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Genistein Can Mitigate the Effect of Radiation on Rat Lung Tissue

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Abstract

We investigated whether genistein could protect the lung from radiation-induced injury. We hypothesized that genistein would reduce the levels of inflammatory cytokines and ROS after irradiation and therefore lead to reduced DNA damage and functional deficits. Whole lungs of Sprague-Dawley rats were irradiated with 18 Gy at ~0.5 Gy/min. At 28 weeks a micronucleus assay was used to examine DNA damage and, using immunohistochemical analysis, expression of IL-1α, IL-1β, IL-6, TNF-α and TGF-β, macrophage activation, oxidative stress (8-OHdG) and collagen levels were measured. A TBARS assay was used to measure the level of malondialdehyde. Functional damage was assessed by measuring the breathing rate of the rats over the course of the experiment. The increase in breathing rate after irradiation was damped in rats receiving genistein during the phase of pneumonitis (6–10 weeks), and there was a 50–80-day delay in lethality in this group. Genistein treatment also decreased the levels of the inflammatory cytokines TNF-α, IL-1β and TGF-β and led to a reduction in collagen content, a reduction in 8-OHdG levels, and complete protection against DNA damage measured in surviving rats at 28 weeks after irradiation. These results demonstrates that genistein treatment can provide partial protection against the early (pneumonitis) effects of lung irradiation and reduce the extent of fibrosis, although not sufficiently to prevent lethality at the radiation dose used in this study.

INTRODUCTION

The lung is a highly radiosensitive organ and therefore poses a problem for radiation exposures to the thoracic region (1). The mechanisms involved in the initiation and perpetuation of radiation-induced lung injury remain incompletely understood; however, many studies have implicated a cyclic inflammatory response associated with inflammatory cells, reactive oxygen species (ROS) and the up-regulation of pro-inflammatory cytokines as major factors in causing lung damage (2–7). The functional effects of radiation on the lung

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are generally thought to occur in two separate phases. Approximately 2 to 3 months after irradiation, radiation pneumonitis can occur followed by radiation fibrosis 4 months to 1 year postirradiation (8). These effects are dependent on the volume and the region irradiated (9–15). In addition, the inclusion of the heart in the radiation field has been reported to increase functional damage seen in the lung (5, 16, 17); however, this effect was not observed in other studies (14, 18). Our previous studies (4, 9) using partial-lung irradiation demonstrated DNA damage in and out of the radiation field, supporting the idea that some DNA damage may be caused by the action of inflammatory cytokines and the resultant production of ROS and/or reactive nitrogen species (RNS) such as peroxynitrite. Consistent with this idea, treatment with superoxide dismutase (MnSOD, CuZn-SOD) reduced out-of-field DNA damage (4). Furthermore, mice overexpressing a transgene for human MnSOD (19, 20) and transgenic mice overexpressing extracellular (EC)-SOD in alveolar and airway epithelial cells (21) were found to be protected against radiation-induced lung injury.

The out-of-field effect demonstrated previously in our laboratory (4, 9) is of interest in elucidating possible mechanisms underlying the inflammatory response stimulated by irradiation of the lung, since we reported in our previous paper (22) that inflammatory cytokines and activated macrophages were up-regulated to an equal degree in and out of the field, whereas DNA damage was induced to a greater degree in field than out of field. We examined cytokine up-regulation, macrophage activation and micronucleus formation at several times over 16 weeks and observed a cyclic pattern. When Eukarion-189 (EUK-189), a SOD-catalase mimetic, was administered after irradiation, much of the DNA damage was scavenged both in and out of field (23), indicating that much of this damage is probably caused by ROS (and/or RNS).

In the current work we examined whether genistein, a soy isoflavone that is a non-specific protein kinase inhibitor and acts as a scavenger for ROS, could protect the lung from radiation-induced injury. Genistein is reported to block the activity of NFκB, a transcription factor that regulates the expression of many cytokines, chemokines, immune receptors and cell adhesion molecules that are activated by different stress conditions, including oxidative stress (24). Based on work demonstrating that genistein administered at 50 mg/kg by intraperitoneal injection could prevent the acute inflammatory response in lung induced by LPS treatment (25), we hypothesized that genistein would reduce the levels of inflammatory cytokines and ROS after irradiation and therefore lead to reduced DNA damage and functional deficits.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats (Charles River Laboratories International, Inc., Wilmington, MA) aged 7–8 weeks and weighing 180–200 g were used in all experiments. The animals were housed in facilities accredited by the Canadian Council on Animal Care and were treated in accordance with approved protocols. In the main experiment, the rats were divided into four experimental groups: radiation and control diet (n = 16), radiation and genistein diet (n = 16), no radiation and control diet (n = 8), and no radiation and genistein diet (n = 8). The AIN-76A diet (Harlan Teklad, Madison, WI), a semi-purified casein-based diet containing no detectable phytoestrogens (limit of detection 5 pmol/liter) was selected as the control diet. The genistein diet was formulated from the control diet, supplemented with 750 mg/kg of genistein, which has been used by other investigators (26). This concentration has been reported to yield serum genistein levels in mice (1–2 μmol/liter) similar to those observed in humans consuming a diet containing modest amounts of soy products (27). Genistein was chemically synthesized (Toronto Research Chemicals Inc, Toronto, Ontario) and incorporated into the AIN-76A diet at Harlan Teklad. Food intake per rat was measured

to be 25–30 g/day, representing a genistein intake of 18.5–22.5 mg/rat. Assuming an oral bioavailability of approximately 20–25% the rats should have absorbed a dose of 20–25 mg/kg.

Radiation

All irradiated animals received a single dose of ^{60}Co γ radiation to the whole lung using a well-established protocol (4, 9) described previously (22). The rats were anesthetized with halothane and put into Perspex jigs. An X ray was taken to define the position of the lungs, and then 10-cm-thick lead blocks, were used to define the radiation field. A dose of 18 Gy (dose rate approximately 0.5 Gy/min) was given to all animals, which were irradiated in groups of four. It was anticipated from previous studies (23, 28, 29) that, at the dose rate used, this dose of 18 Gy would be approximately equivalent to 14 Gy at a dose rate above 1 Gy/min.

Breathing Rate

We measured the breathing frequency of rats as a measure of functional damage. The rats were acclimatized to the method several times in the 2 weeks preceding the start of the experiment. Breathing frequencies were measured once in week 2 and week 4 and then weekly from 6 to 28 weeks postirradiation using a respiration rate monitor (Columbus Instruments, Columbus, OH). The breathing rate of each animal was measured for 2 min after an initial 45-s acclimatization period. Breathing rates were determined by taking the mean of a maximum of five 6-s intervals of calm breathing within the 2-min measurement period. The rats were weighed before each breathing rate measurement was taken, and their food consumption was measured every week. Animals were euthanized if their body weight dropped below 80% of their initial weight or if they became moribund.

Micronucleus Assay

This assay was used to assess radiation-induced DNA damage in rat lungs after irradiation. The protocol was described in detail by Khan *et al.* (4, 9). Briefly, upon killing, the lungs of the each animal were perfused with Hanks' balanced salt solution through the inferior vena cava and were then divided into four quadrants (left upper, left lower, right upper and right lower). In our initial studies all four quadrants were analyzed separately for micronucleus formation, whereas in the main part of the study one quadrant from each rat was used. The tissue was minced and digested with 0.25% trypsin for 80 min at 37°C. The digest was mixed with an equal volume of culture medium, filtered, centrifuged and suspended in culture medium. The cell suspension was plated on single-chambered sterile Permanox slides for 24 h. The culture medium was then supplemented with 3 μg/ml Cytochalasin B (Sigma, Mississauga, Ontario, Canada) to halt cytoplasmic division to produce binucleated cells. After 72 h the cells were incubated with a hypotonic solution for 10 min, fixed with 95% methanol, stained with Acridine Orange (Difco, Burlington, Ontario, Canada), and scored under a fluorescence microscope. The results were recorded as number of micronuclei per 1000 binucleate cells.

