Apoptosis/Cytokine/Enteritis/Ileum/Irradiation/Punica granatum/Pomegranate/Radioprotective.

Radiation-induced enteritis is a well-recognized sequel of therapeutic irradiation. Therefore we examined the radioprotective properties of Punica granatum peel extract (PPE) on the oxidative damage in the ileum. Rats were exposed to a single whole-body X-ray irradiation of 800 cGy. Irradiated rats were pretreated orally with saline or PPE (50 mg/kg/day) for 10 days before irradiation and the following 10 days, while control rats received saline or PPE but no irradiation. Then plasma and ileum samples were obtained. Irradiation caused a decrease in glutathione and total antioxidant capacity, which was accompanied by increases in malondialdehyde levels, myeloperoxidase activity, collagen content of the tissue with a concomitant increase 8-hydroxy-2'-deoxyguanosine (an index of oxidative DNA damage). Similarly, pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) and lactate dehydrogenase were elevated in irradiated groups as compared to control. PPE treatment reversed all these biochemical indices, as well as histopathological alterations induced by irradiation. Furthermore, flow cytometric measurements revealed that leukocyte apoptosis and cell death were increased in irradiated animals, while PPE reversed these effects. PPE supplementation reduced oxidative damage in the ileal tissues, probably by a mechanism that is associated with the decreased production of reactive oxygen metabolites and enhancement of antioxidant mechanisms. Adjuvant therapy of PPE may have a potential to support a successful radiotherapy by protecting against radiation-induced enteritis.

INTRODUCTION

Radiotherapy is widely used for the treatment of solid organ malignancies in the abdomen and pelvis. The highly radiosensitive intestine is an important dose-limiting organ in both total body and abdominopelvic radiation[1,2] because radiation-induced enteritis with several deleterious intestinal symptoms such as bleeding, anorexia, nausea, vomiting and diarrhea can be a challenging clinical problem.[3] It is well known that acute radiation over a threshold dose of 8 Gy causes an immediate but potentially reversible effect on the rapidly renewing intestinal mucosal cells. After 5–8 days of irradiation with a dose of 8 Gy and higher, the inflammatory cascade, denudation of epithelium, and other morphological changes of the intestinal surface develop. The damaged intestinal epithelium loses its function and septic shock becomes the most common cause of death.[3,4]

Although the pathogenesis of radiation enteritis is not clear, it is presumed to be an inflammatory process in which various mediators such as eicosanoids, cytokines, and reactive oxygen metabolites (ROM) take place.[3,5] An extensive literature implicates cellular DNA as the primary target for the biological and lethal effects of ionizing radiation. Besides DNA, lipids and proteins are also attacked by free radicals induced by ionizing radiation.[6,7] Drugs that scavenge or inhibit the formation of ROM may have relevance to cancer patients by ameliorating damage of normal tissues exposed to ionizing irradiation therapy.

Punica granatum L. (Punicaceae), commonly called pomegranate, is a plant used in folkloric medicine for the treatment of various diseases, such as ulcer, hepatic damage and snakebite.[8] Over the past few decades, scientific investigations have provided evidence for the antioxidant, anti-

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inflammatory and anticancer effects of the pomegranate, laying a credible basis for some of its traditional ethnomedical uses.[9] Juice and peels of pomegranate possess potent antioxidant properties and anticancer activities, including interference with tumor cell proliferation, cell cycle, invasion and angiogenesis.[10,11] Pomegranate peel extract (PPE) with an abundance of flavonoids and tannins has been shown to have a high antioxidant activity.[12] Recently, we have demonstrated that PPE, through its antioxidant effects, protected the liver against oxidative injury and fibrosis induced by biliary obstruction in rats.[12] Similarly, in several studies on the therapeutic use of pomegranate, protection of rat gastric mucosa from ethanol or aspirin toxicity,[8,13] protection of neonatal rat brain from hypoxia,[14] prevention of male rabbit erectile dysfunction[15] were all attributed to its antioxidant effects.[16]

Therefore, PPE has been examined for its possible antioxidant and radioprotective effects against irradiation-induced ileal tissue damage using biochemical and histopathological approaches.

MATERIALS AND METHODS

All experimental protocols were approved by the Marmara University Animal Care and Use Committee. Male Sprague Dawley rats (200–250 g) were housed in a light- and temperature-controlled room on a 12:12-h light-dark cycle, where the temperature (22 ± 2°C) and relative humidity (65–70%) were kept constant.

