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Protective Effects of *Nigella Sativa* on Gamma Radiation-Induced Jejunal Mucosal Damage in Rats

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A B S T R A C T

Introduction: The aim of this study was to compare the efficacy of *Nigella sativa* in protection of jejunal mucosa against harmful effects of gamma radiation.

Methods: Radiotherapy group received abdominal gamma radiation of 15 Gy in addition to physiological saline. Radiotherapy + *Nigella sativa* treatment group received abdominal gamma radiation of 15 Gy in addition to *Nigella sativa* treatment in the amount of 400 mg/kg. Radiotherapy and treatment groups were sacrificed 3 days after the exposure to irradiation. Then, jejunum samples were harvested for biochemical and histological assessment of mucosal injury.

Results: *Nigella sativa* treatment was found to significantly lower elevated tissue malondialdehyde (MDA) levels and, to raise reduced glutathione peroxidase (GSH-Px) and superoxide dismutase (SOD) activity in intestinal tissues samples. Single dose 15 Gy gamma-irradiation was noted to result in a marked jejunal mucosal injury. Three days after exposure to irradiation, the villi and Lieberkühn crypts were observed as denuded, and villous height diminished. Concomitantly with inflammatory cell invasion, capillary congestion and ulceration were observed in the atrophic mucosa. *Nigella sativa* treatment significantly attenuated the radiation induced morphological changes in the irradiated rat jejunal mucosa. **Conclusion:** *Nigella sativa* has protective effects against radiation-induced damage, suggesting that clinical transfer is feasible.

Key words: Jejunal mucosa· Radiation injury· *Nigella sativa*· Apoptosis· Oxidative stress

INTRODUCTION

Radiotherapy is known to be one of the most common and important methods for cancer treatment[1]. Killing action of ionizing radiation is mainly mediated through the free radicals generated from the radiolytic decomposition of cellular water[2],including superoxide radical, hydroxyl radical, and hydrogen peroxide [3].These free radicals can stimulate chain reactions by interacting with proteins, lipids and nucleic acids, causing cellular dysfunction and even death[4].Previous studies have indicated that radiation-mediated oxidative stress can induce apoptosis[5].

The intestine is an important dose-limiting organ during radiation therapy of tumors in the pelvis or abdomen. In animal studies, intestinal radiation toxicity (radiation enteropathy) is, by convention, classified as early or delayed, depending on when it occurs relative to the time of radiation therapy [6].It was previously believed that the severity of intestinal radiation toxicity depended directly on cell death in intestinal crypts. This view has been supplanted by the recognition that radiation- induced changes in cellular function and alterations secondary to cell death contribute substantially to the intestinal radiation response[7,8].Because these functional and secondary changes develop over time after radiation exposure, they are particularly promising targets for interventions aimed at preventing or reducing intestinal radiation toxicity.

Biologic modifiers targeting oxidative damage for radioprotection have been studied for decades with limited success. Hence, there is a need for better and more potent compounds, especially on herbal origin to boost antioxidant defence.

The black seed, *Nigella sativa* family Ranunculaceae has been shown to contain > 30% of fixed oil and 0.4-0.45% wt/wt of volatile oil. The volatile oil has been shown to contain 18.4-24% thymoquinone (2-isopropyl-5-methyl-1,4-benzoquinone) and 46%

monoterpenes such as p- cymene and α-piene[9].Recently, clinical and animal studies have shown that the extracts of the black seeds have many therapeutic effects such as immunomodulative, antibacterial, antidiabetic, hepatoprotective, gastroprotective, antihistaminic and antioxidative and neuroprotective ones. [10,11,12-17].

In this study, antiapoptotic effects of *Nigella sativa* in rat jejenum exposed to gamma radiation were examined by light microscopy, proliferating cell nuclear antigen (PCNA) and terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate in situ nick end-labeling (TUNEL) technique.

METHODS

Animals

Thirty 12-14-week-old Male Wistar albino rats weighing between 250 and 300 g (Trakya University Animal Care and Research Unit, Edirne, Turkey) were housed under constant temperature (21^{o}C) and photoperiod (12 h light/dark cycle). They had free access to standard rat chow diet. All animals received human care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences and published by the National Institutes of Health. The study was approved by the Institutional Animal Ethical Committee of the Trakya University, Edirne, Turkey.

Plant material and extraction procedure

The NS seeds were purchased from a local herb store in Eskisehir, Turkey. Sample specimens have been kept at the Plant, Drug and Scientific Researches Center, Anatolian University Faculty of Pharmacy, Eskisehir, Turkey for future reference. The seeds of *Nigella sativa* were powdered in a mixer. 20 g of the powdered seeds were added to 400 ml of distilled water and extraction was carried out by steam distillation. The distillation process

was continued until about 200 ml of distillate was collected. The distillate was extracted three times with chloroform. Moisture was removed by anhydrous sodium sulphate and the resultant extract was evaporated using a 40°C water bath leading to the appearance of the volatile oil. 500 mg of the volatile oil were dissolved in 1 ml of dimethyl sulphoxide (DMSO) then 9 ml of physiological saline was added to yield a concentration of 50 mg volatile oil per 1 ml solution[18].

Experimental Design and Drug Preparation

Twenty-four rats were randomly divided into three groups of eight rats each. At day 7, group 1 (control group) received sham irradiation plus on each day physiological saline (2 ml / in a dose of 400 mg/kg body weight intragastric). Group 2 (radiotherapy group) received abdominal gamma-irradiation of 15 Gy on day 7 as a single dose plus on each day physiological saline (2 ml / in a dose of 400 mg/kg body weight intragastric) as placebo. Group 3 (radiotherapy + *Nigella sativa* group) received abdominal gamma-irradiation of 15 Gy on day 7 and plus on each day *Nigella sativa* (in a dose of 400 mg/kg body weight intragastric) as treatment. All groups were decapitated 3 day after exposure (at day 10) and jejunum samples were removed for histological and biochemical assessment of mucosal injury.