Immunohistochemical Staining

In the majority of the animals we injected a volume (0.5–1.0 ml) of 10% formalin into the quadrant of the lung used for staining to separate the alveolar walls (this was not possible in a few animals). The lungs were then placed in 10% formalin for at least 48 h for fixation, the lungs were embedded in paraffin, and sections 5 μm thick were cut and placed on slides in preparation for immunohistochemical staining. Following a well-established protocol (30) that is described in detail in our previous paper (22), slides were deparaffinized and rehydrated in xylene and alcohol. Endogenous peroxidase activity was blocked by

incubation in 3% hydrogen peroxide for 10 min, and then the slides were incubated in citrate buffer for antigen retrieval. Slides were incubated with primary antibodies to the activated macrophage marker ED-1 (MCA341, 1:100, Serotec), IL-1α (sc-1254, 1:100, Santa Cruz Biotechnology), IL-1β (AAR15G, 1:1000, AbD Serotec, Oxford, UK), IL-6 (sc-1265, 1:200, Santa Cruz Biotechnology), TNF-α (sc-1357, 1:200, Santa Cruz Biotechnology), TGF-β (MCA797, 1:50, AbD Serotec) and 8-OHdG (MOG-110P, 1:1000, JaICA, Shizuoka, Japan) for 1 h at 37°C in a moist chamber. After rinsing with PBS, slides were incubated with the appropriate secondary antibodies: biotinylated anti-mouse IgG (BA-2001, 1:200, Vector Laboratories, Burlingame, CA), biotinylated anti-rabbit IgG (BA-1000, 1:200, Vector Laboratories) and biotinylated anti-goat IgG (BA-5000, 1:300, Vector Laboratories) for 30 min at room temperature. A streptavidin enzyme complex (ID Labs Inc, London, Ontario, Canada) was added to the slides and incubated for 20 min at room temperature. Finally the slides were incubated with a Novared™ substrate followed by counterstaining with Mayer's hematoxylin and mounted with cover slips.

Masson's Trichrome Staining

Slides were deparaffinized and rehydrated as described above. After a wash in distilled water, the slides were stained with Weigert's iron hematoxylin working solution for 10 min. The slides were rinsed in warm running tap water for 10 min and then washed in distilled water. Biebrich scarlet-acid fuchsin solution was added for 15 min, and then the slides were washed in distilled water. The slides were then incubated in phosphomolybdic-phosphotungstic acid solution for 15 min and then transferred immediately to aniline blue solution for 5–10 min. After a rinse in distilled water, the slides were differentiated in 1% acetic acid solution for 5–10 min and then washed again in distilled water. The slides were finally dehydrated quickly through an alcohol series and cleared in xylene before being mounted with a cover slip.

Image Analysis

After staining, the slides were scanned using the ScanScope XT (Aperio Technologies, Vista, CA). This bright-field scanner digitizes the whole microscope slide at 20× and 40× magnification and provides high-resolution images that can be viewed with ImageScope (Aperio Technologies) for quantitative analysis. Using the Positive Pixel Algorithm, the whole slide was analyzed and the numbers of positive pixels/numbers of positive and negative pixels × 100% were recorded (percentage positivity).

TBARS Assay

This assay was used to measure the level of malondialdehyde (MDA) that is produced after lipid peroxidation as a measure of oxidative damage in membranes. One quadrant from each rat lung was used for the TBARS assay. The sample was immediately flash-frozen in liquid nitrogen and kept at −80°C until assayed. The tissue was then sonicated for 15 s in RIPA buffer (Cayman Chemical Company, Ann Arbor, MI) with protease inhibitors (Roche Applied Science, Laval, Quebec) and then centrifuged. The supernatant was used for the assay. Using a TBARS assay kit (Cayman Chemical Company), 100 μl of SDS solution was added to 100 μl of MDA standards (ranging from 0–50 μ*M*) or sample. To these vials, 4 ml of color reagent was added forcefully, and then the vials were capped and boiled for 1 h. The vials were removed and immediately placed on ice for 10 min. The vials were then centrifuged for 10 min. A volume of 150 μl from each vial was placed in a plate, and the absorbance at 530–540 nm was read using a plate reader.

Sircol Assay

The Sircol assay (Biocolor LTD, County Antrim, UK) is a colorimetric procedure that uses a dye reagent containing Sirius Red in picric acid, which specifically binds to soluble collagen. It measures recently synthesized collagen. One lobe from each lung was frozen in liquid nitrogen and kept at −80°C until assayed. The samples were then defrosted and homogenized in PBS. A set of collagen standards was prepared containing 0 to 50 μg of collagen. Then 1 ml Sircol Dye reagent was added to each tube containing test sample or standard and mixed by a mechanical shaker for 30 min. The tubes were then centrifuged for 10 min, and the unbound dye was removed by carefully inverting and draining the tubes. Then 1 ml of Alkali reagent was added to each tube and mixed using a vortex mixer. The absorbance was measured using a spectrophotometer set at 540 nm. A standard curve was derived and the collagen content of the samples was calculated.

Statistical Analysis

One-way analysis of variance (ANOVA) with Tukey's post test was used for multiple comparisons of treated and untreated groups for the cytokine levels, Masson Trichrome, markers of oxidative damage and number of micronuclei. Statistical analyses of survival and breathing rate were done using the Wilcoxon signed rank test and linear mixed modeling, respectively. The linear mixed modeling with autoregressive covariance was done using SAS software. In all statistical analyses, $P < 0.05$ was considered as significant. In all figures, the values are means ± SEM.

RESULTS

Initial studies examined the effect of different doses of genistein given intraperitoneally (dissolved in 1.4 μmol DMSD) immediately after lower or whole-lung irradiation. DNA damage (MN assay) was assayed at 18–20 h after irradiation. These results (Fig. 1) demonstrated that complete protection against DNA damage both in and out of field could be obtained with doses of 12.5 mg/kg and above. Preliminary studies also suggested that four doses of 50 mg/kg genistein given i.p. immediately after irradiation and then daily for the next 3 days provided some protection against increased breathing rates after both 15.5 and 18 Gy (data not shown). Based on these findings, we designed our experiment to examine the effects of putting the animals on a diet containing 750 mg/kg genistein immediately after 18 Gy to the whole lung, a dose that was chosen to achieve significant functional effects. The diet was chosen to be more appropriate for clinical application and was expected to be equivalent to a dose of approximately 20–25 mg/kg (see Methods section). Measurements in a separate group of 17 rats after 4, 8 or 14 weeks on the diet gave a mean plasma level of free genistein of 55.9 (SD 52.2) nmol/liter with no significant differences between the times.

The percentages of animals surviving after the 18 Gy whole-lung irradiation with either the control diet or genistein diet are shown in Fig. 2. The rats on the control diet showed a sharp decrease in survival between 40 and 80 days (approximately 6–12 weeks), with almost 50% of the rats having to be euthanized between 40 and 60 days. Rats on the genistein diet did not become moribund and were not euthanized until later, with the main effect on survival occurring between 70 and 150 days (approximately 10–21 weeks). At the end of the experiment, 28 weeks postirradiation, there was no difference in survival between the two groups.