EXPERIMENTAL DESIGN

As published in our previous study,[12] the peel, juice and seed of pomegranate were extracted with methanol and the free radical scavenging abilities of different concentrations were assessed by DPPH *(1,1-diphenyl-2-picrilhydrazyl)* method. The peel extract, which had the highest antioxidant activity (83% activity for 500 mg/L PPE vs 98% activity of ascorbic acid), was used for the treatment of the irradiated rats. This extract was subjected to HPLC, UV and mass spectra analysis for its content. Three main compounds were identified as punicagalin 1, punicagalin 2 and ellagic acid. For details see Toklu *et al.*, 2007.

Prior to irradiation, rats were pretreated orogastrically with either saline (n = 8) or PPE (n = 8; 50 mg/kg) for 10 days and the treatments were repeated for the following 10 days after the irradiation. Each rat received a single whole-body X-ray irradiation (IR) of 800 cGy under ketamine anesthesia (100 mg/kg intraperitoneally) with a LINAC producing 6 MV photons at a focus. Animals were returned to their home cages following irradiation. Control rats were treated with either saline (n = 8) or PPE (n = 8) in a similar manner as the irradiated groups, but they were not exposed to irradiation. On the 11^{th} day of the post-treatments, the rats were decapitated and trunk blood was obtained for the measurement of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6), lactate dehydrogenase (LDH), total antioxidant capacity (AOC), 8-hydroxy-2'-deoxyguanosine (8-OHdG), an index of oxidative DNA damage and leukocyte apoptosis. Ileum samples were obtained for the determination of tissue malondialdehyde and glutathione levels, myeloperoxidase activity and collagen contents. Formation of reactive oxygen species in the ileal samples was monitored by using chemiluminescence (CL) technique. Ileum samples were also examined microscopically.

BLOOD ASSAYS

Lactate dehydrogenase (LDH) activity, an indicator of tissue damage, was determined spectrophotometrically using an automated analyzer (Bayer Opera biochemical analyzer, Germany). Plasma levels of TNF-α, IL-1β and IL-6 were quantified using enzyme-linked immunosorbent assay (ELISA) kits specific for the rat cytokines according to the manufacturer's instructions and guidelines (Biosource Europe S. A., Nivelles, Belgium). The 8-OHdG content in the extracted DNA solution were determined by enzyme-linked immunosorbent assay (ELISA) method (Highly Sensitive 8-OHdG ELISA kit, Japan Institute for the Control of Aging, Shizuoka, Japan). The total antioxidant capacity in plasma was measured by using colorimetric test system (ImAnOx, cataloge no.KC5200, Immunodiagnostic AG, D-64625 Bensheim, Germany), according to the instructions provided by the manufacturer. These particular assay kits were selected because of their high degree of sensitivity, specificity, inter- and intraassay precision and small amount of plasma sample required conducting the assay.

EVALUATION OF APOPTOSIS AND CELL DEATH IN LEUKOCYTES

Apoptosis was induced by using phorbol myristate acetate (PMA, Sigma-Aldrich, Taufkirchen, Germany) as previously described with some modifications.[17] Briefly, erythrocytes from heparinized blood samples of the groups were discarded using ELS. White blood cells were washed and resuspended in PBS. For each apoptosis experiment, 10^5 cells/ml were distributed into two tubes. One tube was induced for apoptosis using 100 ng/ml of PMA at 37°C for 2 hours, while the other was incubated at the same temperature without stimulation (control). To demonstrate early apoptosis, cells were washed with PBS following stimulation and were labeled with annexin V (Biovision, Mountain view, CA, USA) according to manufacturer's instructions. Briefly 1 µl of annexin V was added to the tubes and cells were incubated at dark for 15 minutes. Once propidium iodide (20 ng/ml, Sigma-Aldrich, Taufkirchen, Germany) was added to label late apoptosis and cell death, cells were immediately acquired by flowcytometry. For analysis, lymphocytes and

neutrophils were separately gated according to their granularity and size on forward scatter (FSC) versus Side Scatter (SSC) plot. Early apoptosis, late apoptosis, necrosis and cell death were evaluated on Fluorescence 1 (FL1 for Annexin V) versus Fluorescence 3 (FL3 for propidium iodide) plots. The percentage of cells stained with only Annexin V was evaluated as early apoptosis, while the percentage of cells stained with both Annexin V and propidium iodide was evaluated as late apoptosis and the percentage of cells stained only with propidium iodide was evaluated as cell death and/or necrosis. Comparisons between the groups were performed by using ratios of apoptosis and cell death. Ratios of apoptosis and cell death were calculated by dividing the percentage values of after-stimulation to that of prior to stimulation in each individual case.

