ORIGINAL ARTICLE

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Melatonin as a Radioprotective Agent: Assessing Its Efficacy in Preventing Lung Injury Induced by Radiation Therapy

Melatoninin Radyasyona Bağlı Akciğer Hasarına Karşı Doza Bağımlı Etkileri: Deneysel Bir Çalışma

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ABSTRACT

Aim: The purpose of our study was to investigate whether melatonin (MLT) can protect against radiation-induced lung injury (RILI) in a rat model, with particular emphasis on dose-dependent effects in preventing pneumonitis and pulmonary fibrosis following radiotherapy (RT).

Materials and Methods: Treatment groups received MLT at a dose of 100 mg/kg, 50 mg/kg, and 5 mg/kg before 12 Gy RT in a single fraction. At the end of week 8, rats were sacrificed, and hematoxylin and eosin stained lung tissues were evaluated using a semi-quantitative scoring method based on the alveolar septal area.

Results: The mean alveolar septal area was 38+11.07 μm^2 in the control group. RT group displayed deteriorated alveolar space and increased alveolar septal thickening with a mean of 42.3+6.60 μm^2. 100 mg/kg group displayed the greatest septal thickening and the most alveolar space decrease with a mean of 49.0+12.37 μm^2. MLT 50 mg/kg and 5 mg/kg groups displayed preserved alveolar morphology and less septal degradation with a mean of 35.0+9.08 μm^2 and 31.1+5.73 μm^2 respectively. The alveolar septal area was significantly greater in the 100 mg/kg compared with the 50 and 5 mg/kg groups (p=0.034 and 0,003, respectively). This finding suggests that the 100 mg/kg MLT dose triggered greater profibrotic changes in lung tissues, leading to reduced alveolar space, potentially due to pro-oxidant effects at high doses.

Conclusion: These findings highlight the importance of MLT dosage in managing RILI <50 mg/kg doses seem to have potentially less alveolar septum degradatation offering protective effects against lung injury.

Keywords: Melatonin, oxidative stress, pneumonitis, radiation-induced lung injury, radioprotection, rat

ÖZ

Amaç: Çalışmamızın amacı, melatonin (MLT) tedavisinin, radyoterapi (RT) sonrasında oluşan pnömonit ve pulmoner fibrozis gibi radyasyon kaynaklı akciğer hasarını (RILI) engelleyip engellemeyeceğini araştırmak ve özellikle doz bağımlı etkilerini incelemektir.

Gereç ve Yöntem: Tedavi gruplarına 12 Gy RT'yi tek bir dozda uygulamadan önce sırasıyla 100 mg/kg, 50 mg/kg ve 5 mg/kg MLT verilmiştir. 8. haftanın sonunda sıçanlar feda edilmiş ve hematoksilin ve eozin ile boyanmış akciğer dokuları alveoler septum alanına dayalı yarı-quantitatif bir puanlama yöntemiyle değerlendirilmiştir.

Bulgular: Kontrol grubunda ortalama alveoler septum alanı 38+11,07 μm^2 bulunmuştur. RT grubunda alveoler boşlukta bozulma ve alveoler septum kalınlaşmasında artış gözlenmiş ve ortalama 42,3+6,60 μm^2 olmuştur. 100 mg/kg grubu, en büyük septum kalınlaşması ve alveoler boşlukta en fazla

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azalmayı göstermiştir ve ortalama 49,0+12,37 μm² bulunmuştur. MLT 50 mg/kg ve 5 mg/kg grupları ise alveolar morfolojiyi korumuş ve daha az septum bozulması göstermiştir, ortalama sırasıyla 35,0+9,08 μm² ve 31,1+5,73 μm² olmuştur. 100 mg/kg grubunun alveolar septum alanı, 50 ve 5 mg/kg gruplarına göre anlamlı şekilde daha büyük bulunmuştur (p=0,034 ve 0,003). Bu bulgu, 100 mg/kg MLT dozunun, yüksek dozda pro-oksidan etkiler nedeniyle, akciğer dokusunda daha fazla profibrotik değişikliklere yol açarak alveoler boşluğun azalmasına neden olduğunu göstermektedir.