Figure 3 shows the average breathing rates for rats over the 28 weeks after irradiation. The rats irradiated with 18 Gy and put on the control diet demonstrated two waves of increase in breathing rate. The first increase occurred between 40 and 80 days (approximately 6–12

weeks) with a peak (approximately 310 bpm) at approximately 75 days postirradiation. The second increase in breathing rate occurred between 100 and 170 days postirradiation with a shallower peak (approximately 300 bpm) at 140 days. The rats on the genistein diet after irradiation also showed two waves of increase in breathing rate. However, the first increase, occurring between 40 and 70 days after irradiation, was damped compared to that seen in the control rats (peak at approximately 250 bpm at 55 days). The second wave occurring between 100 and 185 days postirradiation was similar to that for the rats on the control diet (shallow peak at approximately 310 bpm). With no irradiation, the breathing rates for rats on both the control and the genistein diet stayed at baseline levels, between approximately 180 and 220 breaths per minute, over the 28 weeks. The overall effect of genistein on breathing rate was not significant. The effect was also insignificant for the pneumonitis phase. However, the overall breathing rate of both the irradiated only and irradiated with genistein groups were significantly different from the control and control with genistein groups (P = 0.019 and P = 0.03, respectively). For the rats that survived to the end of the experiment in both irradiated groups during the pneumonitis phase, the effect was not significant.

The results of a quantitative analysis of DNA damage (micronuclei) in the lung cells for the animals that survived to 28 weeks are shown in Fig. 4. Unirradiated rats on either the control or genistein diet had background levels of DNA damage (approximately 26 micronuclei per 1000 binucleate cells). After 18 Gy whole-lung irradiation, there was a high level of DNA damage in rats on the control diet. However, rats fed the genistein diet had very low levels of DNA damage, with micronucleus formation at control (background) levels.

The inflammatory cytokines IL-1α, IL-1β, IL-6, TNF-α and TGF-β were analyzed at the protein level by immunohistochemical staining in lung tissue from rats that survived to 28 weeks, as shown in Fig. 5. No differences were observed in the expression of any of the cytokines between rats on the genistein and control diets after sham irradiation, so the control indicates the average value of these two groups. At 28 weeks after irradiation there were significantly higher levels of IL-6 and IL-1α relative to control values, and no difference was seen between the radiation only and radiation plus genistein groups. For IL-1β, TGF-β and TNF-α, a significant increase was observed after irradiation compared to the control groups; however, when genistein was given in the diet, the irradiated group showed levels similar to those in the control rats.

Results of the analysis of macrophage activation at 28 weeks after 18 Gy whole-lung irradiation are shown in Fig. 6. No difference was seen between the genistein and control diet groups after sham irradiation, so the control represents both of these groups. After irradiation, the percentage positivity of ED-1 staining was significantly increased compared to control values. When genistein was given in the diet after irradiation, the activated macrophages levels were significantly reduced to levels similar to those seen for the control rats.

Masson’s Trichrome stains collagen fibers and is therefore a marker of collagen deposition and fibrosis. Results using this stain on lung tissue from rats that survived to 28 weeks after irradiation are shown in Fig. 7. No difference was seen between the genistein and control diet groups after sham irradiation, so the (old) control represents both of these groups. These (old) control rats, however, showed significantly more staining than was seen in the control rats killed at the beginning of the experiment (young). The radiation-alone group demonstrated significantly more staining than the control groups. When genistein was given after irradiation, a significant decrease in positive staining was observed, although the levels remained above those seen in the control (old) rats.

Results from the Sircol Assay are also shown in Fig. 7. This method can be used to quantify the amount of soluble collagen (recently synthesized collagen) in tissue. As with the Masson's Trichrome results, the old control rats had more soluble collagen than the young control rats. After irradiation, the quantity of soluble collagen increased significantly to well above the levels in the young control rats; however, the levels were not significantly different from those of the (old) control rats that were killed 28 weeks after irradiation. After genistein administration, a decrease in the quantity of soluble collagen was observed; however, this decrease was not significant.

Figure 8 shows analysis of 8-oxo-deoxyguanosine (8-OHdG) staining. 8-OHdG is a specific marker for oxidative DNA damage and is used to assay for oxidative stress. As in previous figures, no difference was seen between the genistein and control diet groups after sham irradiation. No difference in the percentage positivity of staining was seen between control rats killed at the end of the experiment (old) and control animals killed at the beginning of the experiment (young). After irradiation the percentage positivity of 8-OHdG was significantly higher than that for control groups. When genistein was administered after irradiation, the amount of oxidative damage decreased significantly to levels similar to those seen in control rats.

The concentrations of MDA are also shown in Fig. 8. MDA is a product of lipid peroxidation and therefore can be used as a marker of oxidative damage to membranes. The MDA concentrations were similar for the old and young controls. After irradiation the concentration of MDA increased compared to control values, though not significantly. When genistein was given in the diet after irradiation, the MDA concentrations levels were slightly higher (not significant) than those after irradiation alone.

DISCUSSION

Our results show that after irradiation the initial wave of increase in the breathing rate during the pneumonitis phase (40–100 days) showed a trend to be reduced by treatment with genistein. A second wave of increased breathing frequency that occurred later, at a time consistent with the early stages of fibrosis, was not affected by the genistein treatment, despite the fact that both measures of collagen content in the lungs of the rats surviving to 28 weeks was reduced by the genistein treatment. The reduction in breathing rate during the pneumonitis phase coincided with improved survival during the same period for rats treated with radiation and genistein. However, this effect was only transient, because the genistein-treated rats died about 50–80 days later. This suggests that the genistein treatment may have delayed the pneumonitis phase, but the breathing rate data in Fig. 3 do not indicate any delay in the increase in breathing rate in the treated rats. Regardless of any improvement in breathing frequency during the pneumonitic stage, there was no overall improvement in survival at 28 weeks.

Several studies (4, 9, 13, 31) have suggested that infield DNA damage after lung irradiation is caused by both the direct effects of radiation and the indirect effects of the inflammatory response, whereas the out-of-field damage may be caused by the indirect effects of the inflammatory response alone. The exact mechanisms involved in the inflammatory response to radiation are unknown; however, it has been suggested that the generation of ROS (and RNS) immediately after irradiation, together with a cyclic (and chronic) up-regulation of inflammatory cytokines and the recruitment of inflammatory cells such as macrophages and neutrophils, is responsible for the damage seen in the lung after irradiation (32).

Evidence for a role of ROS has been demonstrated in several studies using antioxidants. Langan *et al.* (23) demonstrated that Eukarion-189 (a salen-manganese compound that acts

as a SOD/catalase mimetic) given both before and after irradiation dramatically reduced the micronuclei (by at least 50%) seen in lung fibroblasts after irradiation. Vujaskovic *et al.* (6) looked at the functional end point of breathing rate after irradiation and treatment with the SOD mimetic AEOL 10113 and found that this compound gave significant protection against changes in early breathing rate and late fibrosis. Further studies by this group (21) demonstrated that AEOL 10150 given at a high dose (5 mg/kg) significantly reduced structural lung damage and collagen deposition at late times (26 weeks postirradiation). They also saw a reduction in TGF-β1 levels and 8-OHdG and ED-1 staining levels. Gauter-Fleckenstein *et al.* (33) showed that two Mn porphyrin-based mimics of SOD administered for 14 days after a dose of 28 Gy to the right lung significantly reduced the elevation in breathing rate from 4–16 weeks after irradiation.

In our present study we demonstrated that DNA damage in the form of micronucleus formation was reduced to background levels by administering genistein after irradiation. This provides further evidence that production of ROS is a major contributor to DNA damage. It is of particular interest that even at 28 weeks after irradiation significant DNA damage was detected in lung cells from the surviving irradiated rats that was completely abrogated if the rats were fed the genistein diet (Fig. 4). These results suggest that the DNA damage detected does not influence long-term survival. To assess this issue further, we examined the protein levels of certain inflammatory cytokines as well as the activation of macrophages. At 28 weeks, all cytokines were increased by radiation; however, the levels of TNF-α, IL-1β and TGF-β, but not those of IL-1α and IL-6, were reduced by the administration of genistein. Chen *et al.* (34) found that serum levels of the cytokines Il-1α and IL-6 are during radiation pneumonitis in humans and suggested that they can be used as markers of radiation pneumonitis in patients receiving thoracic radiation.