CHEMILUMINESCENCE (CL) ASSAY

To assess the role of reactive oxygen species (ROS) in radiation-induced tissue damage, luminol and lucigenin chemiluminescences were measured in the ileal samples as indicators of radical formation. Measurements were made at room temperature using Junior LB 9509 luminometer (EG&G Berthold, Germany). Specimens were put into vials containing PBS-HEPES buffer (0.5 M PBS containing 20 mM HEPES, pH 7.2). ROS were quantitated after the addition of enhancers such as lucigenin or luminol for a final concentration of 0.2 mM. Luminol detects a group of reactive species, i.e. $^{\bullet}OH$, H_2O_2, HOCl radicals and lucigenin is selective for O^{-}_{2}. Counts were obtained at 1 min intervals and the results were given as the area under curve (AUC) for a counting period of 5 min. Counts were corrected for wet tissue weight (rlu/mg tissue).[18]

MALONDIALDEHYDE AND GLUTATHIONE ASSAYS

Tissue samples were homogenized with ice-cold 150 mM KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously.[19] Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$ and results were expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure.[20] Briefly, after centrifugation at 2000 g for 10 min, 0.5 ml of supernatant was added to 2 ml of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 ml solution of dithiobisnitrobenzoate (0.4 mg/ml 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of $1.36 \times 10^4\ M^{-1}\ cm^{-1}$. Results were expressed in μmol GSH/g tissue.

MEASUREMENT OF MYELOPEROXIDASE ACTIVITY

Myeloperoxidase (MPO) is an enzyme that is found predominantly in the azurophilic granules of polymorphonuclear leukocytes (PMN). Since tissue MPO activity was shown to correlate significantly with the number of PMN determined histochemically in tissues,[21] it is frequently utilized to estimate tissue PMN accumulation in inflamed tissues. MPO activity was measured in tissues in a procedure similar to that documented by Hillegas *et al.* in 1990.[22] Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH 6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide (HETAB). After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41.400 g for 10 min. Aliquots (0.3 ml) were added to 2.3 ml of reaction mixture containing 50 mM PB, o-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount

Table 1. Plasma lactate dehydrogenase (LDH) activity, TNF-α, IL-1β, and IL-6 levels, total antioxidant capacity (AOC) and 8-hydroxy-2'-deoxyguanosine (8-OHdG) of the saline– or pomegranate peel extract (PPE)-treated irradiation groups. Each group consists of 8 rats.

	Control		Irradiation	
	Saline-treated	PPE-treated	Saline-treated	PPE-treated
LDH (U/I)	1609 ± 143	1750 ± 259	4252 ± 321***	2413 ± 243^{+++}
TNF-α (pg/ml)	5.1 ± 0.5	4.2 ± 0.5	27.4 ± 3.3***	10.0 ± 1.5^{+++}
IL-1β (pg/ml)	18.56 ± 2.45	19.64 ± 2.88	70.76 ± 9.54***	32.1 ± 4.1^{+++}
IL-6 (pg/ml)	4.28 ± 0.70	4.53 ± 0.66	15.45 ± 1.86***	7.68 ± 0.94^{+++}
8-OHdG (ng/ml)	0.79 ± 0.13	0.89 ± 0.1	4.96 ± 0.61***	2.07 ± 0.41^{+++}
AOC (pg/ml)	317 ± 15	327 ± 20	84 ± 10***	229 ± 43^{++}

***$p < 0.001$; compared to saline-treated control group; $^{++}p < 0.01$; $^{+++}p < 0.001$ compared to saline-treated irradiation group.