Abdominal gamma (c)-radiation

Prior to radiotherapy, the rats were anesthetized with xylazine/ketamine (10/90 mg/kg, i.p.) and immobilized by fixing from their 4 extremities on a tray. Irradiation was delivered through abdomen by a cobalt-60 (60Co) teletherapy unit (Cirus, cis-Bio Int., Gif Sur Yvette, France) at a source-surface distance of 100 cm with protection of thorax and extremities (62.41 cGy/min). Control group received equal-field sham irradiation.

Biochemical analysis of antioxidant status

Frozen intestine tissue was homogenized. The homogenates were filtered and centrifuged using a refrigerated centrifuge at 4 ˚C and supernatant were frozen at -20 ˚C in aliquots until used for biochemical assays. The protein content of the supernatant was determined using the method of Lowry et al [19].Malondialdehyde (MDA), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) levels were determined according to the methods of Draper and Hadley[20], Sun et al. [21] and Paglia and Valentine[22], respectively.

Light microscopy prodecures

For light microscopical observation, jejunum specimens were embedded in the paraffin blocks after they had been fixed in Bouin's solution. Five micrometer (µm) sections were obtained and stained with hematoxylin+eosin (H+E).

Histopathological analysis

Jejunum sections were stained with H+E, and mucosal injury, inflammation and hyperemia/hemorrhage were assessed and graded in a blinded manner by a histologist using the histological injury scale previously defined by Chiu et al.[23].Briefly, mucosal damage

was graded from 0 to 5 according to the following criteria: grade 0, normal mucosal villi; grade 1, development of subepithelial Gruenhagen's space at the apex of the villus, often with capillary congestion; grade 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; grade 3, massive epithelial lifting down the sides of villi, possibly with a few denuded tips; grade 4, denuded villi with lamina propria and dilated capillaries exposed, possibly with increased cellularity of lamina propria; and grade 5, digestion and disintegration of the lamina propria, hemorrhage, and ulceration. The sum of the scores for the individual alterations constitutes the radiation injury score (RIS).

Immunohistochemistry

The harvested intestinal tissues were fixed in Bouin's, embedded in paraffin and sectioned at 5 µm thickness. Immunohistochemical reactions were performed according to the ABC technique described by Hsu et al. [24].The procedure involved the following steps: (1) endogenous peroxidase activity was inhibited by 3% H_2O_2 in distilled water for 30 min; (2) the sections were washed in distilled water for 10 min; (3) non-specific binding of antibodies was blocked by incubation with normal goat serum (DAKO X 0907, Carpinteria, CA) with PBS, diluted 1:4; (4) the sections were incubated with specific mouse monoclonal anti-poliferating cell nuclear antigen (PCNA) antibody (Cat. # MS-106-B, Thermo LabVision, USA), diluted 1:50 for 1h at room temperature; (5) the sections were washed in PBS 3 × 3 min; (6) the sections were incubated with biotinylated anti-mouse IgG (DAKO LSAB 2 Kit); (7) the sections were washed in PBS 3 × 3 min; (8) the sections were incubated with ABC complex (DAKO LSAB 2 Kit); (9) the sections were washed in PBS 3 × 3 min; (10) peroxidase was detected with an aminoethylcarbazole substrate kit (AEC kit; Zymed Laboratories); (11) the sections were washed in tap water for 10 min and then dehydrated; (12) the nuclei were stained with hematoxylin; and (13) the sections were mounted in DAKO

paramount. All dilutions and thorough washes between steps were performed using phosphate buffered saline unless otherwise specified. All steps were carried out at room temperature unless otherwise specified.

Tissue sections were examined, the number of the cells in the crypts was counted, within random high-power fields using an Olympus Bx 51 light microscope incorporating a square graticule in the eyepiece (eyepiece x 10, objective x 40, a total side length of 25 μm). PCNA positive cells in the crypts was counted and recorded in the scale of per μm^2. Positive cell/100 crypts cells ratio was used as the index of proliferation.

TUNEL assay

The terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method, which detects fragmentation of DNA in the nucleus during apoptotic cell death in situ, was employed using an apoptosis detection kit (TdT-Fragel TM DNA Fragmentation Detection Kit, Cat. No. QIA33, Calbiochem, USA). All reagents listed below are from the kit and were prepared following the manufacturer's instructions. Five-μm-thick intestinal sections were deparaffinized in xylene and rehydrated through a graded ethanol series as described previously. They were then incubated with 20 mg/ml proteinase K for 20 minutes and rinsed in TBS. Endogenous peroxidase activity was inhibited by incubation with 3% hydrogen peroxide. Sections were then incubated with equilibration buffer for 10–30 minutes and then TdT-enzyme, in a humidified atmosphere at 37^oC, for 90 minutes. They were subsequently put into pre-warmed working strength stop/wash buffer at room temperature for 10 minutes and incubated with blocking buffer for 30 minutes. Each step was separated by thorough washes in TBS. Labelling was revealed using DAB, counter staining was performed using Methyl green, and sections were dehydrated, cleared and mounted.

TUNEL positive cells were counted, and the TUNEL positive cells/100 intestinal villi cells ratio was used as the index of apoptosis. These analyses were performed using two sections from each animal at 400x magnification in at least ten different regions for each section.

Statistical analysis

All statistical analyses were carried out using SPSS statistical software (SPSS for windows, version 11.0). All data were presented in mean (±) standard deviations (S.D.). Differences in measured parameters among the five groups were analyzed with a nonparametric test (Kruskal-Wallis). Dual comparisons between groups exhibiting significant values were evaluated with a Mann–Whitney U-test. These differences were considered significant when probability was less than 0.05.

