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Histopathological assessment of protective effects of N-acetyl cysteine on rat lung exposed to ionizing radiation

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ABSTRACT: Aim: Radiotherapy (RT) has been used for many years as a curative or adjuvant with chemotherapy and surgery in the treatment of various thoracic malignancies. However, adverse pulmonary effects such as pneumonia and fibrosis can be life-threatening. For this reason, it is very prominent to support RT with compounds that will reduce side effects. This study examines the protective effect of N-acetyl cysteine (NAC), an anti-inflammatory and an antioxidant, against lung damage caused by radiotherapy.
Materials and Methods: Twenty-eight female Wistar albino rats were used. The rats were divided into four groups control (injected with saline), NAC (injected with NAC), RT (exposed to radiation) RT+NAC (injected with NAC+exposed to radiation). After three months of irradiation, rats were sacrificed and lung samples were taken for light and electron microscopic evaluation. The alveolar wall thickness, mast cell count, and hyaline membrane formation were evaluated with light microscopy. Electron microscopy was used to evaluate morphological changes in the lungs.
Results: The results showed a significant increase in scores of alveolar wall thickness and hyaline membrane formation and mast cell count in the RT rats. In the RT+NAC group, administration of NAC to rats caused significant amelioration in the measured parameters. Similarly, in electron microscopic examination, it was observed that the morphological damages in the RT group were significantly reduced in the RT+NAC group.
Conclusion: The results obtained in this study suggested that the use of NAC before thoracic radiotherapy may reduce the damage to the lungs due to radiotherapy.

KEYWORDS: radiotherapy; thoracic irradiation; lung; histopathological evaluation; N-acetyl cysteine; radioprotection

1. INTRODUCTION

Radiotherapy (RT) is one of the cancer treatment modalities for palliative, curative, adjuvant, and prophylactic irradiation [1]. Radiation-induced damage to the lungs can occur as a complication of many thoracic malignancies for lung cancer, breast cancer, esophageal cancer, thymoma, and lymphoma [2]. After RT, chronic complications, such as acute radiation pneumonitis, pulmonary fibrosis and pulmonary insufficiency have been observed and limited quality of life [3]. The effects of lung irradiation are divided into early radiation toxicity, which occurs a few hours to a few days after exposure to radiotherapy, and late radiation toxicity, which occurs months or years after treatment [4]. The histopathological changes of irradiation injury begin right off after radiation exposure, but the clinical findings may not become evident for weeks, months, or even years after treatment [5].

There are several studies in the literature about the structural changes caused by ionizing radiation in the lungs. Perivasculitis, perivascular edema, and exudative lesions in the alveolar space were the prominent light microscopic findings in these studies after two months of irradiation [6]. Cellular infiltration, interstitial expansion, larger, more granular and atypical type I and type II pneumocytes were observed after three months of irradiation. Infiltration, fibroblasts, and hypertrophy of Type II pneumocytes increased after four to six months of irradiation. Although collagen was initially seen in focal areas, it was observed in the alveolar wall and pleura over time. Fibrin deposition was observed by electron microscopy. Ultrastructural examination of the lungs at 12 and 26 weeks after radiation showed dynamic changes such as excessively increased mast cells [6].

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Some drugs can be used to reduce lung damage from radiation. Glucosamine, Ethyl pyruvate, and depolymerized heparin can be used as ameliorator for radiation-induced lung injury [7-9]. N-acetylcysteine (NAC) is a mucolytic, antioxidant, and anti-inflammatory agent [10]. NAC, an analog of the naturally occurring amino acid L-cysteine, is a sulfhydryl compound and has radioprotective properties [11,12]. There are limited studies investigating the effect of NAC after thoracic irradiation. Tarbell et al. investigated the effect of systemic absorption of NAC on the survival characteristics of the hematopoietic stem cell in the irradiated mice. They reported that there was a significant delay in the incidence of death in the 14 Gy dose group as a result of NAC treatment, and no significant protective effect of NAC in the 12, 13, and 15 Gy dose groups [13]. There is no study in the literature examining the protective role of NAC in lung injury as histopathologically after thoracic irradiation. In this study, it was purposed to examine the protective effect of NAC on lungs by histopathological methods three months after thoracic irradiation.

2. RESULTS

2.1. Light Microscopic Findings

In the control and NAC groups' sections of Hematoxylin-Eosin (HE) stained had normal lung morphology (Figure. 1). In the HE sections of the RT group was seen intense inflammatory cell infiltration, edema, and hyaline membrane formation. Inflammatory areas including alveolar macrophages, eosinophils, and the other inflammatory cells were evident in the highly enlarged interstitial space. The alveolar walls were quite thickened. Although the alveolar epithelium was markedly damaged, the number of the alveoli decreased. Clusters of foam cells were observed especially in the sub-pleural regions. Some epithelial cells of the bronchioles had degenerations and there were sloughed epithelial cells debris and the inflammatory cells in the bronchiole lumen (Figure. 2). In the RT+NAC group sections of HE stained had normal lung morphology in addition there were areas of inflammatory cells infiltration and hyaline membrane formation but reduced rate compared to the RT group. Foam cells, increased alveolar macrophages, eosinophils, and the other inflammatory cells were observed in the enlarged interstitial space. Sloughed epithelial cells were seen in some bronchiole's lumen (Figure 3). In addition, the sections were stained with Toluidine Blue (TB) and the mast cells were counted in all groups (Figure. 4).