of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

HISTOPATHOLOGICAL ANALYSIS AND TISSUE COLLAGEN MEASUREMENT

Samples of the tissues were fixed in 10% (vol/vol) buff-

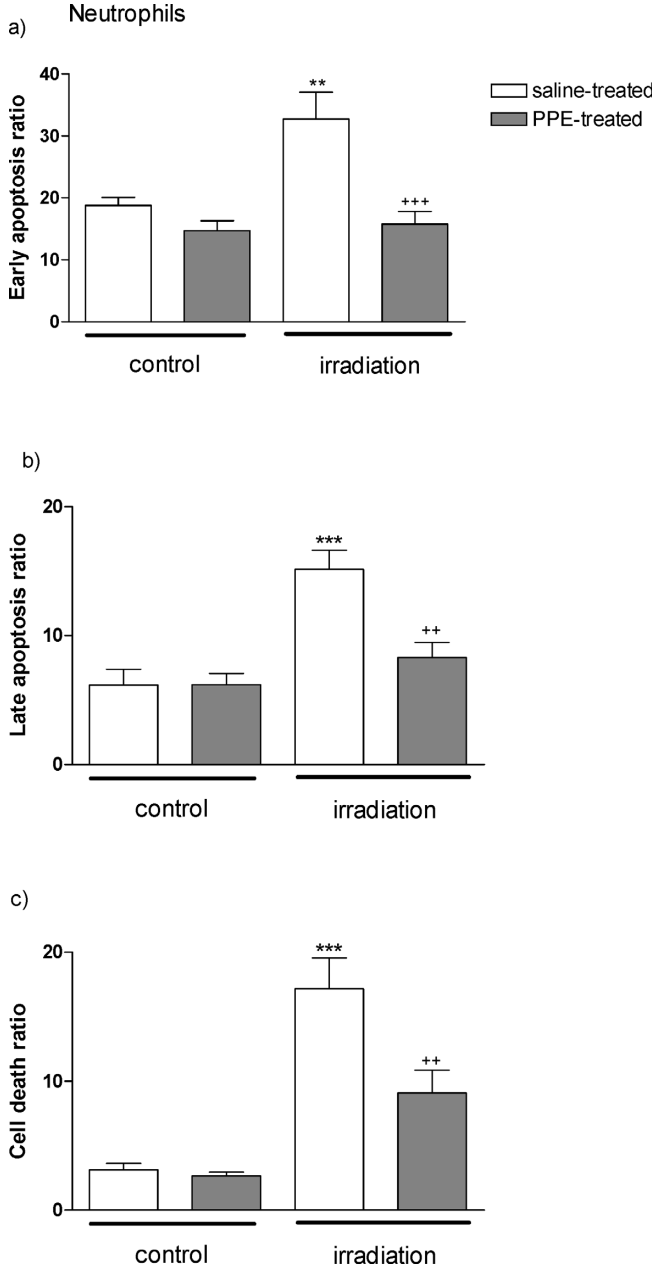

Fig. 1. a) Early apoptosis, b) Late apoptosis and c) cell death of neutrophils obtained from saline- or pomegranate peel extract (PPE)-treated rats induced with irradiation. Early and late apoptosis and cell death ratios were calculated by dividing the values of after-stimulation to the values obtained without phorbol myristate acetate stimulation. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with saline-treated control group; $^{++}P < 0.01$, $^{+++}p < 0.001$ compared with saline-treated irradiation group.