Raschke *et al.* (35) investigated the effects of genistein on oxidative-induced DNA damage using the oxidant H_2O_2 to induce oxidative stress in cells of a prostate carcinoma cell line. They observed that preincubation with genistein for 24 h reduced DNA strand breaks as assessed by the comet assay, consistent with our results for MN formation after irradiation. Landauer *et al.* (36) administered a single subcutaneous dose of genistein either 1 h or 24 h prior to a single lethal dose of γ radiation. For mice treated with genistein (25 to 400 mg/kg) 24 h prior to irradiation, a significant increase in 30-day survival was observed compared to control mice. Day *et al.* (37) examined the effects of 200 mg/kg genistein on 30-day survival and lung injury after 7.75 Gy total-body irradiation. Significant improvement in 30-day survival was observed after genistein pretreatment as well as a reduction in micronuclei 24 h postirradiation and focal distribution of collagen-rich plaques 90 days postirradiation. Genistein also prevented the reduction in TGFβR1 after irradiation.

There is a great deal of evidence that a cyclic cascade of inflammatory cytokines, together with the activation of macrophages, is initiated very early after irradiation. It is believed that the further production of ROS by both cytokines and macrophages perpetuates the inflammatory response, leading to pneumonitis and at a later stage to radiation-induced fibrosis. TGF-β is both a pro- and anti-inflammatory molecule. At the site of injury it activates inflammatory cells such as macrophages to release inflammatory cytokines. TGF-β is also a key player in the formation of fibrosis by stimulating fibroblast differentiation and collagen deposition. As mentioned above, we observed that at 28 weeks postirradiation the levels of TGF-β were reduced after treatment with genistein compared to levels induced after irradiation alone. Consistent with this, we also found that the collagen content of the lungs at this time was reduced after treatment with genistein. The amount of collagen increased with age in the control (nonirradiated) rats, indicating that collagen deposition is dependent on age.

TNF-α and IL-1 are known to stimulate NF-κB, a transcription factor that is also stimulated by oxidative stress. It is a central mediator of the inflammatory response and can induce the expression of inflammatory cytokines. Genistein can block the transcriptional activity of NF-κB and might therefore be expected to reduce the levels of cytokines that would otherwise be induced. Natarajan *et al.* (38) observed that NF-κB is activated within 30 min after low-dose irradiation, with a maximum at 3 h after irradiation, followed by a second wave of activation 24 to 48 h after irradiation. The study was done *in vitro*, and when the cells were incubated with a TNF-α soluble receptor or a TNF-α neutralizing antibody, this second wave of activation was blocked. They further investigated the effect of incubating cells with an NF-κB inhibitor and found that TNF-α was inhibited at both the mRNA and protein level. Our results show that genistein can reduce the increased level of TNF-α at 28 weeks after irradiation and that TNF-α staining was localized to macrophages. In addition, genistein was reduce the level of ED-1 staining, suggesting a reduction of activated macrophages that is consistent with the suggestion that TNF-α may be responsible for initiating the recruitment of macrophages and other inflammatory cells to the site of injury.

An interesting aspect of our results examining the effect of genistein on oxidative stress is that the data for DNA damage (MN assay and 8-oxodeoxyguanosine measurements) indicate a reduction by genistein treatment, but the MDA analysis of lipid (membrane) peroxidation suggests no effect. As discussed in our previous paper (39), one possible explanation for this apparent discrepancy may be that DNA damage is a result of ROS produced internally in the cell (directly by radiation or by leakage from damaged mitochondria) plus some leakage into the cell of ROS produced externally as a result of the inflammatory process, while most of the lipid peroxidation is caused primarily by externally produced ROS. This would be consistent with the much higher levels of ROS (several orders of magnitude) that can be produced by inflammatory cells than would be expected to be generated by the radiation doses used in this study. If this hypothesis is correct, it may explain why the genistein treatment we used is only partially effective in preventing functional damage. The drug level may have been sufficient to scavenge low levels of ROS in the cells but insufficient to block higher levels of extracellular ROS. However, it is also possible that the functional deficit is a result of a slow buildup of damage and, since the inflammatory response is cyclical, the ROS levels at any given time may not necessarily reflect such a buildup.

Conclusion

Our study has demonstrated that genistein provides partial mitigation of the effects of lung irradiation. Functionally we saw a trend for an improvement in breathing rate during the stage of pneumonitis and a delay of lethality during the same phase. At the molecular level we observed a decrease in important pro-inflammatory cytokines (IL-1α, TNF-α and TGF-β) and a reduction in collagen content, a major contributor to fibrosis. Most interestingly, we saw a complete reduction of DNA damage; however, this did not contribute to any improvement in long-term animal survival. This may be due to the levels of drug used in this study. Thus examining the effects of higher doses of genistein will be important for future experiments. Time scheduling for treatment with genistein may also be important; further studies are needed to determine the effects of the genistein treatment on cytokine levels and lipid peroxidation levels at earlier times during the development of the functional damage.

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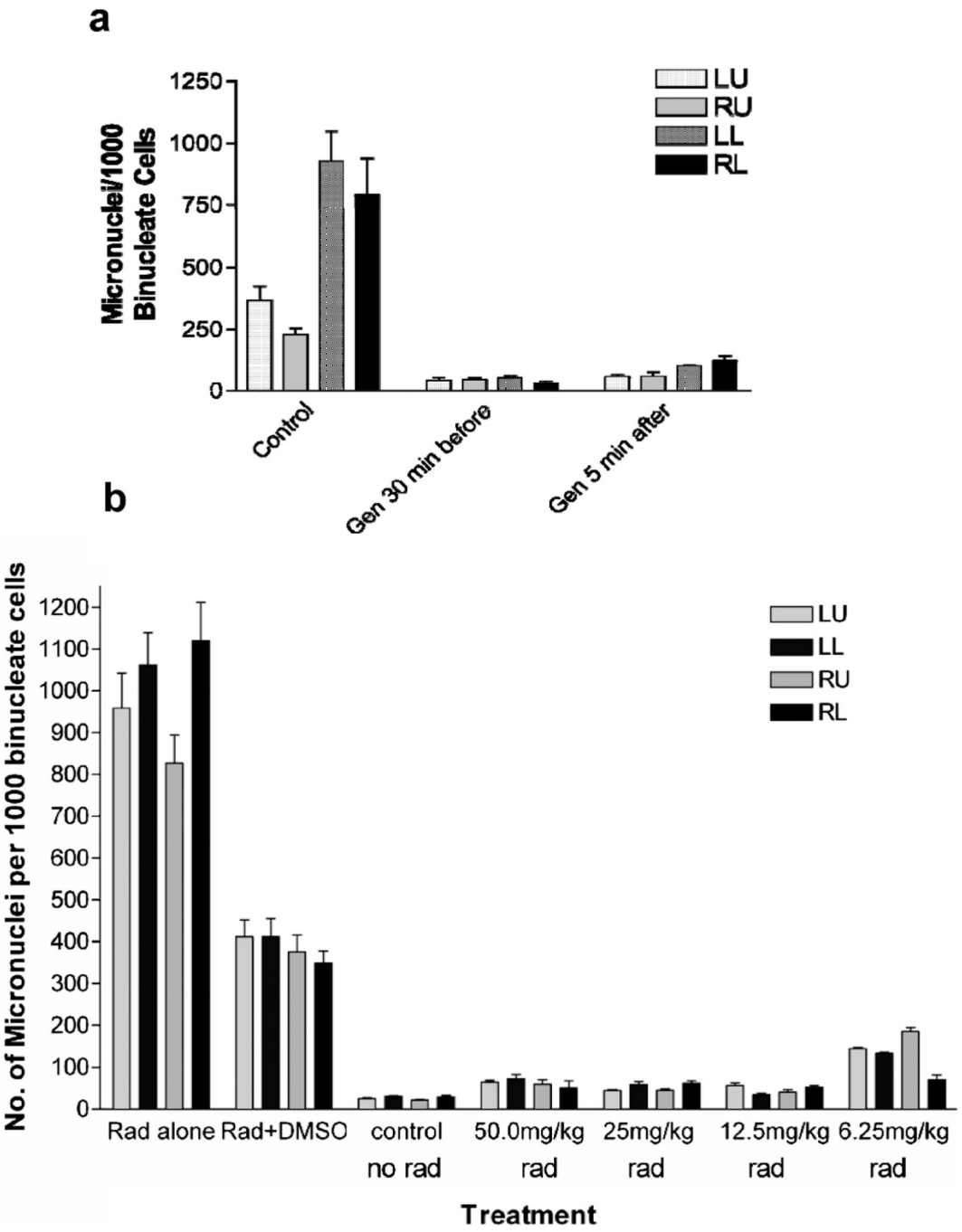