Sonuç: Bu bulgular, RILI yönetiminde MLT dozajının önemini vurgulamaktadır. 50 mg/kg altı dozlar, alveolar septum bozulmasının daha az olmasını sağlayarak akciğer hasarına karşı koruyucu etki gösterebilir.

Anahtar Kelimeler: Melatonin, oksidatif stres, pnömonit, radyasyona bağlı akciğer hasarı, radyoproteksiyon, sıçan

INTRODUCTION

With 2.5 million cases in 2022, lung cancer is a major cause of mortality globally[1]. Approximately 60% of lung cancer patients undergo radiotherapy (RT) either as a standalone treatment or in combination with chemo/immunotherapy[2]. For non-small cell lung cancer, which is the most frequent kind, thoracic RT is recommended for most of the advanced stages[3]. Although RT remains a cornerstone treatment, it is associated with serious adverse effects. One of the complications is radiation-induced lung injury (RILI), which consists of pneumonitis and pulmonary fibrosis. The prevalence of RILI varies depending on the influence of factors such as previous surgeries, pre-existing lung conditions, concurrent use of lung-damaging agents and systemic comorbidities such as diabetes mellitus[4]. It has been reported that up to 30% of patients may experience radiation pneumonitis 1-6 months post-treatment[5,6]. For a higher dose of RT, the incidence rates of RILI may increase up to 40%[5].

The pathophysiology of RILI is a complex process and driven by inflammation. Following exposure to RT, lung undergoes a series of molecular events which cause acute and chronic pulmonary damage. Earliest responses include damage to the vascular endothelium. It leads to increased vascular permeability and infiltration of inflammatory cells into the lung parenchyma. These immune cells release pro-inflammatory cytokines like tumor necrosis factor-alfa, interleukin-6, and transforming growth factor-beta, which increase the inflammatory response and start fibrotic changes[7]. There is currently no established curative treatment of RILI. Eventhough steroids have been used to subside inflammation, serious side effects of steroids are a major problem. Therefore, it is necessary to find alternative therapeutic drugs which has fewer side effects.

Melatonin (MLT), a multifunctional hormone with powerful antioxidant properties, has gained a lot of interest for its potential as a radioprotective agent. It has been shown to reduce oxidative stress and stimulate cell survival by scavenging free radicals and modulating autophagy[8,9]. Although its protective effects appear to be dose dependent, the ideal dosage for lung protection after thoracic RT remains unclear[10]. Previous studies suggested inconsistent results on optimal dose for MLT, emphasizing the need for further research[11].

In our study, we aim to address the current gap in knowledge by systematically investigating the dose-dependent efficacy of MLT in mitigating RILI using an *in vivo* model. By evaluating histopathological changes of alveolar damage, we pursue to identify the minimum effective MLT dose necessary for pulmonary protection. Our findings may help guide future thoracic RT protocols for lung cancer patients.

MATERIALS AND METHODS

Rats

Our study had ethical approval from the Bezmialem Foundation University Experimental Application and Research Center (decision number: 2022-79, date: 27.01.2025) and the experiment was also conducted in the same center. A total of 45 adult male Sprague Dawley rats (200-300 g) were divided into 5 groups (n=9 per group). The rats were housed in standard rat cages at 23±2 °C temperature with 55±10% humidity. Twelve-hour day and night light periods were obtained. The rats were fed with ad-libitum rat chow and drinking water. The study is an animal experiment, and no patient data were used.

Melatonin

For treatment groups, synthetic MLT powder (N-acetyl-5-methyoxytryptamine, ≥98, Sigma-Aldrich, USA) was dissolved in ethanol and diluted in 0.9% saline solution to a concentration of 10 mg/mL.

Radiotherapy Technique

Before irradiation, the rats were anesthetized with 60-90 mg/kg intraperitoneal ketamine hydrochloride (Ketalar; EWL Eczacıbaşı Warner Lambert İlaç Sanayi ve Ticaret A.Ş., İstanbul) and 6-10 mg/kg xylazine hydrochloride (Rompun 2% Bayer Kimya San. Ltd. Şti., İstanbul, Türkiye). After anesthesia, the rats were laid on a foam tray with arms and legs stabilized with cotton bandages in a supine position. Then the simulated computed tomography was performed. In the Varian treatment planning system, the heart and lung volumes were contoured, and a 3D conformal plan was generated. An X-ray linear accelerator (Rapid Arc, Varian Medical Systems, Palo Alto, USA) which produces a 6-megavolt photon beam at 100 cm was used to irradiate the thoracic region with 12 Gy in 1 fraction at a dose rate of 300 monitor units. Elastic-gel bolus was used to maximize the cardiac point dose[12].