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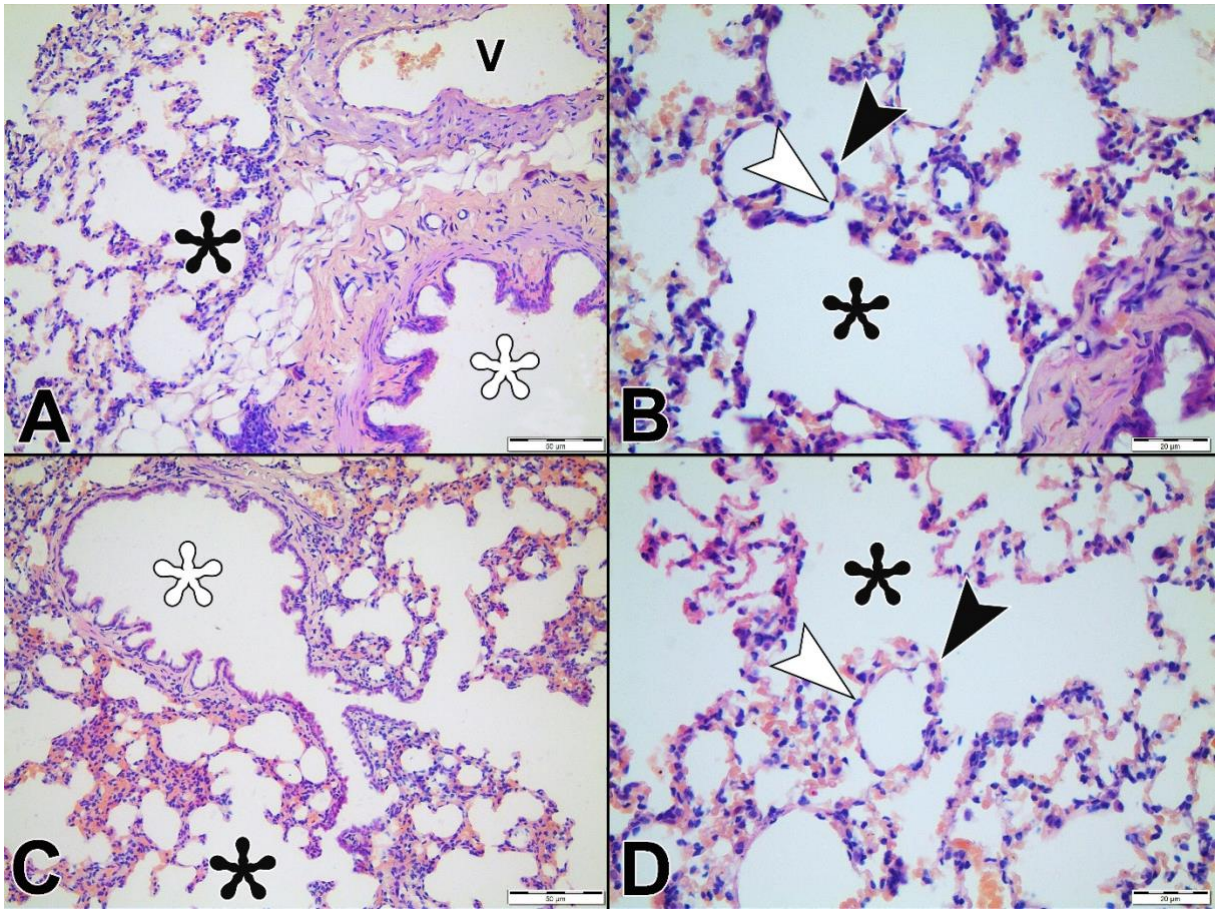

Figure 1. HE stained of control (A ve B) and NAC (C ve D) groups. Normal alveolar lumen (black asterisk), terminal bronchiole (white asterisk), Type 1 (white arrowhead) and Type 2 (black arrowhead) pneumocytes, V: vessel. A and C X200, B and D X400.