RESULTS

Biochemical findings

The values of the tissue MDA level SOD and GSH-Px activities and statistical differences of these measurements are shown in Figure 1. Tissue MDA levels ($p < 0.001$) were significantly increased, SOD ($p < 0.01$) and GSH-Px activities ($p < 0.001$) were significantly decreased in radiation exposed rats in comparison to control. *Nigella sativa* treatment significantly ($p < 0.05$) decreased the elevated tissue MDA levels comparison to control group and increased the reduced GSH-Px ($p < 0.05$) and SOD activities ($p < 0.05$) in comparison to radiation group.

Histopathologic findings

Normal histopathology presented in control group (Fig. 2a). The typical histological features that occurred in the radiation treated group were characterized by

shortening of the villi, loss of villous epithelium, multiple erosions, inflammatory cell infiltration, necrosis, and hemorrhage into the intestinal wall (Fig. 2b). The histological structure of the intestinal mucosa was markedly improved after administration of *Nigella sativa* (Fig. 2c). Data related to scoring obtained by means of H+E staining as well as the microphotographs are shown in Figure 2a-c. As expected, no mucosal injury was observed in the control group. According to the Chiu et al. [23].system, injury in the radiation treated group increased as compared to the control group, $p < 0.0001$). It was determined that *Nigella sativa* administration significantly prevented the mucosal injury caused by radiation (radiation+ Nigella sativa, $p < 0.001$, Fig. 2d).

Immunohistochemical findings

In control group, PCNA-positive cells were mainly observed in the crypt regions (Fig. 3a). After radiation exposure, PCNA positive cells were markedly decreased in the crypt regions (Fig. 3b). Treatment with Nigella sativa, the number of PCNA positive cells significantly increased (Fig. 3c) (Immunoperoxidase and haematoxylin counterstain). Sequential changes in the proliferation index are indicated in control, radiation and radiation+*Nigella sativa* groups (Fig. 3d).

TUNEL findings

TUNEL-positive cells tended to be observed in the upper part of the villi (Fig. 4a). In control group, a few TUNEL-positive cells are observed in the intestinal villi (Fig. 4b). TUNEL positive cells increased after radiation exposure in the detached epithelial cells from the villous tip in the radiation group (Fig. 4c). Treatment of *Nigella sativa* markedly reduced the number of TUNEL positive cells. Sequential changes in the

apoptotic index are indicated in sham, radiation and radiation+*Nigella sativa* groups (Fig. 4d).

DISCUSSION

The gastrointestinal tract is, after the bone marrow, the organ most sensitive to the effects of radiation. As a result of ionizing radiation, acute morphological changes of the intestine are observed within 24–48 h. [25].Early radiation enteropathy develops during radiation therapy as a result of intestinal crypt cell death, disruption of the epithelial barrier, and mucosal inflammation. [6].

Several chemical compounds and their analogues have been screened for their radioprotective ability; however, their high toxicity at optimum protective doses precluded their clinical use[26].Patients might tolerate dietary agents better than other drugs because humans consume many of these dietary ingredients daily[27].

Radiation of the abdomen in experimental animals induces major morphological and functional changes in the intestinal epithelium (mucosal atrophy and ulceration and toxin and microbial translocation to the mesenteric lymph nodes) while treatment with GH and IGF-I largely inhibits these histological lesions. [28].In this study, the typical histological features that occurred in the radiation treated group were characterized by shortening of the villi, loss of villous epithelium, multiple erosions, inflammatory cell infiltration, necrosis, and hemorrhage into the intestinal wall. Our data are corroborated by previous studies reported by other investigators on radiation induced intestinal damage in animals. To date, no information on the effect of *Nigella sativa* on radiation-induced intestinal damage in rats has been reported. Our data indicate that histologically, in the radiation treated group, villous atrophy, subepithelial edema, and epithelial damage were more pronounced than in the *Nigella sativa* -treated group

Normally, there are few apoptotic cells at the tips of the villi as a consequence of the differentiation and senescence of the epithelium. The programmed cell death (PCD) seems to occur while the cells move up along the villus to the tips before being shedded into the gut lumen[29].

In a study [30], the small intestine sustained more severe damage than the large intestine. The hallmark occurrence of apoptosis was apparent early on in treatment in both the small and large intestine, beginning at week 1, continuing throughout the treatment, and peaking by week 5. The increase in apoptosis was accompanied by an increase in expression of nuclear p53 in the crypts of the small and large intestine affected by radiation. These findings are consistent with their previous studies showing that the earliest effect of chemotherapy is the rise of apoptosis in intestinal crypts[31]. Apoptosis is an inherent protective mechanism used by cells to regulate proliferation and occurs at a low level in the healthy, normal small intestine. However, apoptosis is a rare occurrence in the colon[32].In this rat irradiation model, they have shown that the fractionated dosing of ionizing radiation greatly increases the occurrence of apoptosis in both the small and large intestine.

It is well known that radiation, depending on the dose, induces DNA damage[33-34].which sequentially triggers PCD via different molecular pathways or leads to cell necrosis[33,35,36]. In order to determine whether *Nigella sativa* could maintain these proliferating cells population and regeneration after gamma radiation, the distribution and the number of their apoptosis and proliferative ability were evaluated by TUNEL and PCNA immunostaining respectively. Likewise, in previous studies, the data from our experiments indicate that the histological and functional lesions observed in the irradiated rats were due to the induction of PCD in intestinal epithelial cells as well as in crypt and submucosal cells. In the irradiated rats increased numbers of apoptotic cells across the villi were observed with the

TUNEL stain in histological sections of intestine whereas in the control samples few apoptotic nuclei existed, and these were found only at the tips of the villi.