Treatment Groups

For the experiment, 5 groups of Sprague-Dawley rats were used. Each group had 9 adult male rats. Prior to sham irradiation, 1 cc of saline solution was administered to the control group. G2 group had 1 cc saline injection intraperitoneally 30 min before 12 Gy thoracic RT. For other groups, MLT was administered at a dose of 100, 50 and 5 mg/kg respectively[8,11]. Eight weeks later, histological changes in the lung were examined. Experimental design and workflow is depicted in Figure 1.

Histological Evaluation

Eight weeks later, rats were anesthetized with ketamine and then the animals were euthanized by exsanguination. After thoracotomy, the lung tissue was harvested and fixed for 24 hours in 10% neutral buffered formalin. Following standard tissue preparation, 5 µm transverse slices were obtained to display the lungs' alveoli and bronchial structure. Hematoxylin and eosin (H&E) was used to stain the obtained sections.

Lung samples were assessed with a standardized, semi-quantitative alveolar septal area-based scoring method which measures alveolar septum area with a rapid and automated algorithm designed to segment alveolar space images across entire tissue selection[13]. The extent of tissue damage was quantitatively evaluated using a mathematical segmentation-based image analysis approach. Five photographs taken at 10X magnification from the alveolar area of each tissue were analyzed with the Fiji program[14]. Through image segmentation, the alveolar space and alveolar wall was identified as distinct regions of interest. A numerical score was then calculated by determining the area ratio of the alveolar wall relative to the alveolar space. Segmentation relied on pixel color differences, as alveolar spaces exhibited more uniform coloration compared to the heterogeneous tones found in the alveolar walls.

In addition to H&E staining, a separate set of paraffin sections was stained with Masson's trichrome, which specifically highlights type I collagen and is commonly used to assess fibrotic areas.

Statistical Analysis

The Shapiro-Wilk test was used to analyze the variables' appropriateness for a normal distribution. The one-way analysis of variance with post-hoc Tukey's multiple comparison test (comparisons between all groups) was employed to compare the numerous independent groups. All statistical analyses were conducted using SPSS version 29 for Mac (IBM Corp. Armonk, NY), and a significance level of $p<0.05$ was accepted.

RESULTS

In the semi-quantitative evaluation of H&E-stained lung sections, the control group (G1) exhibited a normal lung parenchymal architecture characterized by uniformly thin alveolar septa and well-preserved alveolar spaces. The