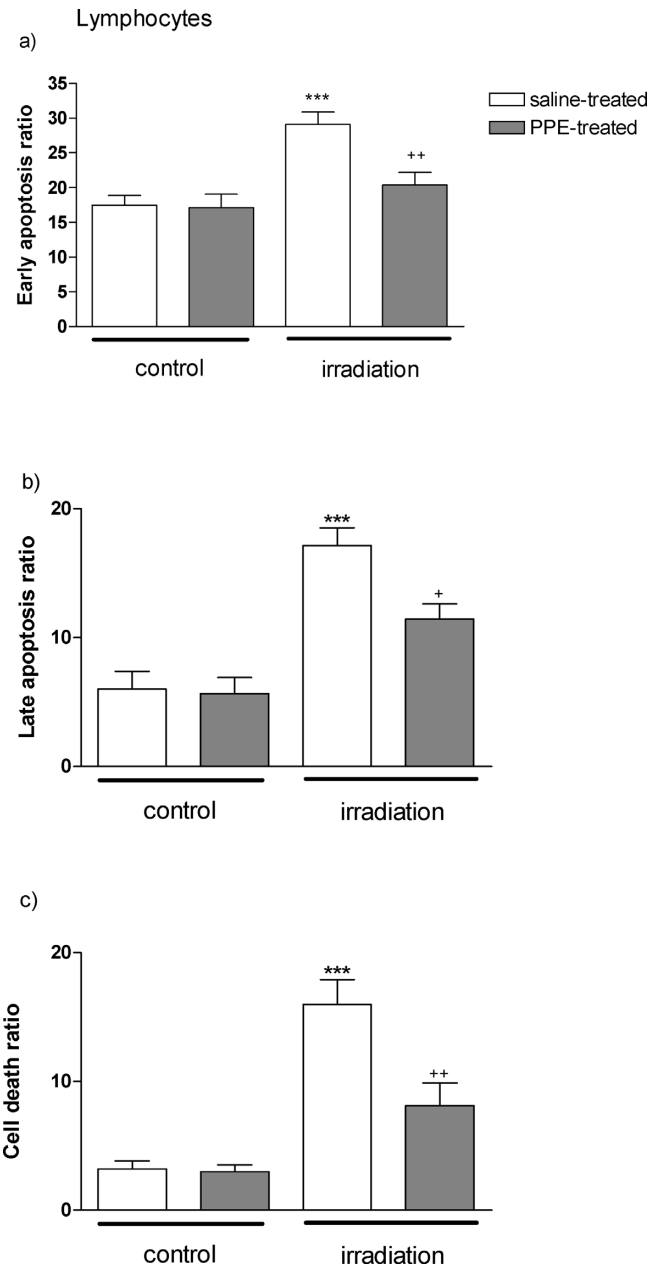

Fig. 2. a) Early apoptosis, b) Late apoptosis and c) cell death of lymphocytes obtained from saline- or pomegranate peel extract (PPE)-treated rats induced with irradiation. Early and late apoptosis and cell death ratios were calculated by dividing the values of after-stimulation to the values obtained without phorbol myristate acetate stimulation. $^{***}P < 0.001$ compared with saline-treated control group; $^{+}P < 0.05$, $^{++}p < 0.01$ compared with saline-treated irradiation group.

ered p-formaldehyde and were prepared for routine paraffin embedding. Tissue sections (6 μm) were stained with Hematoxylin and Eosin (H&E) and examined under a light microscope (Olympus-BH-2, Tokyo, Japan) by an experienced histologist, who was unaware of the treatment conditions.

Extra tissue samples that were fixed in p-formaldehyde and embedded in paraffin were cut (15 μm) and used for tissue collagen measurement as a free radical-induced fibrosis marker. Evaluation of collagen content was made according to the method published by Lopez de Leon and Rojkind,[23] based on selective binding of the dyes Sirius Red and Fast Green FCF to collagen and non-collagenous components, respectively. Both dyes were eluted readily and simultaneously by using 0.1 N NaOH-methanol (1:1, v/v). Finally, the absorbances at 540 and 605 nm were used to determine the amount of collagen and protein, respectively.

STATISTICS

Statistical analysis was carried out using GraphPad Prism 3.0 (GraphPad Software, San Diego; CA; USA). All data are expressed as means ± SEM. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. Values of $p < 0.05$ were regarded as significant.

RESULTS

In saline-treated irradiated (IR) animals, plasma lactate dehydrogenase activity, as a marker of generalized tissue damage, and proinflammatory cytokines TNF-α IL-1β and IL-6 were significantly increased ($p < 0.001$; Table 1), while PPE administration abolished these elevations ($p < 0.001$). Similarly, in the plasma of saline-treated IR groups, oxidative DNA marker 8-OHdG was increased ($p < 0.001$), while total antioxidant capacity (AOC) was significantly reduced ($p < 0.001$). On the other hand, PPE treatment applied before and after the irradiation reduced the elevation in plasma 8-OHdG level and prevented the reduction in AOC ($p < 0.01$ and 0.001).

Annexin V stainings alone were evaluated as early apoptosis, while annexin V along with propidium iodide stainings were evaluated as late apoptosis. Both early and late apoptosis ratios in neutrophils were significantly higher in the saline-treated IR groups when compared to control groups ($p < 0.01$–0.001, Fig. 1a and b). Furthermore, neutrophil death was significantly increased in saline-treated irradiated group ($p < 0.001$; Fig. 1c). Similar results were observed in the apoptosis and cell death ratios of lymphocytes obtained from saline-treated IR rats ($p < 0.001$, Fig. 2a, b and c). However, PPE administration prevented the apoptotic response of both neutrophils and lymphocytes to irradiation and reduced the cell death ratio in both types of leukocytes ($p < 0.01$, Fig. 1 and 2).

Chemiluminescence levels in the ileal samples detected by both luminol and lucigenin probes showed significant increases in the saline-treated IR group as compared to the CL levels of the control group ($p < 0.01$; Table 2). On the other hand, in the IR group that has received PPE treatment, the elevations in CL values were significantly decreased ($p < 0.05$–0.01). In accordance with the CL values, the MDA content in the ileum of the saline-treated irradiated rats was also significantly elevated ($p < 0.001$) as compared to non-radiated control group, while a concomitant decrease in ileal GSH level was observed ($p < 0.05$; Table 2). On the other hand, when animals were treated with PPE before and after the irradiation, MDA levels were decreased ($p < 0.05$) and depleted GSH levels were replenished ($p < 0.01$). Myeloperoxidase activity, which indicates the extent of neutrophil infiltration to the tissue, was significantly higher in the ileal tissues of saline-treated IR group than that of the control group ($p < 0.001$; Table 2), while it was significantly

Table 2. Luminol and lucigenin chemiluminescence, malondialdehyde (MDA) and glutathione (GSH) levels, myeloperoxidase (MPO) activity, collagen content, in the ileal tissues of saline– or pomegranate peel extract (PPE)-treated irradiation groups. Each group consists of 8 rats.

