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Original Article

Investigation of the effects of melatonin on lung tissue through the NLRP3/TLR2/NEK7 pathway in an experimental endotoxemia model

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Abstract

Sepsis, a severe clinical syndrome, arises from pro-inflammatory and apoptotic processes. Its rapid progression from sepsis to severe stages necessitates timely intervention. The lipopolysaccharide (LPS) agent triggers pro-inflammatory mediator release through Toll-like receptors, particularly TLR-2, a vital biomarker in sepsis with multiple organ failure. In LPS-induced septic shock, the NEK7-mediated NLRP3 inflammasome pathway, linked to acute lung injury, is suppressed. This pathway is implicated in sepsis-induced platelet activation and septic shock development. Antioxidants like melatonin may positively impact reducing septic shock. In microbial-induced sepsis, melatonin can regulate pro-inflammatory mediator transcriptional activation, potentially controlling the pro-inflammatory state.

In the project, the histopathological impact of melatonin in lung tissue during endotoxic shock induced by the LPS agent in Sprague-Dawley rats, and its immunoreactivity to NLRP3/NEK7/TLR-2 molecules, were assessed. Lung volumes were evaluated using micro-computed tomography (Micro-CT). While bleeding, cell infiltration, and thickening of the alveolar wall were observed in the lungs of the LPS group, a reduction in these symptoms was noted in the LPS+Melatonin group. Expressions of NEK7, TLR2, and NLRP3 increased in both the LPS and LPS+Melatonin groups compared to the control group. It was determined that in the LPS+Melatonin group, levels of NEK7, TLR2, and Malondialdehyde (MDA) decreased compared to the LPS group. Additionally, a decrease in the total volume of lung tissue was observed in the LPS group. In this context, our study reported the therapeutic effect of melatonin on sepsis-related acute lung injury. Our study suggests that melatonin administration in the experimental endotoxemia model melatonin may help reduce lung damage by inhibiting NEK7 and TLR2 expressions.

Keywords: Endotoxemia, Inflammation, LPS, Lung Injury, Sprague-Dawley, Micro-CT.

1. Introduction

Sepsis is a heterogeneous syndrome characterized by a highly intricate interplay between various biological systems that results in deregulation of the inflammatory networks. Our understanding related to the key mechanisms involved in the pathogenesis of sepsis has tremendously evolved. İrregular production of pro-inflammatory chemicals is interconnected with pathophysiological mechanisms of inflammation during sepsis. These specific pleiotropic multifaceted mediators that connect and rewire different cascades of the immune responses can be viewed as "central hubs" in the inflammatory networks [1, 2]. It has also become significantly evident that the complement, fibrinolysis and coagulation systems interact in a complex network through various bidirectional connections. Therefore, in sepsis, crosstalks between complement and coagulation pathways contribute to the disease aggressiveness [3, 4].

Sepsis is an important cause of acute lung injury and its more severe form, acute respiratory distress syndrome [5]. Acute lung injury caused by sepsis a major reason of refractory hypoxemia in patients. Changes in lung permeability following damage to alveolar epithelial and endothelial cells due to sepsis can lead to pulmonary edema [6]. In this context, melatonin may have protective properties against tissue damage caused by sepsis [7].

Melatonin, an antioxidant and anti-inflammatory molecule, is an endogenous hormone that is secreted at night

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from the gland called pineal or pineal and plays contributory role in the modulation of various biological functions such as sleep, reproduction, immunity and biological rhythms. Melatonin has various functional effects such as blood pressure regulation [8, 9], regulatory effect on endocrine rhythm, regulating immune functions, and protection from free oxygen radicals [10, 11].

Melatonin has pleiotropic roles in the regulation of different cellular process including the sleep-wake cycle and its receptors are widely distributed. A study conducted in 2014 reported that melatonin regulates mitogen-activated protein kinase (MAPK) and affects pro-inflammatory mediators in microbial-induced sepsis [12]. Melatonin appears to be associated with the sepsis state through its anti-inflammatory [13, 14] and immunomodulatory effects. Melatonin intervention may contribute to better survival in a septic animal model [15–17]. In this regard, melatonin may have a positive effect on multiple organ failure and septic shock due to sepsis.

Inflammasomes are supramolecular complexes are assembled in the cytosol in response to damage-associated and pathogen-associated stimuli as well as other danger signals. NLRP3 (NOD-like receptor protein 3) assembles a cytosolic innate immunological complex. Pyroptosis is highly inflammatory principally because of the release of inflammasome-dependent cytokines IL-1β and IL-18 [18]. Assembly of NLRP3 inflammasome triggers the activation of several cellular processes that stimulate additional surge of pro-inflammatory cytokines, triggering innate and adaptive immune responses. Therefore, NLRP3 activation appears to be associated with various inflammatory conditions such as sepsis [19]. In addition, it is known that the NIMA-related kinase 7 (NEK7) molecule is involved in the stimulation of the NLRP3 inflammasome in sepsis-related lung injury [20]. In this context, molecules affecting the NLRP3 inflammatory pathway may have a protective effect against septic shock and organ damage.

NEK7, a kinase is a critical and selective upstream regulator of NLRP3 inflammasomes [21]. NEK7-mediated stimulation of the NLRP3 inflammasome can be coordinated during Staphylococcus aureus-associated septic infection. In this context, the NEK7 molecule is involved in NLRP3 activation in sepsis-related acute lung injury [20].