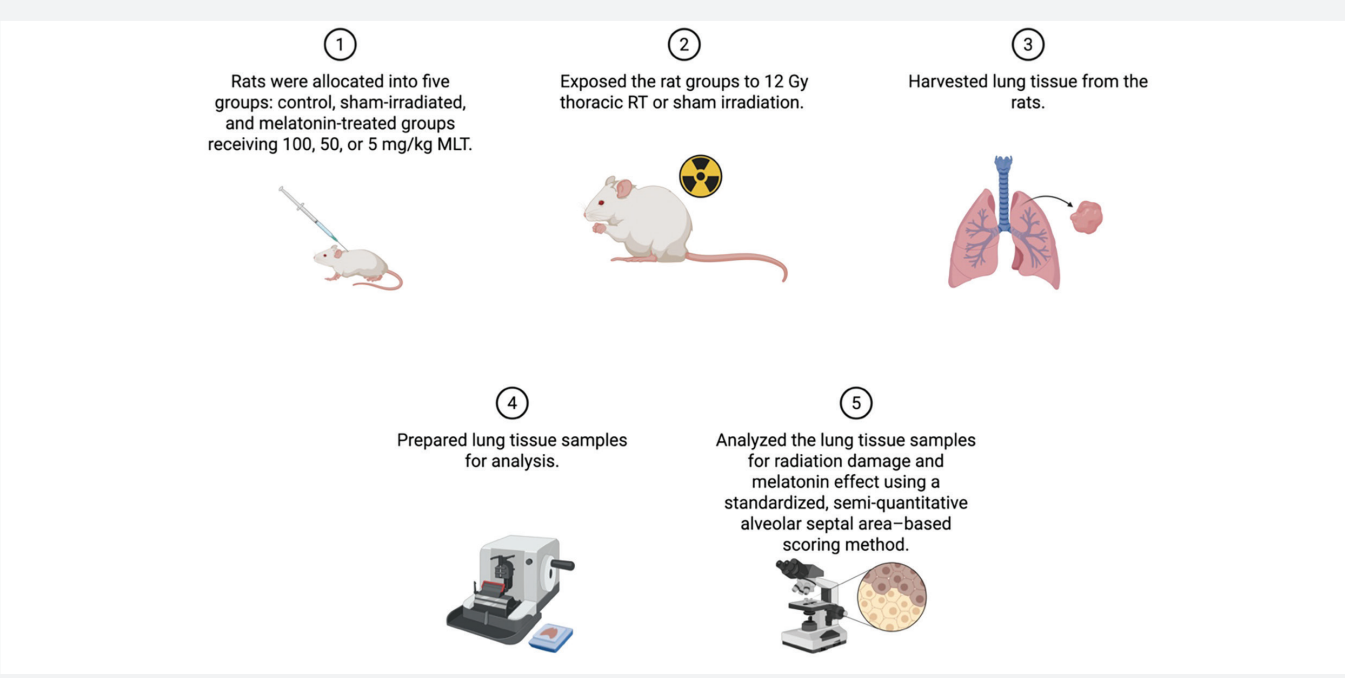

Figure 1. Experimental design and workflow of melatonin treatment and thoracic radiotherapy in a rat model of RILI

RILI: Radiation-induced lung injury, MLT: Melatonin, RT: Radiotherapy

alveoli were regularly shaped, evenly distributed, and free of structural distortion, collapse, or over-distension. The alveolar walls appeared delicate and continuous, with intact epithelial lining and no evidence of septal thickening, edema, inflammatory cell infiltration, or hemorrhage (Figure 2a). Quantitative assessment confirmed these observations, with a mean alveolar septal area of 38±11.07 μm² (Figure 2f). RT-only group (G2) demonstrated deteriorated alveolar space and increased alveolar septa thickening with a mean septal area of 42.3±6.60 μm², although the difference from control was not statistically significant (Figure 2b-f). MLT 50 mg/kg (G4) and 5 mg/kg (G5) groups showing preserved alveolar morphology and reduced septal thickening, with mean areas of 35.0±9.08 μm² and 31.1±5.73 μm², respectively (Figure 2d-f, Table 1). Even though the difference to the control group was not statistically significant, it suggests the antioxidant MLT application even in low doses has a protective effect against profibrotic changes in lung alveoli (Figure 2e-f).

In contrast, MLT 100 mg/kg group (G3) exhibiting the greatest septal thickening and the most alveolar space decrease, with mean septal area (49.0±12.37 μm²). Compared to this group, both the 50 and 5 mg/kg MLT groups had significantly smaller alveolar septal areas (Group 3 vs. 4 p=0.034; Group 3 vs. 5 p=0.003). These results suggest that high-dose MLT may promote profibrotic changes through potential pro-oxidant effects, while lower doses demonstrate protective effects.

Masson trichrome staining was used to evaluate fibrosis and associated morphological changes in the alveolar septa and bronchial structures of lung tissue. No marked difference was observed among the treatment groups in the extent of light green-stained areas, indicating comparable levels of type I collagen deposition and, consequently, fibrosis. Representative histological images illustrating morphological features were shown in Figure 3.