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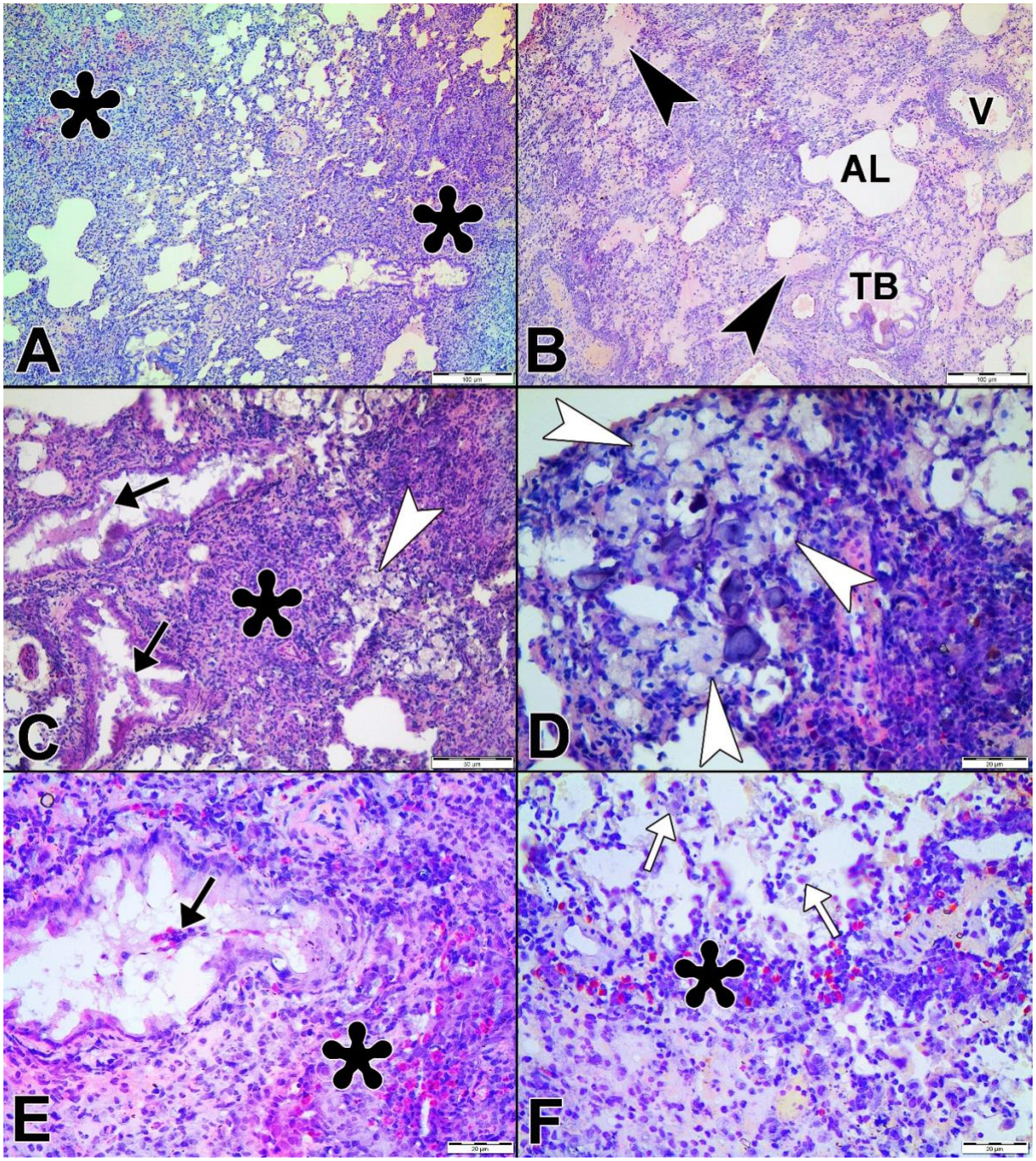

Figure 2. HE stained of RT group. Intense inflammation and thickening in the alveolar wall (black asterisk), edema in the interstitium (black arrowhead), damaged epithelial cells debris and inflammatory cells in the bronchiole lumen (black arrow), foam cells (white arrowhead), increased alveolar macrophages (white arrow), V: Vessel, AL: Alveolar lumen, TB: Terminal bronchiole. A and B X100, C X200, D, E, and F X400.

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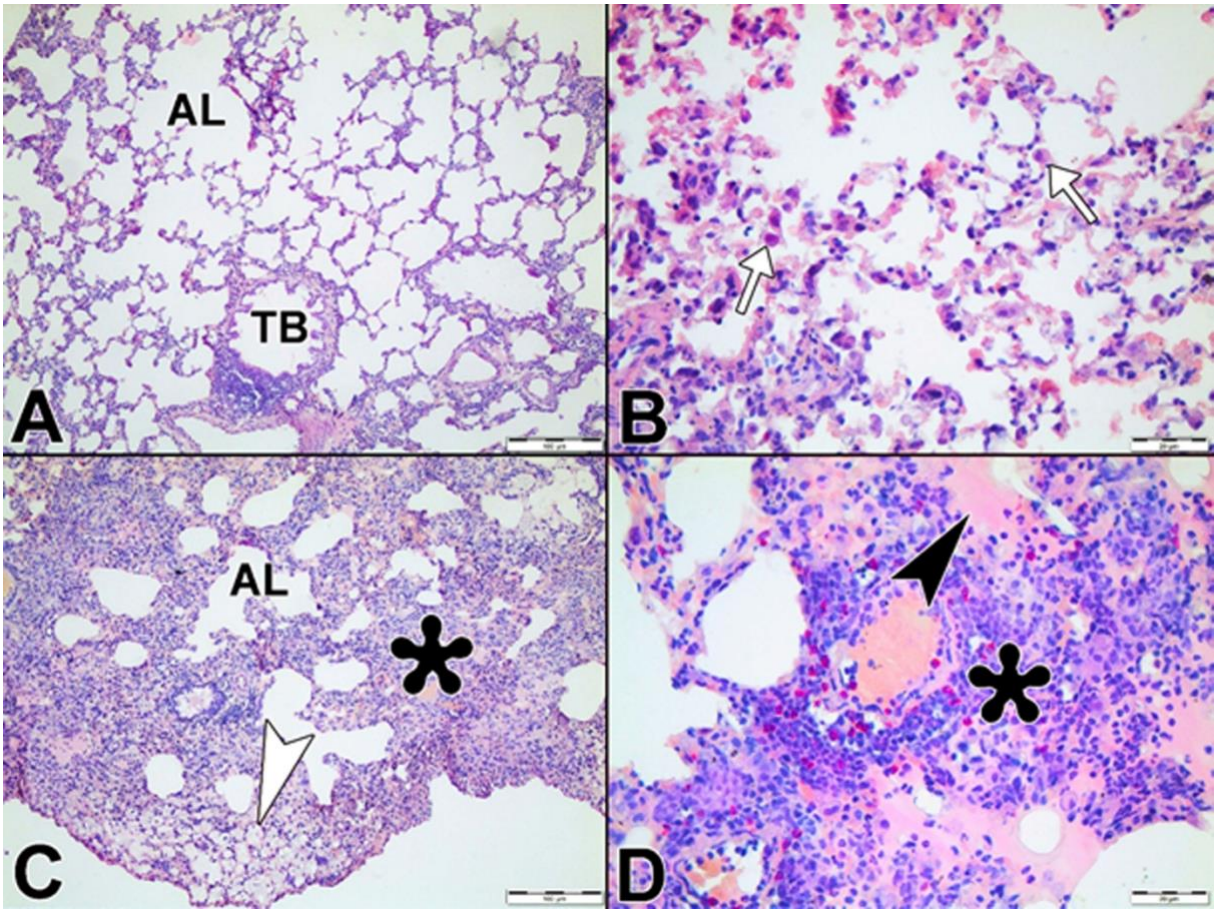