	Control		Irradiation	
	Saline-treated	PPE-treated	Saline-treated	PPE-treated
Luminol (rlu/mg)	10.76 ± 0.6	6.73 ± 0.4	19.64 ± 2.4**	12.83 ± 1.5^{+}
Lucigenin (rlu/mg)	8.63 ± 0.9	8.50 ± 0.7	15.60 ± 1.8**	10.88 ± 0.7^{+}
MDA (nmol/g)	36.6 ± 3.0	35.5 ± 1.5	63.8 ± 3.0***	48.1 ± 5.0^{+}
GSH (μmol/g)	1.10 ± 0.03	1.23 ± 0.04	0.85 ± 0.08*	1.20 ± 0.07^{++}
MPO (U/g)	6.40 ± 0.5	6.30 ± 0.6	19.35 ± 1.5***	13.65 ± 1.0^{++}
Collagen (mg/mg prot.)	14.60 ± 1.4	13.80 ± 1.4	21.60 ± 1.7*	14.20 ± 1.4^{+}

*$p < 0.05$, **$p < 0.01$,***$p < 0.001$; compared to saline treated control group; $^{++}p < 0.01$; compared to saline-treated irradiation group.

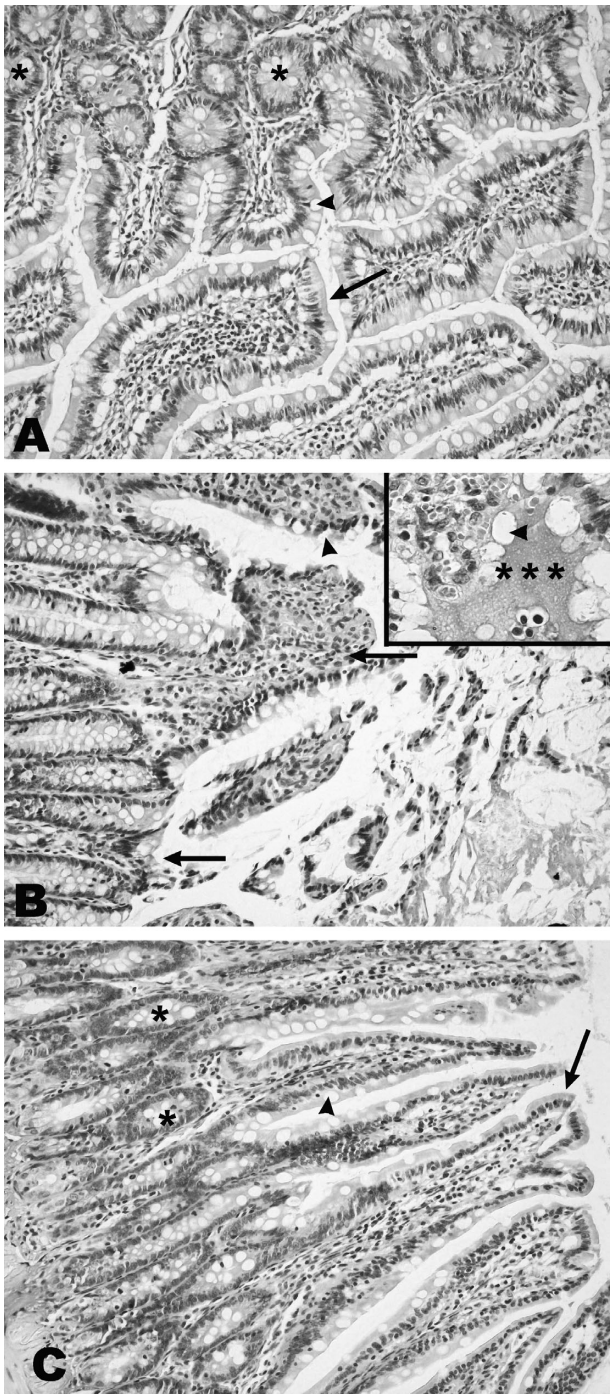

Fig. 3. Photomicrographs of ileum. a) control group, regular epithelium (arrow) with goblet cells (arrowhead) and glands (*), b) saline-treated irradiation group, note severe detachment of epithelium (arrows) and goblet cells (arrowhead), and severe edema in the lamina propria (***, inset), c) PPE-treated irradiation group, the prominent regeneration of epithelium (arrow) with re-established goblet cells (arrowhead) and glands (*) HE, X 200 and inset X 400.

depressed in the PPE-treated IR group ($p < 0.05$).