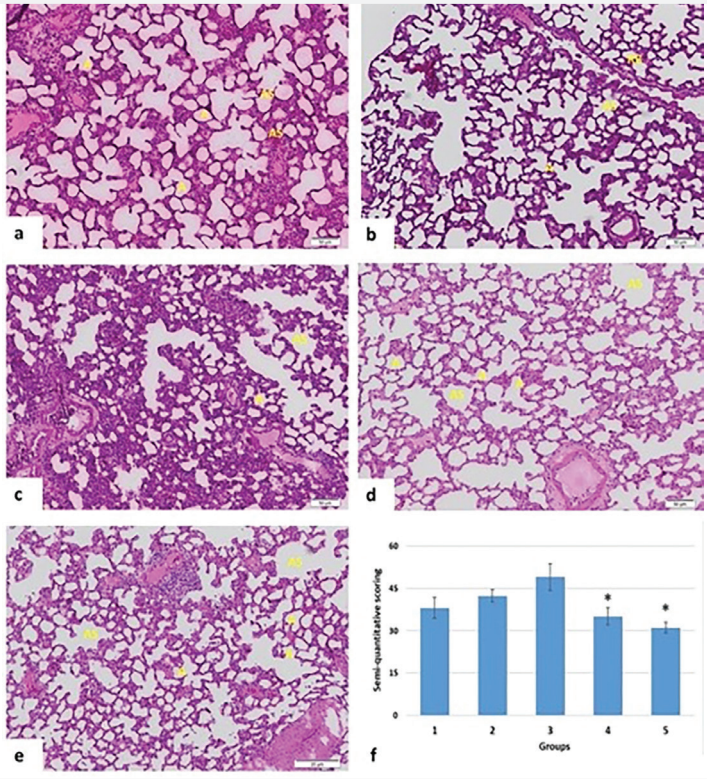

Figure 2. Representative hematoxylin and eosin-stained lung tissues (a-e) and semi-quantitative evaluation of alveolar septal area (f). (a) Control group showing normal lung parenchyma with thin alveolar septa and well-preserved alveoli (mean septal area: 38.0±11.07 μm²). (b) RT-only group demonstrating deteriorated alveolar space and increased alveolar septa thickening with a mean septal area of 42.3±6.60 μm², although the difference from control was not statistically significant. (c) MLT 100 mg/kg group (G3) exhibiting the greatest septal thickening and the most alveolar space decrease, with mean septal area (49.0±12.37 μm²). (d, e) MLT 50 mg/kg (G4) and 5 mg/kg (G5) groups showing preserved alveolar morphology and less septal degradation, with mean areas of 35.0±9.08 μm² and 31.1±5.73 μm², respectively. (f) Quantitative analysis showing significantly greater septal thickening in the 100 mg/kg group compared with the 50 and 5 mg/kg groups (p=0.034 and 0.003, respectively). 10X magnification