Figure 3. HE stained of RTN group. Infiltration in the interstitium (black asterisk) and edema (black arrowhead), foam cells (white arrowhead) and alveolar macrophages (white arrow), AL: Alveolar lumen, TB: Terminal bronchiole. A and C X100, B and D X400.

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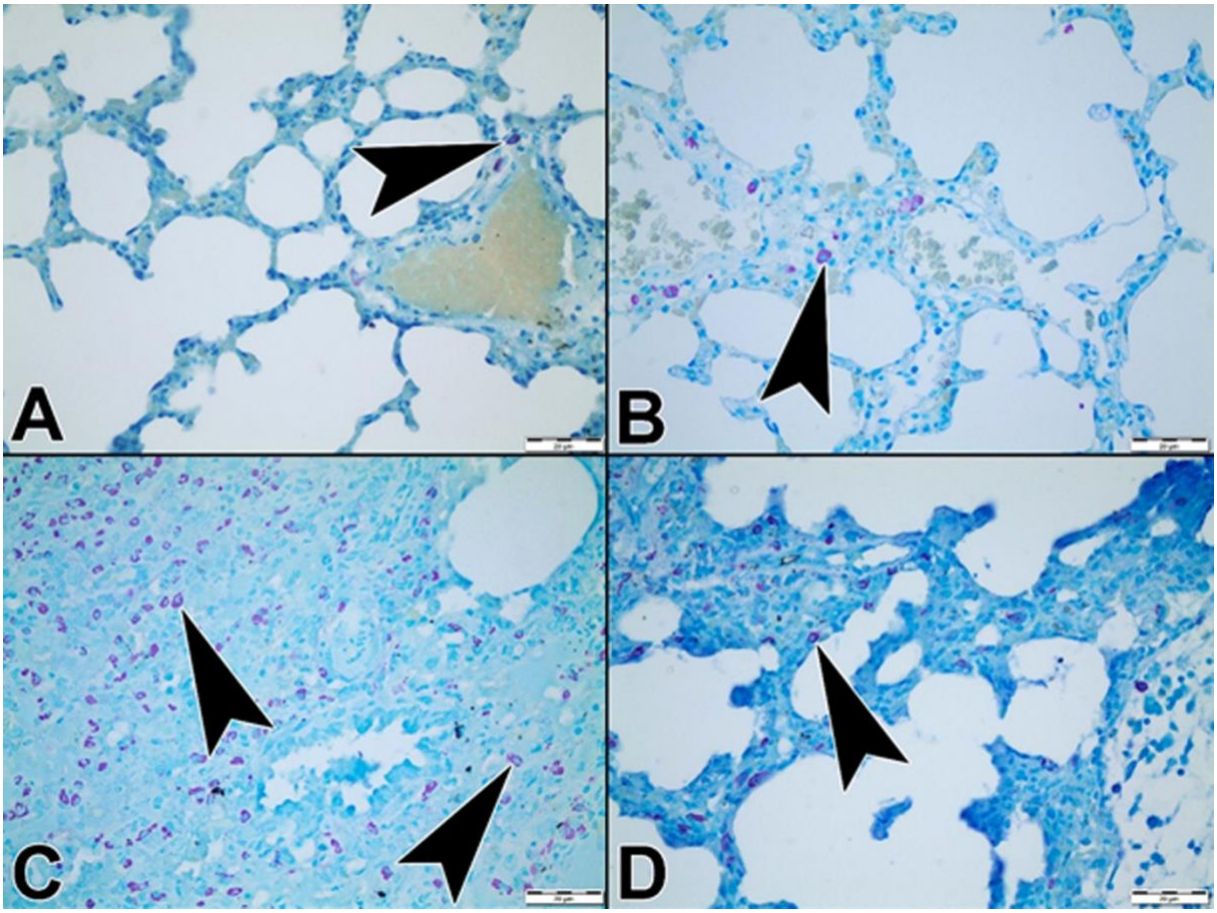

Figure 4. TB stained of C (A), NAC (B), RT (C), RTN (D) groups. Mast cells (black arrowhead). A, B, C, and D X400.