In the present study, we found that after radiation exposure, a few PCNA positive cells were observed in the crypt regions; treatment with *Nigella sativa* significantly increased the number of PCNA positive cells. TUNEL positive cells increased after radiation exposure in the detached epithelial cells from the villous tip in the radiation group; treatment of *Nigella sativa* markedly reduced the number of TUNEL positive cells. Our results are in agreement with previous studies on radiation induced intestinal injury in rats.

The damage from oxygen free radicals is an important mechanism of tissue injury induced by irradiation[37].In a study, Sun et al. [38] showed that MDA level and SOD activity was a good indicator of the degree of tissue peroxidation. They found that MDA level increased, while the SOD activity decreased significantly after whole body irradiation. Treatment with Omegaven increased SOD activity and decreased MDA level. Therefore, the effect of Omegaven in reducing peroxidation may play a important role in suppressing irradiation-induced intestinal injury and improving survival advantage. In the present study, we found that *Nigella sativa* treatment significantly decreased the elevated tissue MDA levels comparison to control group and increased the reduced GSH-Px levels and SOD activity in comparison to radiation group. Our results support findings of the above-mentioned literature reports on oxidative stress in terms of intestinal survival.

In conclusion, this is first study to demonstrate that intragastric administration of *Nigella sativa* maintains antioxidant defenses and thus reduces intestinal mucosal injury, oxidative damage and apoptosis in radiaton treated rats. However, more investigations are required to evaluate *Nigella sativa*'s antioxidant and anti-apoptotic effect in clinical and experimental models.

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Figure Legends

Figure 1. Tissue MDA level SOD and GSH-Px activities in sham, radiation and radiation+*Nigella sativa* groups. Tissue MDA levels ($^{a}p < 0.001$) were significantly increased, SOD ($^{a}p < 0.01$) and GSH-Px activities ($^{a}p < 0.001$) were significantly decreased in radiation exposed rats in comparison to sham. *Nigella sativa* treatment significantly ($^{b}p < 0.05$) decreased the elevated tissue MDA levels comparison to sham group and increased the reduced GSH-Px ($^{b}p < 0.05$) and SOD activities ($^{b}p < 0.05$) in comparison to radiation group.

Figure 2. Light microscopy of intestinal tissue in different groups. **(a)** Normal histopathology presented in sham group; **(b)** after radiation exposure, rat intestine shows severe histopathological injury including shortening of the villi, loss of villous epithelium, multiple erosions, inflammatory cell infiltration, necrosis and hemorrhage into the intestinal wall; **(c)** treatment with *Nigella sativa* greatly reduced all of these changes associated with radiation exposure (H&E, scale bar, 100 μm); **(d)** intestinal mucosal injury evaluated in sham, radiation

and radiation+*Nigella sativa* groups, $^{a}p < 0.0001$ compared to sham group, $^{b}p < 0.001$ compared to radiation group.

Figure 3. Light microscopy of intestinal tissue in different groups. **(a)** In sham group, PCNA-positive cells were mainly observed in the crypt regions; (**b)** after radiation exposure, PCNA positive cells were markedly decreased in the crypt regions; (**c)** treatment with *Nigella sativa* significantly increased the number of PCNA positive cells, (Immunoperoxidase and haematoxylin counterstain, scale bar, 50 μm). (**d)** Sequential changes in the proliferation index are indicated in sham, radiation and radiation+*Nigella sativa* groups, $^{a}p < 0.0001$ compared to sham group, $^{b}p < 0.001$ compared to radiation group.

Figure 4. Light microscopy of intestinal tissue in different groups. TUNEL-positive cells tended to be observed in the upper part of the villi. (**a)** In sham group, a few TUNEL-positive cells are observed in the intestinal villi; (**b)** TUNEL positive cells increased after radiation exposure in the detached epithelial cells from the villous tip in the radiation group; (**c)** treatment of *Nigella sativa* markedly reduced the number of TUNEL positive cells (Scale bar, 50 μm); (**d)** sequential changes in the apoptotic index are indicated in sham, radiation and radiation+*Nigella sativa* groups, $^{a}p < 0.0001$ compared to sham group, $^{b}p < 0.001$ compared to radiation group.