Control ileum tissues demonstrated a regular morphology (Fig. 3a) with the normal epithelium and glands, while enlargement of goblet cells, epithelial desquamation and prominent edema in lamina propria were prominent in the irradiated group (Fig. 3b). However, in the PPE-treated IR group (Fig. c), the morphology was nearly the same as the control group with well-established epithelium and glands. Irradiation caused a significant increase in the ileal collagen content of saline-treated animals ($p < 0.05$; Table 2), implying the presence of enhanced tissue fibrotic activity. On the other hand, in the PPE-treated group tissue collagen content was significantly decreased ($p < 0.05$) and was not different than that of the non-irradiated control animals.

DISCUSSION

Since tissue injury from ionizing radiation is believed to be a consequence of a cascade of cytokine activity due to oxidative stress from radiolytic hydrolysis and formation of reactive oxygen metabolites (ROM),[6] agents such as PPE which are directed on the inhibition of oxidative stress appear to have a potential in radioprotection. The results of the present study demonstrate that whole-body irradiation causes oxidative tissue damage in the ileum of the rats, as assessed by increased lipid peroxidation, neutrophil infiltration and fibrosis concomitant with a decrease in ileal GSH. Treatment with PPE depresses lipid peroxidation, neutrophil infiltration and replenishes GSH content in the tissue, verifying the protective effect of PPE against oxidative injury. In addition, irradiation caused both early and late apoptosis in the leukocytes, whereas PPE treatment effectively prevented this oxidant-induced blood cell damage. Furthermore, PPE treatment alleviated irradiation-induced elevations in serum LDH activity and pro-inflammatory cytokines.

The results demonstrating the protective effect of PPE in radiation-induced oxidative damage suggest that PPE may be considered as a choice of adjuvant therapy in reducing radiation enteritis, which otherwise limits the efficacy of the radiotherapy in 0.5–15% of patients treated with abdominal radiotherapy.[24] Because of the rapid turnover of intestinal mucosa, the acute phase with the symptoms of nausea, vomiting, and diarrhea is usually self-limited and persists for hours to several days after exposure.[24,25] The chronic phase, which may occur months to several years after exposure, is a result of damage to the submucosal vessels and is associated with inflammation, stricture formation, and obstruction. It has been hypothesized that the incidence of adverse chronic effects is proportional to the severity of acute symptoms, because the early response to ionizing radiation makes the tissues more susceptible to subsequent inflammatory events. Therefore, it is of great importance to protect the intestine from the acute phase of radiation injury by ameliorating the radiation-induced oxidative injury.

It is well known that X-ray irradiation-induced inflammatory response in the small intestine involves the recruitment of activated inflammatory cells.[26] These immune cells synthesize and release several different cytokines, inflammatory mediators and reactive oxygen metabolites.[1] In the present study, the intestinal MPO activity in the irradiated rats was increased significantly, indicating that radiation-induced oxidative injury in the tissue involves the contribution of neutrophil accumulation. There is substantial evidence that activated neutrophils can exacerbate tissue injury through the production of oxygen metabolites, initiating the deactivation of antiproteases and activating cytotoxic enzymes including elastase, proteases, lactoferrin and MPO. MPO is an essential enzyme for normal neutrophil function, released into the extracellular fluid as a response to various stimulatory substances. On the other hand, PPE treatment effectively prevented the neutrophil infiltration, suggesting that protective effect of PPE against radiation-induced injury may involve its inhibitory effect on tissue neutrophil infiltration. Furthermore, as evidenced in the present study, ionizing radiation resulted in increased serum TNF-α, IL-1β, and IL-6 indicating the role of these cytokines in radiotherapy-induced toxicity, while PPE depressed the cytokine response. Accordingly, it seems likely that the amelioration of IR-induced oxidative damage by PPE, in part, may involve the suppression of a variety of pro-inflammatory mediators produced by leukocytes and macrophages.