Table 1 showed the histopathological changes according to the scoring scale in the lung. There was a statistically significant difference in the mast cell count, leukocyte score, thickness of alveolar wall score, and hyaline membrane formation score in the RT group in comparison with the control, NAC, and RT+NAC group ($p<0.05$). Similarly, measured overall scores were significantly different in the RT+NAC group in comparison with the control and NAC groups ($p<0.05$). However, the differences in the RT+NAC group were smaller than RT group ($p>0.05$).

Table 1. Means of measured histological variables in lung tissue. All data were presented as mean±SD.

Variables	Control	NAC	RT	RT+NAC
Number of mast cells	18.0±3.89	18.83±2.92	86.83±6.30^{abc}	66.75 ±4.27^{ab}
Leukocytes score	0.35±0.10	0.60±0.06	3.65±0.083^{abc}	2.32±0.05^{ab}
Hyaline membrane formation score	0.47±0.16	0.58±0.075	3.46±0.20^{abc}	2.35±0.10^{ab}
Thickness of alveolar wall score	0.5±0.10	0.53±0.16	3.66±0.16^{abc}	2.40±0.28^{ab}

^{a}Difference is statistically significant from control group; ^{b}Difference is statistically significant from NAC group; ^{c}Difference is statistically significant from RT+NAC group; Post-hoc: Tukey test; α:0.05; Anova

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2.2. Electron Microscopic Findings

Normal lung morphology was observed in the thin sections of the control and NAC groups (Figure 5). Type 1 and Type 2 pneumocytes, blood-air barrier, and the other structures were normal. In the RT group, increased alveolar macrophages, mast cells, and inflammatory cells were seen in the interstitial space. Collagen fibers had increased considerably in the enlarged interstitium. Also, in this group damaged Type 2 pneumocyte cell debris, alveolar macrophages, and erythrocytes were observed in the alveolar lumen (Figure 6). When RT+NAC group was examined, it was observed that the findings similar to the RT group significantly decreased. There were locally increased collagen fibers, inflammatory cells, and mast cells in the interstitial space. Some alveolar lumen had degenerated epithelial cells debris (Figure. 7).

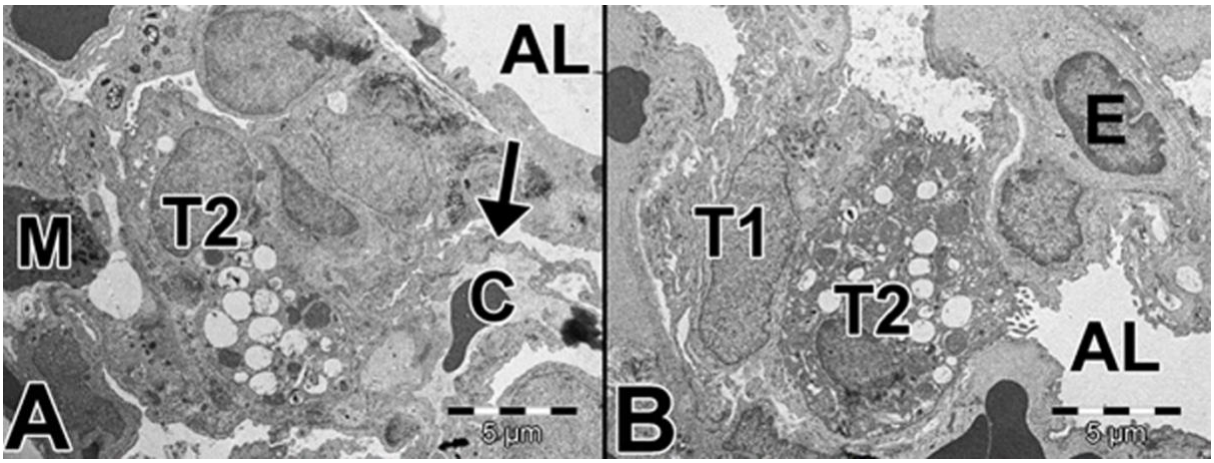

Figure 5. Electron microscopic findings of Control (A) and NAC (B) groups. Normal Type 1 (T1) and Type 2 (T2) pneumocytes, blood-air barrier (black arrow), endothelial cell (E), alveolar lumen (AL), alveolar macrophage (M), capillary (C). A and B X5000.