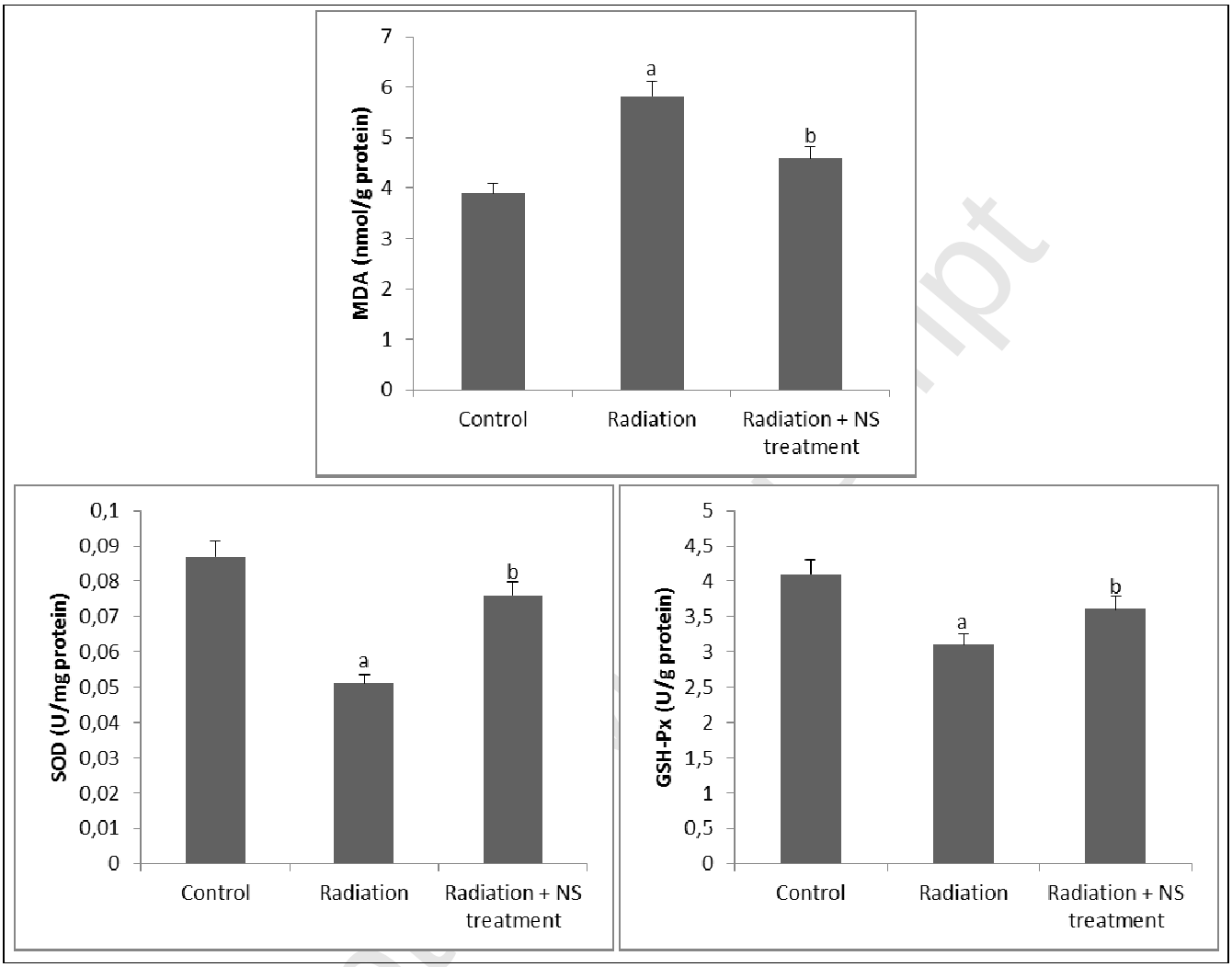


Fig. 1. Tissue MDA level SOD and GSH-Px activities in sham, radiation and radiation+Nigella sativa groups. Tissue MDA levels ($^{a}P < 0.001$) were significantly increased, SOD ($^{a}P < 0.01$) and GSH-Px activities ($^{a}P < 0.001$) were significantly decreased in radiation exposed rats in comparison to sham. Nigella sativa treatment significantly ($^{b}P < 0.05$) decreased the elevated tissue MDA levels comparison to sham group and increased the reduced GSH-Px ($^{b}P < 0.05$) and SOD activities ($^{b}P < 0.05$) in comparison to radiation group.