FIG. 1.

Micronucleus yields after lower (panel a) or whole (panel b) lung irradiations (10 Gy) with or without genistein treatment assayed at 18–20 h after irradiation. Panel a: Genistein was injected i.p. (50 mg/kg) 30 min prior to irradiation or 5 min postirradiation. The control data are significantly different from those for both treatment groups. Panel b: Genistein was injected at various doses immediately after irradiation. Each bar represents the mean ± SE (*n* = 4 for all treatment groups) for the individual quadrants; LU = left upper, LL = left lower, RU = right upper, RL = right lower.

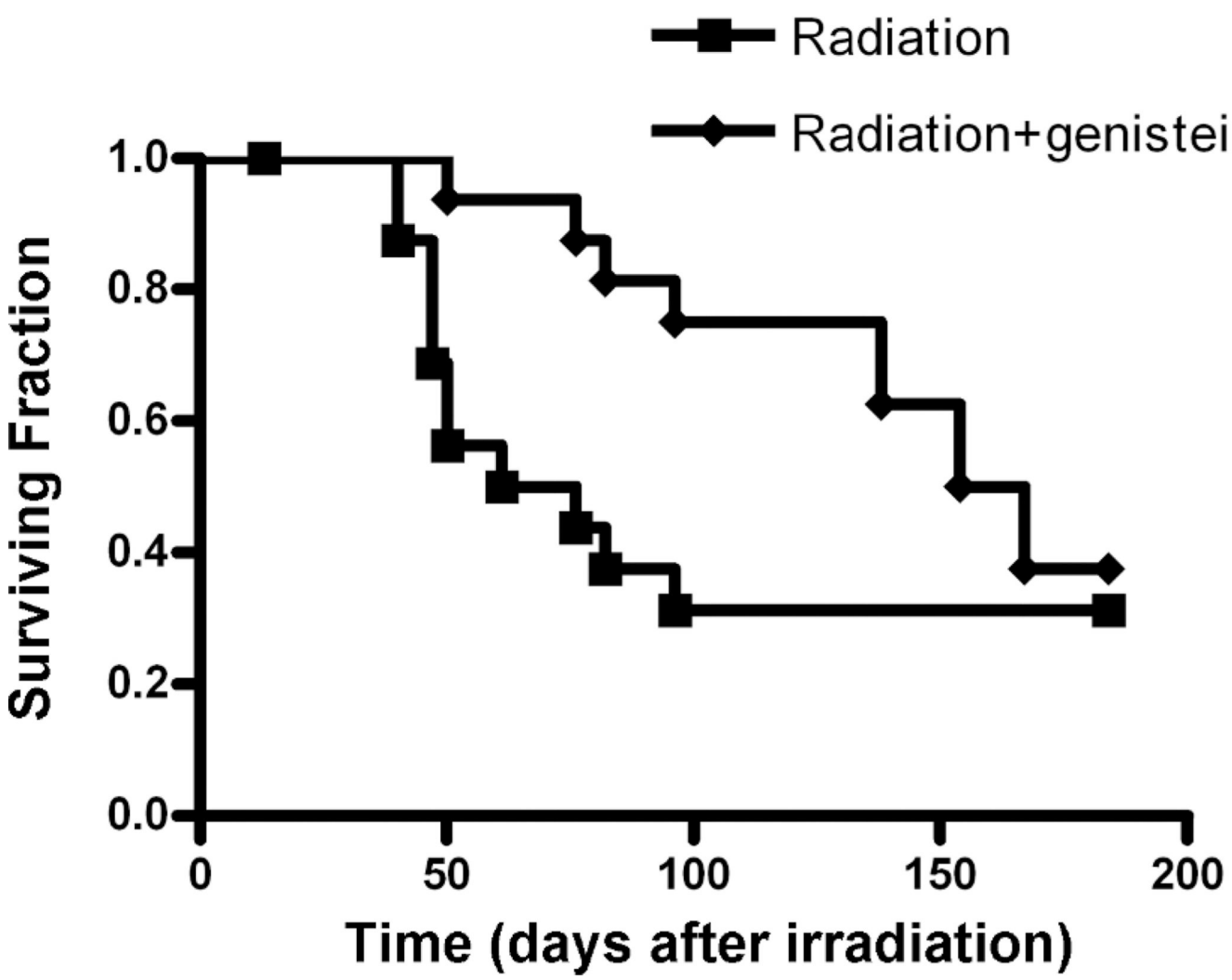

FIG. 2.
Survival after 18 Gy whole-lung irradiation. The squares represent rats on the control (soy-free) diet and the diamonds represent rats on the genistein diet.