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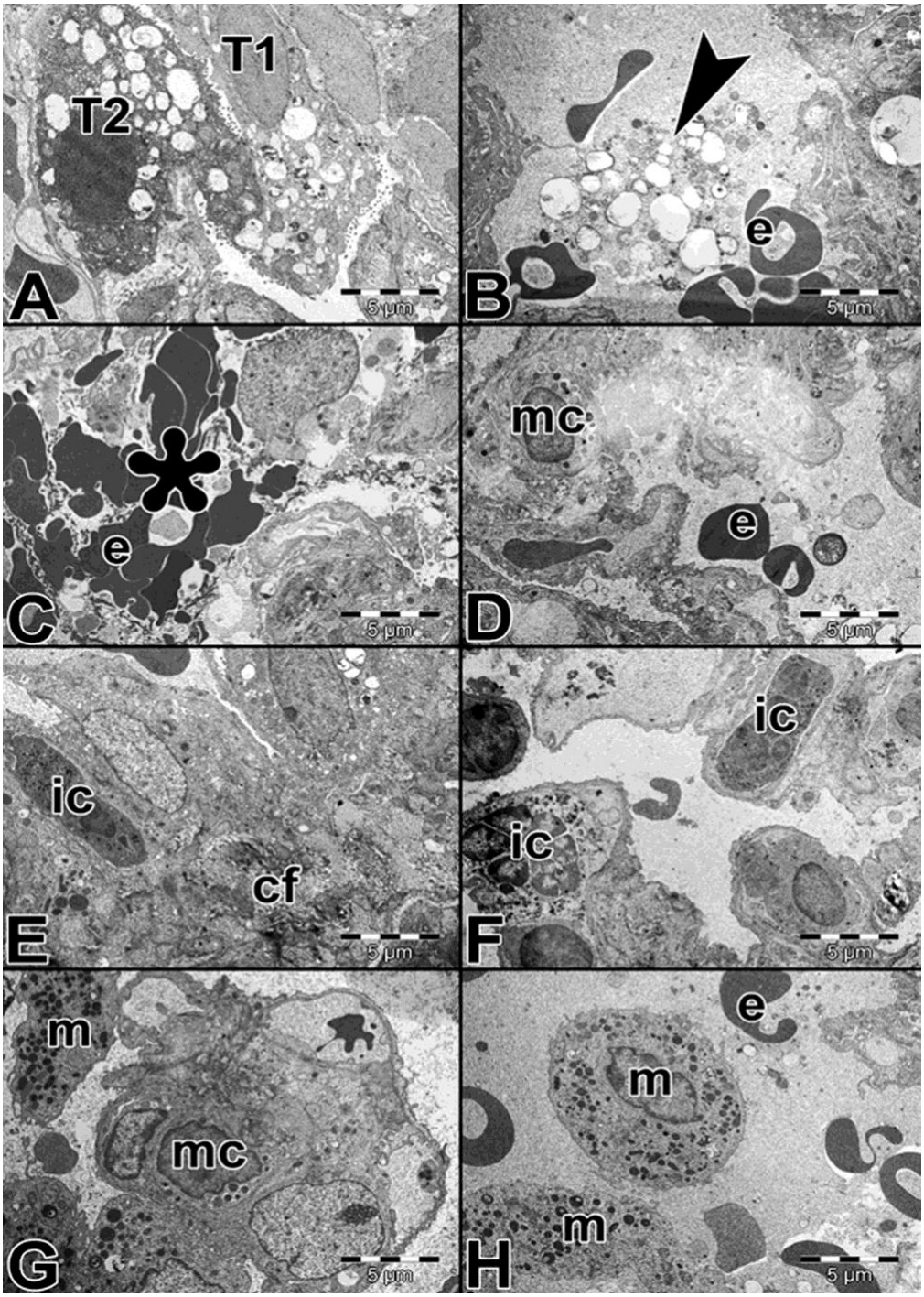

Figure 6. Electron microscopic findings of RT group. Type 1 (T1) and Type 2 (T2) pneumocytes, sloughed Type 2 cell debris in the alveolar lumen (black arrowhead), erythrocytes (e) and cell debris in the alveolar lumen (black asterisk), mast cells (mc), inflammatory cells (ic), alveolar macrophage (M), collagen fibers (cf). A, B, C, D, E, F, G, and H X5000.

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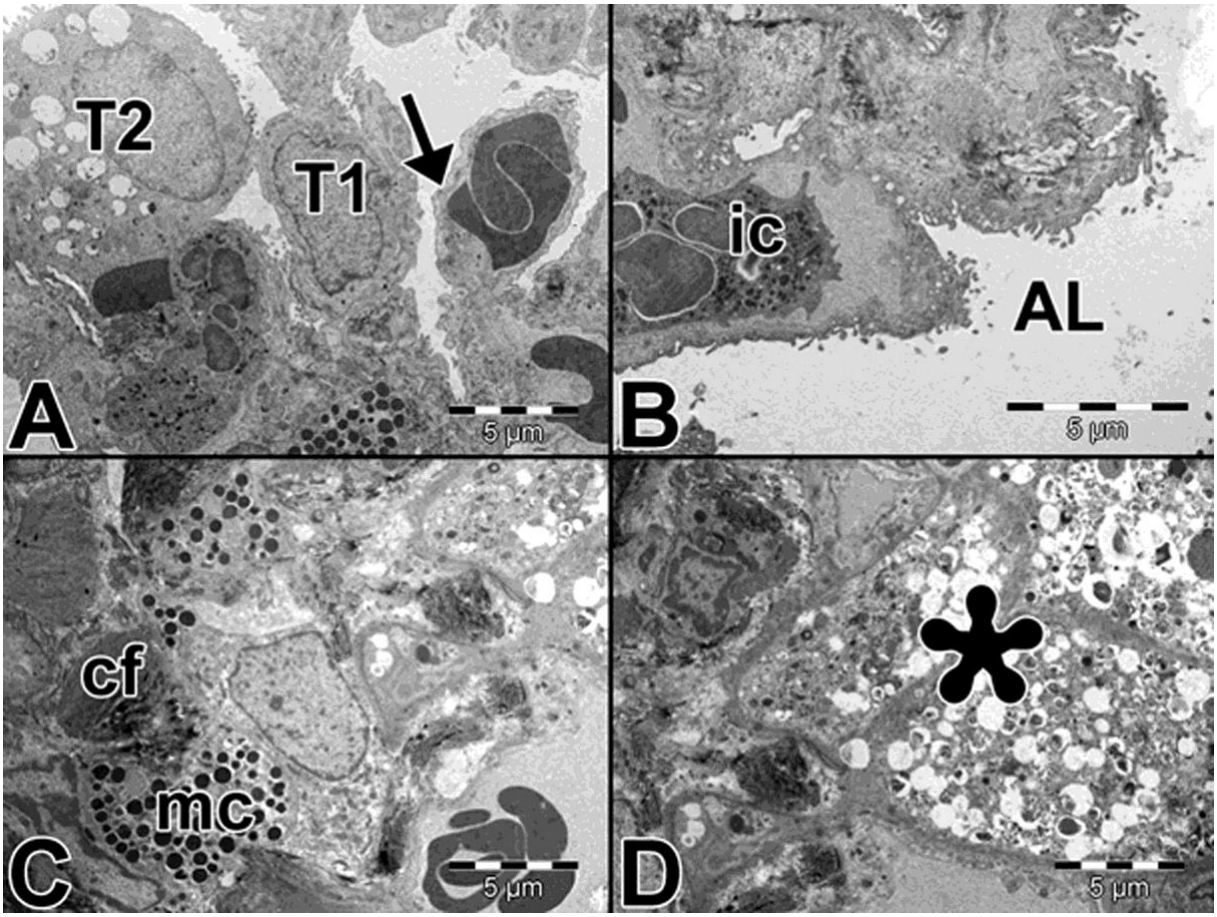