AS: Alveolar septal area, A: Alveoli, MLT: Melatonin, RT: Radiotherapy

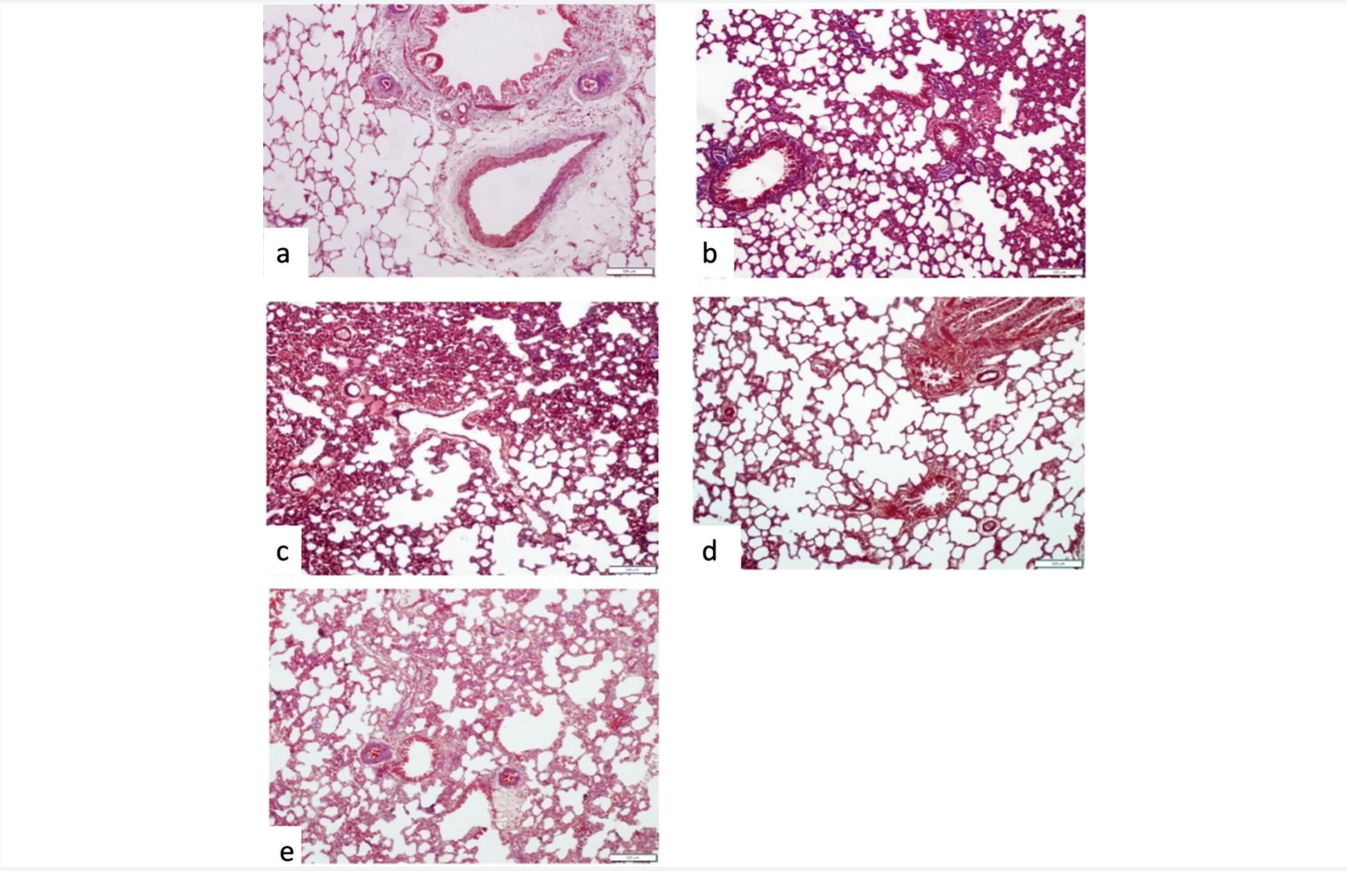

Figure 3. Representative histological images of lung tissues stained with Masson trichrome to evaluate fibrosis and morphological alterations in the alveolar septum and bronchial structures. (a) Group 1 (control), (b) Group 2 (RT only), (c) Group 3 (RT + MLT 100 mg/kg), (d) Group 4 (RT + MLT 50 mg/kg), and (e) Group 5 (RT + MLT 5 mg/kg). 10X magnification. Masson trichrome staining did not reveal a marked difference in fibrotic changes among the groups, with representative morphological appearances shown here

MLT: Melatonin, RT: Radiotherapy

Table 1. Mean alveolar septal areas of among control, radiation-only, and radiation plus MLT treatment groups and multiple comparisons among groups with post-hoc Tuckey test

Treatment groups	Mean of alveolar septal area (μm²) ± SD error	Comparisons among groups	Mean difference (μm) ± SD error	p-value	95% CI (lower bound-upper bound)
G1 (control)	38.07±3.69	G2	-4.27±4.31	0.85	-16.60 to 8.07
		G3	-10.96±4.61	0.14	-24.15 to 2.22
		G4	2.98±4.31	0.95	-9.35 to 15.31
		G5	6.97±4.31	0.49	-5.36 to 19.30
G2 (RT)	42.34±2.20	G1	4.27±4.31	0.85	-8.07 to 16.60
		G3	-6.69±4.61	0.59	-19.88 to 6.49
		G4	7.25±4.31	0.45	-5.08 to 19.58
		G5	11.24±4.31	0.08	-1.10 to 23.57
G3 (RT + 100 mg/kg MLT)	49.03±4.67	G1	10.96±4.61	0.14	-2.22 to 24.15
		G2	6.69±4.61	0.59	-6.49 to 19.88
		G4	13.94±4.61	0.034	0.76 to 27.13
		G5	17.93±4.61	0.003	4.75 to 31.12