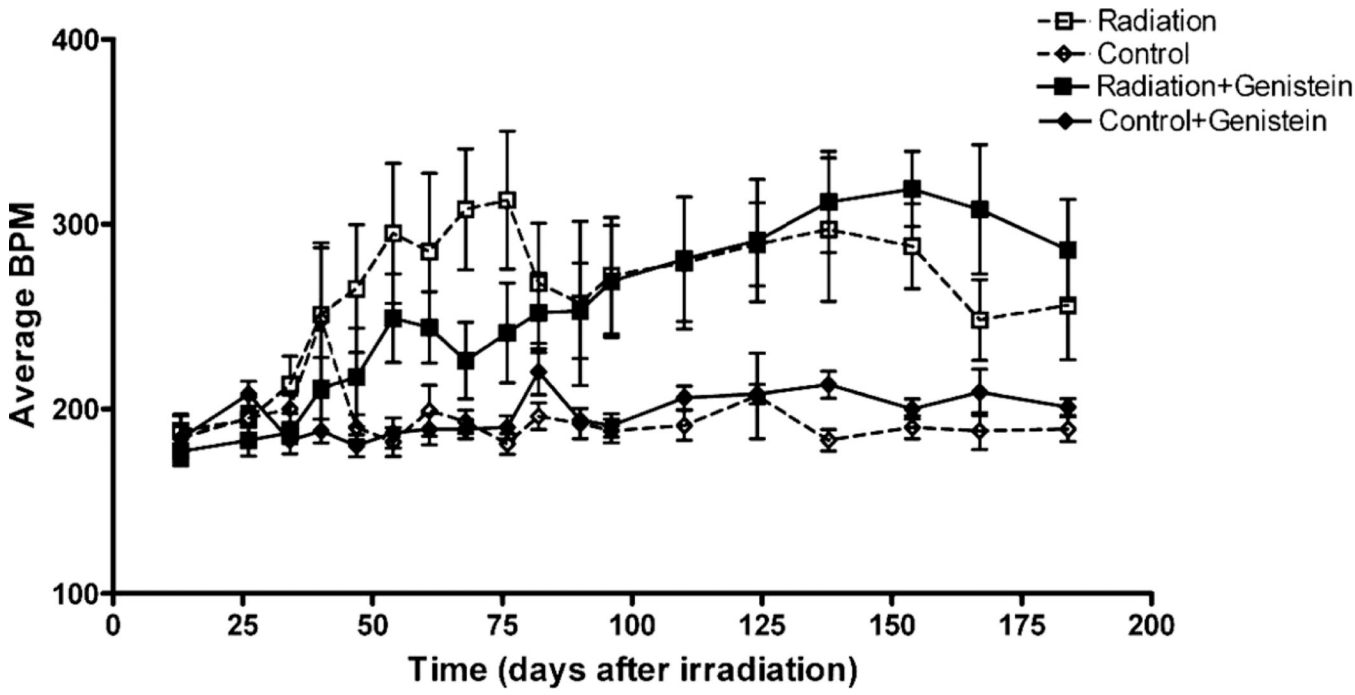

FIG. 3.

The average breaths per minute of unirradiated rats on the control or genistein diet and of rats irradiated with 18 Gy to the whole lung and placed on the control or genistein diet. Each point represents the mean ± SEM.

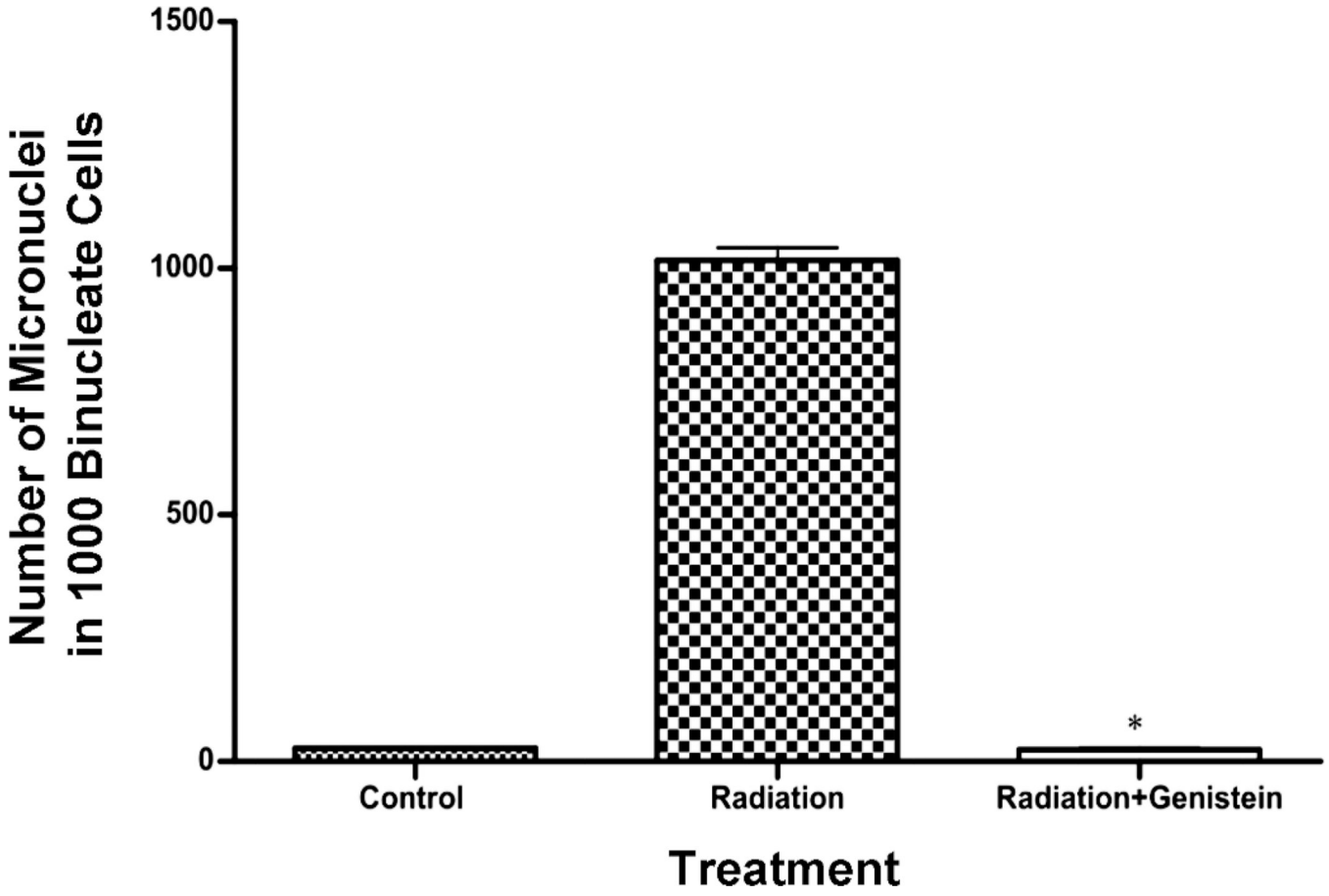

FIG. 4.

Micronuclei per 1000 binucleate cells in lung fibroblasts. Control represents the combined groups of no radiation and control diet and no radiation and genistein diet. The radiation group was given 18 Gy whole-lung irradiation and placed on a control (soy-free) diet, and the radiation plus genistein was given 18 Gy whole-lung irradiation and placed on a diet containing genistein. Bars represent the means ± SEM. The asterisk indicates a statistically significant difference from the radiation-only group.