Figure 7. Electron microscopic findings of RT+NAC group. Type 1 (T1) and Type 2 (T2) pneumocytes, blood-air barrier (black arrow), inflammatory cell (ic) in the capillary lumen, collagen fibers (cf), mast cells (mc), degenerated cells debris in the alveolar lumen (AL) (black asterisk). A, C and D X5000, B X 7500.

3. DISCUSSION

In the present study, rats were irradiated with a single dose of 20 Gy ionizing radiation to induce lung injury and the protective effect of NAC on radiation-induced lung injury was investigated by light and electron microscopic examination. Findings from the present study indicated that 20 Gy ionizing radiation caused a significant injury to the rat lung and NAC partially ameliorated this effect of ionizing radiation three months after irradiation.

In the current study, the number of mast cells was significantly higher in the RT groups than in the control, NAC, and RT+NAC groups. The count of mast cells increased by 4.8-fold in the RT group compared to the control group. Although the number of mast cells increased significantly in the RT+NAC group compared to the control group, it showed a significant decrease compared to the control group. These results showed that ionizing radiation caused mast cell activation three months after irradiation, but administration of NAC reduced this activation. An increase in mast cell count after irradiation has also been reported in previous similar studies. Vergara et al [18] used stereological-morphometric techniques to examine the ultrastructure of the lung of rats after unilateral thoracic irradiation with 30 Gy and showed that the number of mast cells increased 540 times 6 months after irradiation. In the study by Travis et al. [19], the right lung of rats was irradiated with 20 Gy and reported the presence of mast cells in the alveolar wall. In addition, Szabo et al. [20] reported that mast cell number increased by 16-fold after 15 Gy thoracic irradiation in the rats. Although the increase in the number of mast cells in these studies is very dramatically compared to our study, this difference can be explained by the differences between the doses and the time elapsed after irradiation in both studies. The various components released by the mast cell include histamine, cytokines, and proteases such as chymase and tryptase [21]. Some of these components, like histamine and tryptase, induce

inflammatory cell infiltration, encourage cytokine generation, and increase vessel permeability. This further facilitates mast cell migration and exacerbates inflammation [22,23]. The light and electron microscope findings in the current study show that ionizing radiation causes intense inflammation in the lung. While the leukocyte score was 0.35, representing less than 20 leukocytes in each area, in the control group, this value increased to 3.5, representing high than 45 leukocytes in each area, in the RT group. In the RT+NAC group, the score was 2.25, representing 20-45 leukocytes in each area. While the leukocyte count increased significantly in the RT+NAC group compared to the control group, it decreased significantly compared to the RT group. These results show that NAC treatment for 7 days before radiotherapy significantly reduces mast cell activation and inflammation that may be caused by radiotherapy in the lung.

One of the important findings in this study was the increase in lung wall thickness. The thickness of alveolar wall score in the RT group increased by 7.32-fold compared to the control group. Administration of NAC before radiotherapy reduced this value to 4.8 times. Although the thickness of the alveolar wall score increased significantly in the RT+NAC group compared to the control group, it showed a significant decrease compared to the RT group. An increase in alveolar wall thickness is an important indicator of lung injury. Almeida et al. observed increased inflammatory cell infiltration and alveolar wall thickness in the lungs 2 months after 24 Gy irradiation [24]. In this study, electron microscopic findings also indicate an increase in the amount of collagen in the interstitium. Collagen deposition and increased alveolar wall thickness are histological signs of fibrosis [25]. NAC treatment significantly reduced collagen deposition, alveolar wall thickness.