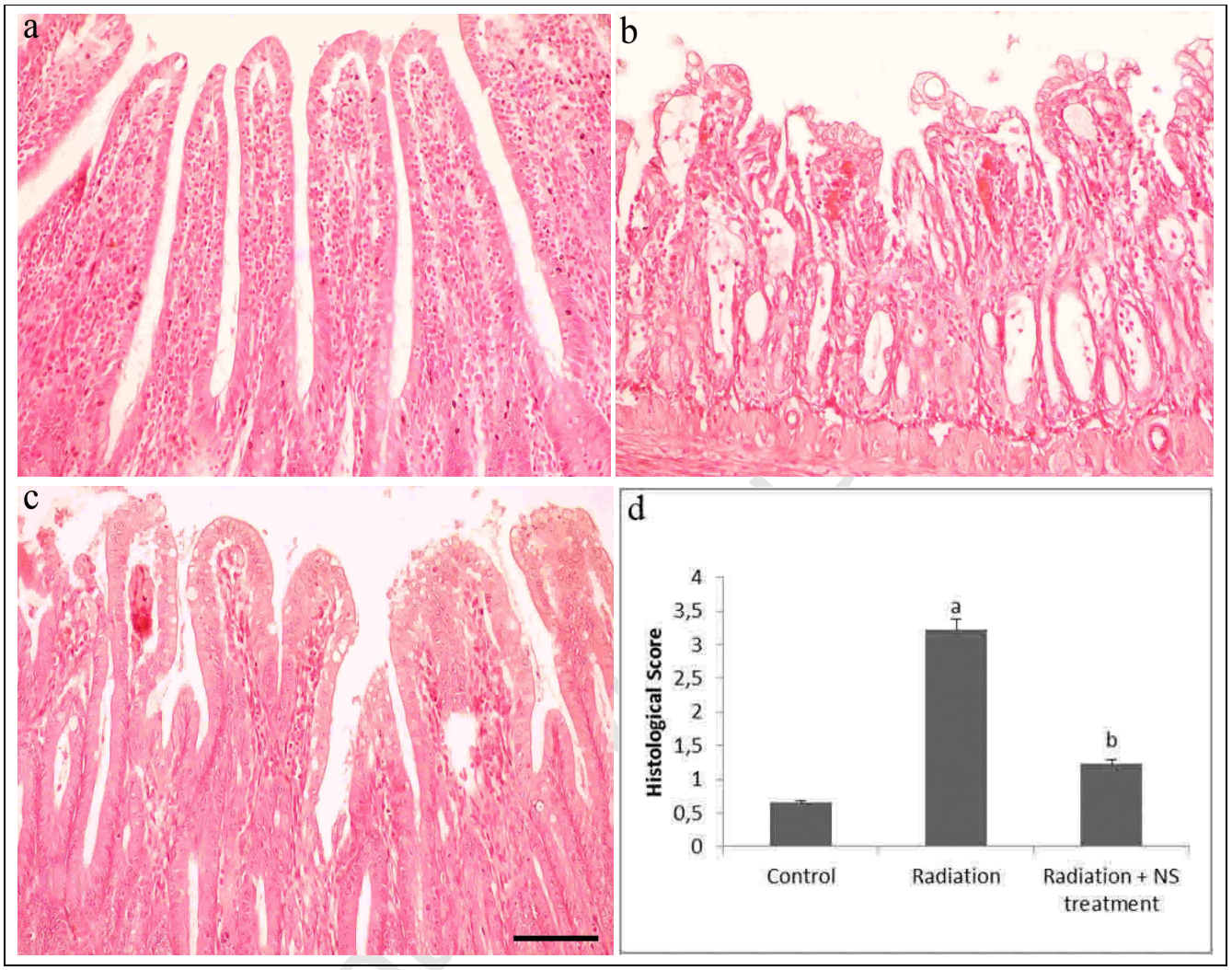
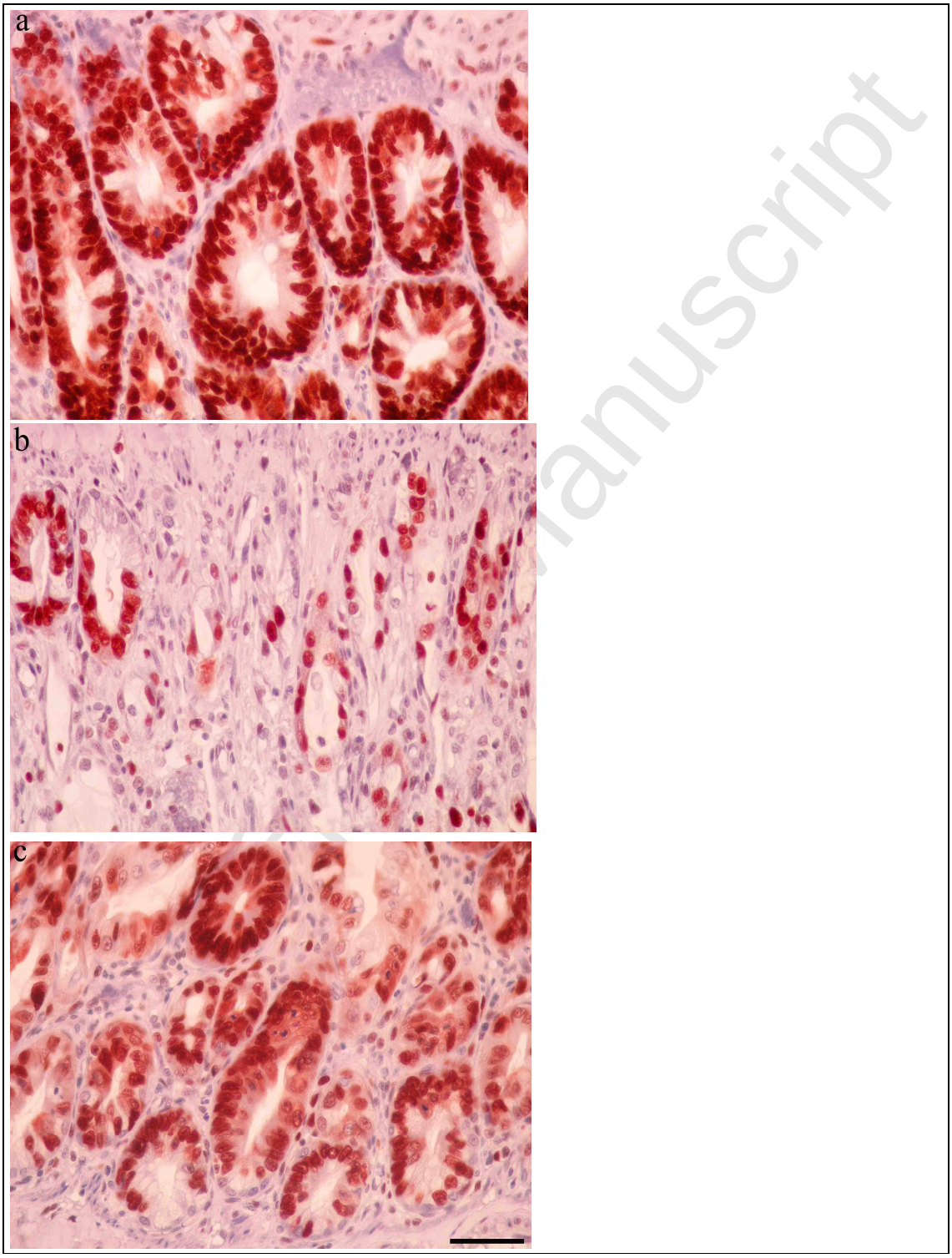
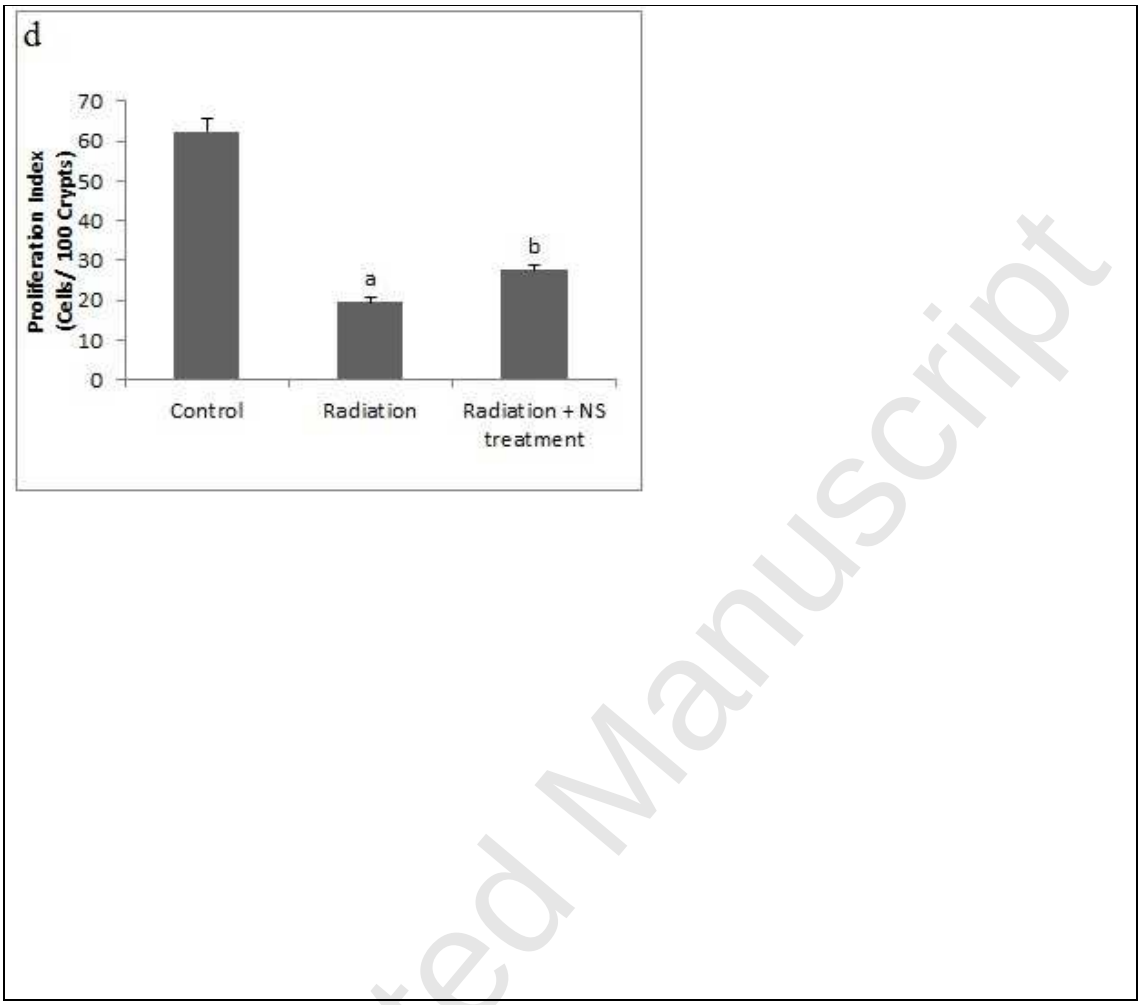