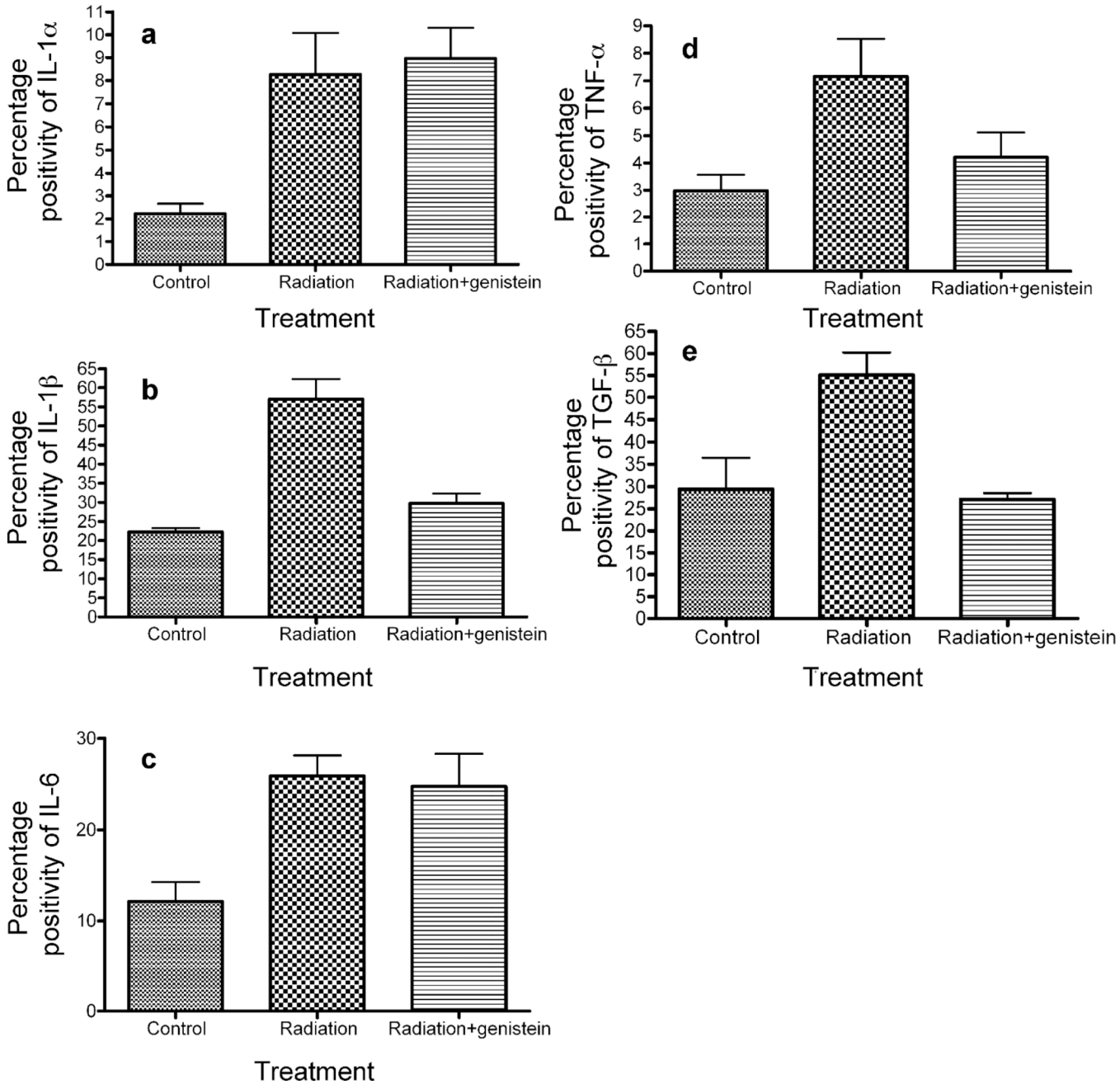

FIG. 5.

Protein expression of IL-1α, IL-1β, IL-6, TNF-α and TGF-β. Percentage positivity is calculated as the number of positive pixels/number of positive and negative pixels. Control represents the combined groups of no radiation and control diet and no radiation and genistein diet. The radiation group was given 18 Gy whole-lung irradiation and placed on a control (soy-free) diet, and the radiation plus genistein was given 18 Gy whole-lung irradiation and placed on a diet containing genistein. Bars represent means ± SEM.

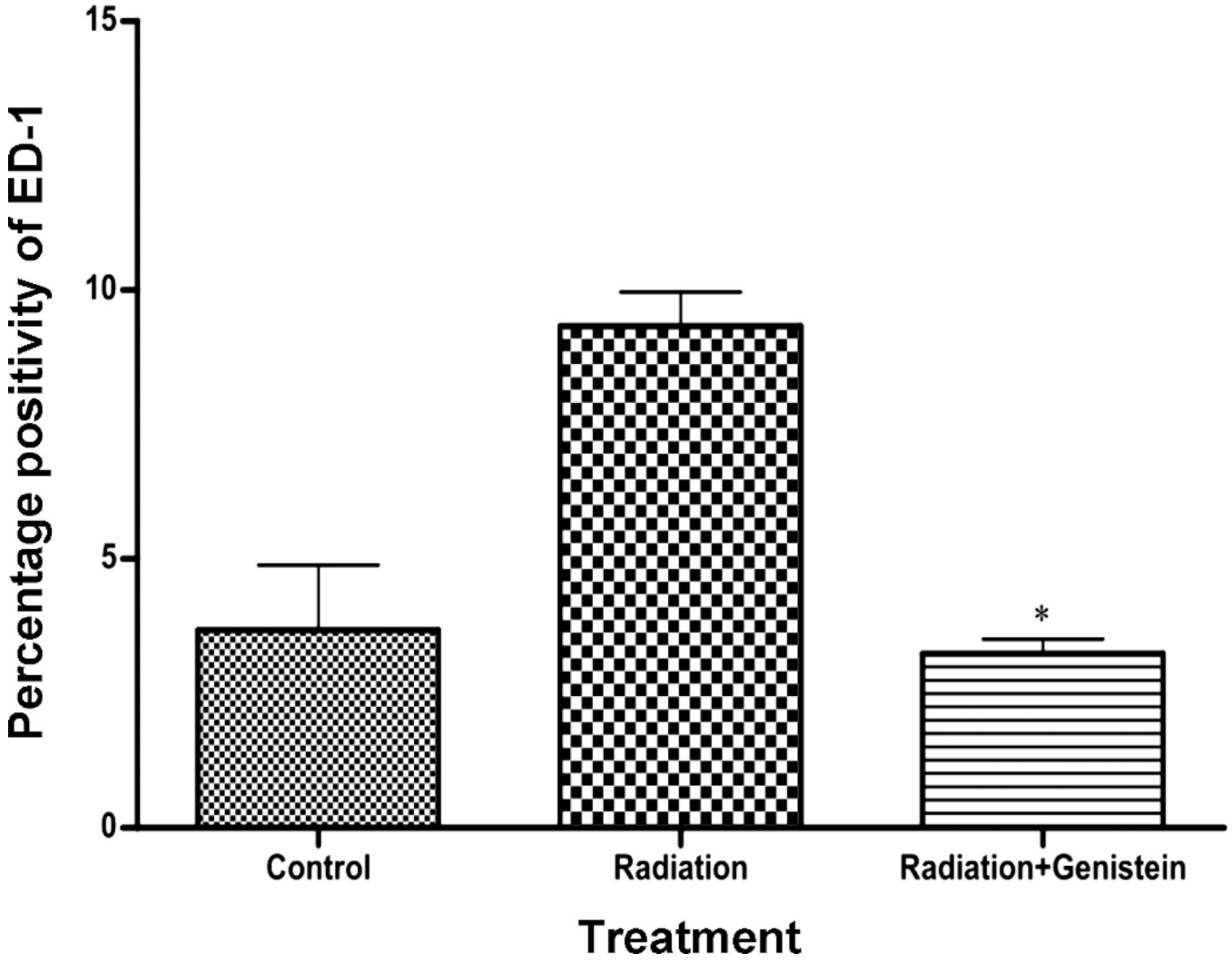

FIG. 6.

Positivity of ED-1, a marker for activated macrophages in the rat. Control represents the combined groups of no radiation and control diet and no radiation and genistein diet. The radiation group was given 18 Gy whole-lung irradiation and placed on a control (soy-free) diet and the radiation plus genistein group was given 18 Gy whole-lung irradiation and placed on a diet containing genistein. Bars represent means ± SEM. The asterisk indicates a significant difference from the radiation-only group.