Table 1. Continued

Treatment groups	Mean of alveolar septal area (μm²) ± SD error	Comparisons among groups	Mean difference (μm) ± SD error	p-value	95% CI (lower bound-upper bound)
G4 (RT + 50 mg/kg MLT)	35.09±3.02	G1	-2.98±4.31	0.95	-2.22 to 24.15
		G2	-7.25±4.31	0.45	-19.58 to 5.08
		G3	-13.94±4.61	0.034	-27.13 to -0.76
		G5	3.99±4.31	0.88	-8.35 to 16.32
G5 (RT + 5 mg/kg MLT)	31.10±1.91	G1	-6.97±4.31	0.49	-19.30 to 5.36
		G2	-11.24±4.31	0.08	-23.57 to 1.10
		G3	-17.93±4.61	0.003	-31.12 to -4.75
		G4	-3.99±4.31	0.88	-16.32 to 8.35

SD: Standard deviation, CI: Confidence interval, MLT: Melatonin, RT: Radiotherapy

DISCUSSION

RILI is one of the most common side effects of RT in lung cancer treatment. It typically begins with acute lung damage and inflammation, eventually progressing to lung fibrosis, which is a chronic, progressive and potentially fatal condition that severely impairs respiratory function. Currently, there is no curative treatment available for RILI. MLT has shown potential to mitigate RILI. While many studies have explored the impact of specific MLT doses on RILI, only a limited number have directly compared varying doses. To address this gap, the primary objective of our study is to find the lowest effective dosage for RILI. We evaluated the impact of RT and varying doses of MLT on lung morphology, focusing on changes in alveolar septal thickness and parenchymal integrity.

The control group served as the baseline, showcasing healthy lung parenchyma with thin alveolar septa and distinct respiratory bronchioles. The mean alveolar septal area of 38±11.07 μm² provided a clear reference for assessing the impact of RT and MLT treatment on lung tissue morphology. This group highlights the expected structural integrity of lung tissue under normal conditions, serving as a crucial comparison point. The RT-only group exhibited moderate morphological changes, characterized by deteriorated alveolar space and thickened alveolar septa, with a mean of 42.3±6.60 μm². Although the difference compared to the control group was not statistically significant, this numerical increase suggests an early tendency toward profibrotic or other pathological changes. The lack of a statistically significant difference between the RT-only and control groups is likely attributable to the relatively low radiation dose and the 8-week evaluation period, which were chosen to capture early lung injury rather than established fibrosis. These findings, while subtle, highlight the potential of RT to induce morphological alterations in lung tissue. Previous studies have shown that radiation can lead to inflammation and subsequent architectural distortion, with various inflammatory mediators contributing to these processes[15-17].

The MLT treatment groups demonstrated significant benefit in terms of alveolar septal preservation, with the MLT 50 mg/kg and 5 mg/kg groups displaying normal alveolar morphology and less septal degradation, reflected by mean alveolar septal areas of 35.0±9.08 μm² and 31.1±5.73 μm², respectively, compared to 42.3±6.60 μm² in the RT-only group. These findings are consistent with previous reports indication that MLT exerts protective effects on lung tissues by preserving alveolar architecture and mitigating structural damage under pathological conditions[11,17]. In bleomycin-induced lung injury models, MLT administration reduced collagen deposition and improved structural integrity, suggesting a role in counteracting fibrotic remodeling[18]. Similarly, MLT has been shown to suppress inflammatory cell infiltration and provide structural protection in lungs exposed to chronic lipopolysaccharide[19]. These studies and our findings emphasize MLT's multifaceted role in lung protection by reducing oxidative stress, inflammation and structural deterioration.

Interestingly in the MLT-treated groups, a clear dose-dependent effect on lung morphology was observed. The 100 mg/kg MLT group exhibited the most significant pathological changes, including marked alveolar space reduction and thickened alveolar septa, with a mean alveolar septal area of 49.0±12.37 μm². This finding suggests that high-dose MLT may have pro-oxidant effects, potentially exacerbating alveolar septal thickening. In contrast, the 50 mg/kg and 5 mg/kg MLT groups displayed near-normal alveolar septal thickness, with mean values of 35.0±9.08 μm² and 31.1±5.73 μm², respectively. Notably, the differences between these lower doses and the 100 mg/kg group were statistically significant, emphasizing

the adverse effects of high-dose MLT. This finding is consistent with prior experimental studies demonstrating that high doses of MLT may exert paradoxical pro-oxidant, pro-inflammatory, or tissue-damaging effects, potentially through mitochondrial dysfunction and dysregulation of redox homeostasis, thereby attenuating its radioprotective benefits at higher dose ranges[20,21]. Given the absence of molecular oxidative stress or inflammatory biomarkers, this finding should be interpreted cautiously and regarded as a preliminary observation rather than definitive evidence of a pro-oxidant or profibrotic effect of high-dose MLT. Furthermore, the low-dose groups exhibited trends suggesting a protective effect against alveolar septal thickening, as their mean alveolar septal areas were slightly lower than the control group, though these differences were not statistically significant.