Fig. 2. Light microscopy of intestinal tissue in different groups. **(a)** Normal histopathology presented in sham group; **(b)** after radiation exposure, rat intestine shows severe histopathological injury including shortening of the villi, loss of villous epithelium, multiple erosions, inflammatory cell infiltration, necrosis and hemorrhage into the intestinal wall; **(c)** treatment with Nigella sativa greatly reduced all of these changes associated with radiation exposure; **(d)** intestinal mucosal injury evaluated by Chiu et al. (1970) scoring system in sham, radiation and radiation+Nigella sativa groups. $^{a}P < 0.0001$ compared to sham group, $^{b}P < 0.001$ compared to radiation group. (H&E, scale bar, 100 μm).





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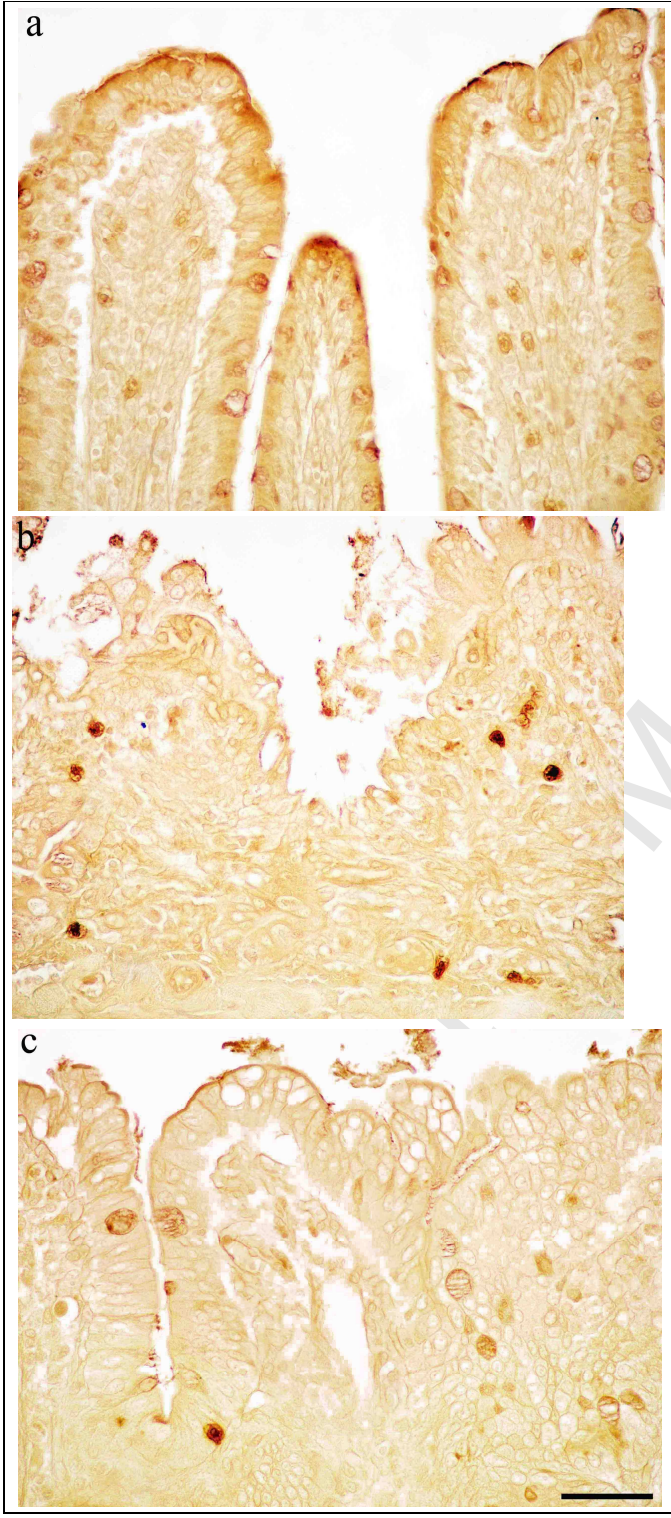