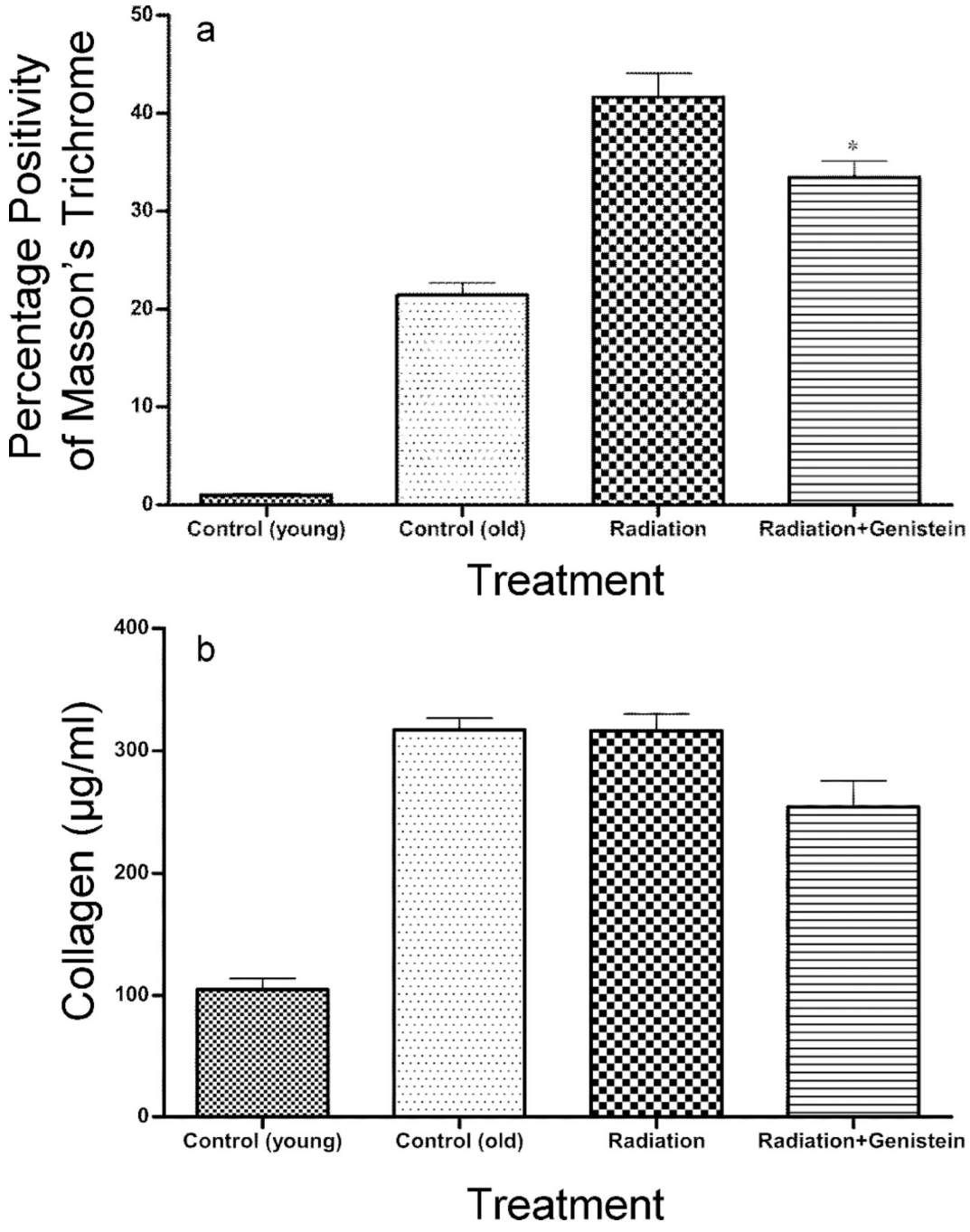

FIG. 7.

Masson's Trichrome staining for collagen content (panel a) and Sircol Assay quantifying soluble collagen (panel b). Control (young) animals were assayed at approximately 8 weeks of age. Control (old) animals approximately 35 weeks of age include the combined groups of no radiation and control diet and no radiation and genistein diet. The radiation group was given 18 Gy whole-lung irradiation and placed on a control (soy-free) diet and the radiation and genistein was given 18 Gy whole-lung irradiation and placed on a diet containing genistein. Bars are means ± SEM. The asterisk indicates a significant difference from the radiation-only group.

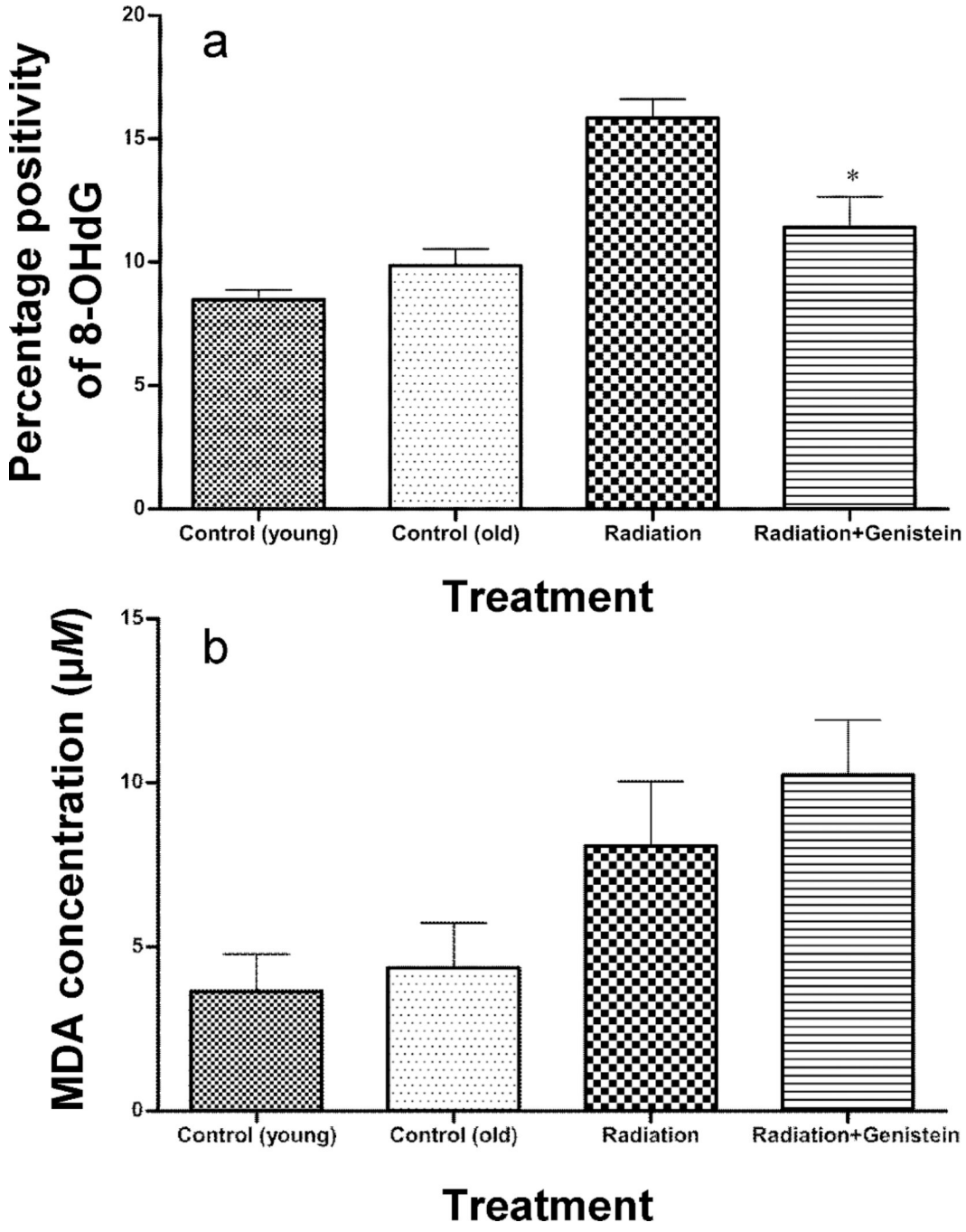

FIG. 8.

Oxidative damage in the lung assessed by the percentage positivity of 8-OHdG expression (panel a) and TBARS assay (panel b). Control (young) animals were assayed at approximately 8 weeks of age. Control (old) animals include the combined groups of no radiation and control diet and no radiation plus genistein diet. The radiation group was given 18 Gy whole-lung irradiation and placed on a control (soy-free) diet and the radiation plus genistein group was given 18 Gy whole-lung irradiation and placed on a diet containing genistein. Bars represent means ± SEM. The asterisk indicates a significant difference from the radiation-only group.