Hyaline membrane formation is one of the characteristics of the exudative phase of lung injury 3-12 weeks after irradiation [26,27]. Gas exchange is dramatically reduced due to hyaline membrane formation [26]. In the present study hyaline membrane formation score in the RT group increased by 7.36-fold compared to control group. Administration of NAC before radiotherapy reduced this value to 5 times. Although the hyaline membrane formation score increased significantly in the RT+NAC group compared to the control group, it showed a significant decrease compared to the RT group. NAC is a drug with well-known anti-inflammatory and antioxidant properties. In addition, it has been used in clinics for 50 years because it is cheap and reliable [28]. Previous studies have demonstrated the protective effect of NAC from radiation damage in the different tissues [11,28,29]. Our findings that NAC reduces the effect of radiation are compatible with previous studies.

4. CONCLUSION

In conclusion, in this study, it was observed that NAC, used at a dose of 240 mg/kg for 7 consecutive days before thoracic irradiation, significantly improved the structural damage to the lungs 3 months after irradiation. NAC is an anti-inflammatory and antioxidant drug and has been used safely for many years without any side effects observed in the clinic. The results obtained in this study suggested that the use of NAC before thoracic radiotherapy may reduce the damage to the lungs due to radiotherapy. However, further clinical studies are needed in this area.

5. MATERIALS AND METHODS

5.1. Animals and Experimental design

Twenty-eight 12-week-old Wistar albino rats were used in the experiments. During the experiment, the rats were housed in galvanized cages at a room temperature of 22-25 °C and average humidity of 55%, with 12 hours of light and 12 hours of darkness. They were fed with a standard rat pellet and their water were given as ad libitum. The rats were divided into four groups control (injected with saline for 7 consecutive days), NAC (injected NAC at a dose of 240 mg/kg day for 7 consecutive days), RT (exposed to radiation at a dose of 20 Gy) RT+NAC (injected with 240 mg/kg day NAC 7 consecutive days + exposed to radiation at a dose of 20 Gy). Three months after the RT, all rats were sacrificed with overdose anesthesia and lung tissue was isolated for histopathological evaluations. The research was approved by the Animal Experiments Local Ethics Committee of the Mersin University (Reference No: 26.12.2016/16/50).
In the present study, RT was applied to the thorax region of RT and NAC+RT groups rats under anesthesia (Ketalar®, 50 mg/kg, Eczacıbaşı, Istanbul, Türkiye and xylazine hydrochloride (Rompun®, 5 mg/kg, Bayer, USA). 6 MV photon energy of the linear accelerator device was used for irradiation. The radiation dose was given to the rats in a single fraction. During the irradiation, the rats were immobilized in the supine position

and a total of 20 Gy radiation dose was administered, 10 Gy from the anterior direction and 10 Gy from the posterior direction. A 1 cm bolus was placed in the anterior area of the rats in order to obtain a more homogeneous dose distribution [14,15].

5.2. Histopathological Evaluation

5.2.1. Light microscopic examination

Lung samples for light microscopic examinations were thrown into freshly prepared buffered 10% formaldehyde. Then the tissues were embedded in paraffin blocks and were cut into 5 µm sections by using a rotary microtome (Leica, 2125RT). The sections were stained with Hematoxylin-Eosin (HE) for the general morphologic evaluations, scoring of infiltration, and the alveolar injury or Toluidine Blue (TB) for the scoring of mast cells. The inflammatory cells were appraised using a histologic leukocyte count-based grading system. Ten separate high-power magnification (X400) fields were studied to score inflammatory cells in each section. Amounts were graded using the following scale: Grade 0: no extravascular leukocytes; Grade 1: less than 20 leukocytes per high magnification field; Grade 2: 20-45 leukocytes per high magnification field; Grade 3: more than 45 leukocytes per high magnification field [16]. The alveolar injury was evaluated by hyaline membrane formation and alveolar wall thickening, as previously described [17]. For this, 5 separate high-power magnification (X400) fields were studied. Each item is scored from zero to four, of which zero points represent normal, one point represents a very small change, two points represent a slight change, three points represent moderate change and four points represent a serious change. Mast cells were counted in five different areas at X200 magnification. All evaluations were done using a light microscope (Olympus BX50) and taken photos with a video camera connected to the same microscope.

5.2.2. Electron microscopic examination

For electron microscopic examinations, tissue samples of 1 mm3 size were taken to freshly prepared 2,5% glutaraldehyde fixative at +4°C and four hours later washed with phosphate buffer and put in to clean the same buffer. Then, samples were processed with appropriate protocols. Firstly, the tissues were embedded in epoxy resin and polymerized in the incubator for a night at 60°C. Then, the 2000 nm semithin sections were cut using an ultramicrotome (Leica, UCT125, Leica GmbH, Germany). When reached the wanted area 70 nm thin sections were cut the same ultramicrotome and taken to the copper grids. The thin sections were contrasted with uranyl acetate and lead citrate for five minutes. The sections were analyzed by transmission electron microscope (TEM, JEOL JEM1011, JEOL Corp., Tokyo, Japan) and taken photos with a digital camera (Megaview III, Olympus GmbH, Germany) connected to the TEM.

5.3. Statistical Analysis

Data were analyzed using SPSS v.20 statistical package program (IBM, Istanbul, Türkiye). The checks of normality of variable are tested with the Kolmogorov-Smirnov test. One-way ANOVA was used to analyze data and TUKEY post hoc multiple comparison test to investigate the difference among groups. P values less than 0.05 were considered as statistically significant.

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