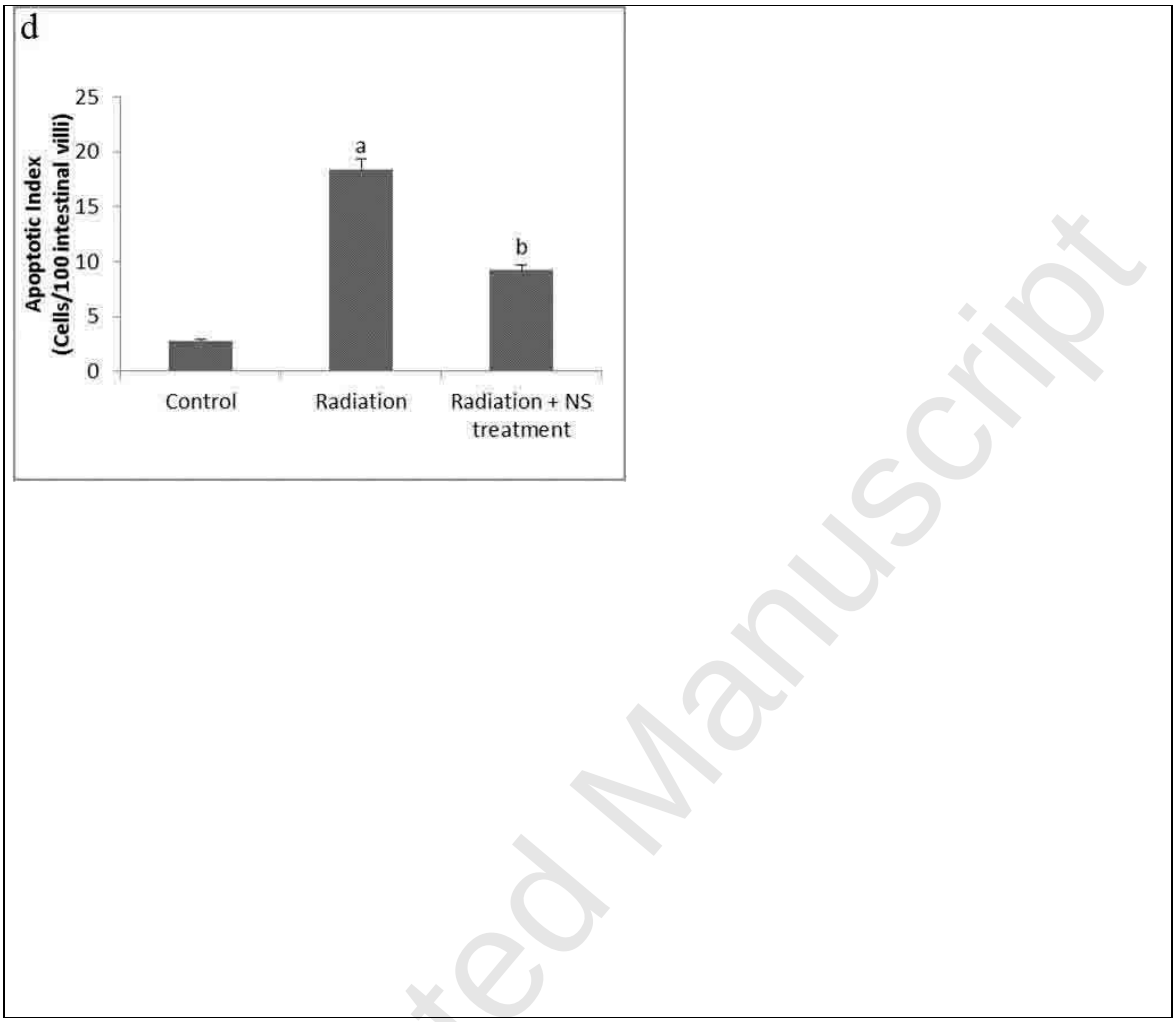

Fig. 4. Light microscopy of intestinal tissue in different groups. TUNEL-positive cells tended to be observed in the upper part of the villi. (**a)** In sham group, a few TUNEL-positive cells are observed in the intestinal villi; (**b)** TUNEL positive cells increased after radiation exposure in the detached epithelial cells from the villous tip in the radiation group; (**c)** treatment of Nigella sativa markedly reduced the number of TUNEL positive cells; (**d)** sequential changes in the apoptotic index are indicated in sham, radiation and radiation+Nigella sativa groups. $^{a}P < 0.0001$ compared to sham group, $^{b}P < 0.001$ compared to radiation group (Scale bar, 50 μm).