It has been reported that irradiating biological material leads to a rapid burst of ROM, such as superoxide ($O_2^{-\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl ($^{\bullet}OH$) generated primarily because of the ionizing of water molecules,[1] which then interact with biological target molecules, causing lipid peroxidation and DNA damage, and subsequently resulting in cell killing and mutations.[27] In this study, we evaluated the generation of oxygen radicals using luminol- and lucigenin-enhanced CL technique. Although the two probes differ in selectivity,[18] our CL data demonstrate that tissue injury induced by irradiation involves toxic oxygen metabolites without any selectivity. In accordance with increases in toxic oxygen metabolites, the ileal MDA level was also significantly increased, indicating the presence of enhanced lipid peroxidation due to irradiation injury. Since PPE treatment prevented elevations in tissue MDA and attenuated the increases in tissue luminol and lucigenin-enhanced CL levels, it seems likely PPE ameliorates radiation-induced oxidative injury, in part, by scavenging the reactive oxygen radicals. Our results are in agreement with the studies which have demonstrated that *P. granatum* peel extract decreased lipid peroxidation in hepatic, cardiac, and renal tissues[28] and had a facilitatory effect on the scavenging ability of superoxide anion and hydrogen peroxide. Previously, we have shown that chronic PPE supplementation alleviated oxidative injury of the liver and improved the hepatic structure and function in rats exposed to bile duct ligation.[12] Another study in rats with carbon tetrachloride-induced liver damage demonstrated that pretreatment with pomegranate peel extract resulted in the reduction of lipid peroxidation, while the free-radical scavenging activity of catalase, superoxide dismutase, and peroxidase were significantly enhanced.[29] In the present study, the GSH level that was decreased due to its consumption during irradiation-induced oxidative stress was increased by PPE treatment, suggesting that it may be an important factor in protecting the tissue against oxidative injury. Since GSH and the activities of glutathione reductase and glutathione peroxidase, which are critical constituents of GSH-redox cycle, provide major protection in oxidative injury by participating in the cellular system of defense against oxidative damage, they play a critical role in limiting the propagation of free radical reactions, which would otherwise result in extensive lipid peroxidation.

There is an extensive literature verifying that cellular DNA is the primary target for the biological and lethal effects of ionizing radiation. Direct ionization in DNA molecule can cause disruption of chemical bonds in the molecular structure, where highly reactive free radicals produced during the radiolysis of water subsequently migrate to and damage to DNA.[7] As an indicator of oxidative DNA damage the measured 8-OHdG in the blood is significantly increased while PPE treatment reversed this effect of radiation. In accordance with these observations, our results also demonstrate that radiation-induced cellular damage is correlated with enhanced leukocyte apoptosis and cell death, while PPE protected both early and late apoptosis ratio in neutrophils and leukocytes and cell death. As it was observed in our previous study,[12] LDH activity was also increased significantly following radiation, while PPE effectively reduced this increase. LDH is an enzyme that does not have a metabolic function upon its release to the extracellular space, but its presence outside the cytoplasm indicates disturbances of the cellular integrity induced by pathological conditions. Thus, the current results show that irradiation-induced generalized tissue damage as assessed by elevated plasma LDH activity was reduced by PPE treatment, suggesting the positive impact of PPE on cellular integrity. Accordingly, histopathologic examination revealed that radiation-induced intestinal damage and inflammation in irradiated animals was ameliorated when the animals were treated with PPE. Moreover, increased collagen content of the ileal tissue, indicating the presence of tissue fibrotic activity, was also attenuated via the antioxidant properties of PPE treatment. Since fibrosis is a common form of tissue damage after exposure to a wide variety of insults including the oxidative stress, inhibition of irradiation-induced intestinal fibrosis is likely to participate in the protective mechanisms of PPE in radiation injury. Indeed, the risk of irradiation-induced fibrosis occurring in the normal tissues several months to years after radiotherapy remains a major factor in limiting the

radiation dose that can be applied safely to cancer patients.

CONCLUSION

Irradiation is an essential therapeutic modality in the management of abdominopelvic tumors, but its acute and delayed side effects on normal tissues limit the effectiveness of the therapy. Our results imply that extract of dried pomegranate peels, which were previously reported to exert antioxidant, anti-inflammatory and antiproliferative activities via significant effects on subcellular signaling pathways,[9,30,31] are also of potential therapeutic value in ameliorating irradiation-induced oxidative organ injury. Considering its low toxicity[32,33] and direct inhibitory effect on the growth of cancer cells[11,34,35] through the inhibition of angiogenesis via downregulation of vascular endothelial growth factor.[36] PPE can be regarded as a potential agent in limiting the intestinal complications due to irradiation without compromising its antitumor activity.

Conflict of interest: None

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