The evaluation of alveolar septal thickening in lung tissues following radiation exposure is a complex process that can yield varying results depending on the methodologies employed. In our study, Masson trichrome staining did not reveal significant differences in fibrosis among the treatment groups, despite the semi-quantitative evaluation indicating dose-dependent morphological changes. It is essential to consider the sensitivity of the staining method and the specific stages of fibrosis being assessed. The limitations of Masson trichrome staining in detecting early or subtle fibrotic processes may contribute to this discrepancy, as the staining technique is primarily designed to highlight collagen deposition rather than the nuanced cellular and molecular changes that precede overt fibrosis[22]. The study by Farhood et al.[23] emphasizes that while traditional histopathological methods may not capture early fibrotic changes, other techniques such as molecular assessments could provide deeper insights into the underlying processes. For instance, the modulation of inflammatory cytokines and signaling pathways by agents like MLT has been shown to play a critical role in mitigating RILI, suggesting that the absence of detectable morphological differences might not reflect a lack of biological activity but rather the early stages of fibrotic processes that are not yet fully developed[16]. Furthermore, the use of advanced imaging techniques, such as micro-CT, has been proposed as a means to detect early RILI, potentially offering a more sensitive approach to evaluating lung tissue changes over time[24]. Moreover, the findings from the study by Dogan highlight the importance of understanding the temporal dynamics of lung injury post-radiation, as early histological changes may not manifest as significant architectural distortion until later stages[15]. This aligns with the notion that the assessment of alveolar septal thickening should incorporate a variety of methodologies, including both histological and molecular techniques, to provide a comprehensive understanding of the tissue response to radiation and treatment interventions[13].

Study Limitations

Although the Sprague-Dawley rat model allows controlled evaluation of RILI, the findings may not be fully generalizable to clinical practice due to interspecies differences and the use of a single-fraction 12 Gy thoracic irradiation, which does not mirror conventional fractionated RT. Second, lung injury was assessed at a single late time point (8 weeks), precluding evaluation of early inflammatory changes or long-term fibrotic progression. Third, histopathological assessment was limited to semi-quantitative alveolar septal area analysis and Masson's trichrome staining, without accompanying molecular or biochemical markers of oxidative stress, inflammation, or fibrosis, which restricts mechanistic interpretation, particularly regarding dose-dependent MLT effects.

CONCLUSION

In conclusion, low-dose MLT (5 and 50 mg/kg) preserved alveolar morphology and limited septal thickening, indicating a protective effect against early radiation- and age-related profibrotic changes in lung tissue. Conversely, high-dose MLT 100 mg/kg) was associated with increased alveolar septal thickening and reduced alveolar spaces, suggesting a possible pro-oxidant and profibrotic effect at higher doses. Despite these dose-dependent histomorphometric differences, Masson trichrome staining demonstrated no significant differences in collagen deposition among groups, indicating the absence of established fibrosis at this stage.

Ethics

Ethics Committee Approval: Our study had ethical approval from the Bezmialem Foundation University Experimental Application and Research Center (decision number: 2022-79, date: 27.01.2025) and the experiment was also conducted in the same center.

Informed Consent: The study is an animal experiment, and no patient data were used.

Footnotes

Authorship Contributions

Surgical and Medical Practices: E.C., K.Y., P.A., M.Ü., Ş.A.E., F.Ö.D., Concept: K.Y., S.Ö., M.Ü., Ş.A.E., Design: E.C., P.A., S.K., Data Collection or Processing: K.Y., P.A., S.Ö., S.K., F.Ö.D., Analysis or Interpretation: E.C., Literature Search: S.Ö., F.Ö.D., Writing: E.C., G.E.Ş.

Conflict of Interest: No conflict of interest was declared by the authors.

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