Toll-like receptors (TLR) are critically important non-catalytic receptors in the immune system. Toll-like receptors (TLR2 and TLR4) are considered the most important pattern recognition receptors covering various antigenic determinants [22]. Stimulation of TLRs induces an acute inflammatory response in the innate immune response following sepsis-induced acute lung injury [23]. TLR2 is a member of a family of immune receptors. TLR2 recognizes a variety of microorganisms, such as viruses, fungi and bacteria and triggers pro-inflammatory responses. TLR2 is secreted primarily by myeloid cells [24]. Plasma levels of TLR2 are increased in various inflammatory conditions [25]. TLR2 is an ideal biomarker and pharmacological target for sepsis in critically ill patients suffering from failure of multiple organs [26].

Micro-CT is a versatile tool for examining diverse samples, including live specimens and various solid or liquid substances. Its applications extend to crucial areas such as imaging and analysis of soft tissues and bones. The technology facilitates in-vivo examinations of small animals, contributing to its widespread use in the health sector. Micro-CT serves as an objective assessment and diagnostic method in animal studies, particularly in the research and development of new drugs and agents [27, 28].

In line with the information presented, the effect of melatonin on NLRP3/NEK7/TLR-2 molecules in the lung was determined in the experimental endotoxemia model. The effectiveness of melatonin in sepsis-related lung injury was examined by analyzing the expression of NLRP3/NEK7/TLR-2 antibodies, MDA determination with Elisa, histopathological and immunohistochemical evaluation, and Micro-CT methods.

2. Material and Methods

2.1. Experimental groups

The protocol of our study was designed carefully. Kayseri Erciyes University Animal Ethics Committee gave the formal approval (Ethical committee decision number: 22/277). Controlled environment was provided to the experimental animals with a temperature of 24 ± 2°C and 60% humidity under a 12-hour light/dark cycle. Experimental animals had free access to standard food and tap water. All procedures were strictly in compliance with the recommendations in the Guide for the Care and Use of Laboratory Animals adopted by NIH (USA) as well as Declaration of Helsinki. Rats were given anesthesia by administering ketamine hydrochloride (50 mg/kg intramuscular (i.m.)/intraperitoneal (i.p.)) and 2% xylazine chloride (10 mg/kg i.m./i.p.).

In our research design, Sprague-Dawley adult male rats (n=28) were sub-divided randomly into 4 equal groups (n = 7), and these groups are listed below. Administration doses of LPS and melatonin to rats were selected according to the previously reported literature [1, 29, 30]. Additionally, experimental mice were moniored daily for any possible morphological or symptomatic change. In the study, all i.p. injection and gavage applications were performed at similar timings of the day. Since the current study determines melatonin-mediated protective effects on lung tissue in sepsis, melatonin was applied to the 3rd and 4th experimental groups for 10 days. Therefore, after completion of melatonin application in the 4th group, at the end of the 10th day, LPS at the determined dose was given intraperitoneally after 1 hour of application.

Group 1 (Control group, n=7): 1 ml physiological saline (SF) (0.9% sodium chloride (NaCl) sol.) was administered to the animals via i.p. every day for 10 days.

Group 2 (Melatonin group, n=7): Animals were given 1 ml i.p. melatonin at a dose of 10 mg/kg. It was applied daily for 10 days [30].

Group 3 (LPS group, n=7): LPS was administered at a dose of 1 ml at a dose of 30 mg/kg was administered to the animals i.p. as a single dose on the last day of the experiment [1].

Group 4 (Melatonin + LPS group, n=7): Animals were given 10 mg/kg melatonin 1 ml i.p. was administered daily for 10 days, and on the last day of the experiment, 30 mg/kg LPS was given intraperitoneally as a single dose of 1 ml.

2.2. Dissection of lung tissue of experimental groups

Sprague-Dawley rats in the experimental groups were sacrificed by the exsanguination methodology while under anesthesia, and lung tissue was dissected.

2.3. Histological analysis

Lung tissues obtained from control, melatonin (MEL), LPS, and LPS + MEL groups were placed in a 10% formaldehyde solution and fixed for 1 day. Following fixation, the tissues were left under running water overnight. Accordingly, next day, it was kept in increasing alcohol series (70%, 80%, and 96%) for one day each. After being left in 100% alcohol for 3 hours, the process was made clear in xylol. Paraffin was left in I, II, and III at intervals of one hour. Then, the samples were embedded in clean paraffin and turned into paraffin blocks. 5 µm thick sections were taken from each paraffin block onto slides. Sections were passed through xylene and decreasing alcohol series and stained with hematoxylin and eosin (H&E, BES LAB). The parameters of bleeding in the lung tissue, cellular infiltration, and alveolar wall thickening were scored according to whether 0 was none, 1 was mild, 2 was moderate, and 3 was severe [31].

2.4. Immunohistochemical analysis

Anti-NEK7 (Elabscience, E-AB-53304), Anti-TLR2 (Elabscience, E-AB-63499), and Anti-NLRP3 (Elabscience, E-AB) were detected in the lung tissues taken from the experimental groups using the Avidin-Biotin peroxidase method. -70161) immunoreactivities were detected by immunohistochemical analysis [29, 32]. After deparaffinization of 5 µm thick sections, citrate buffer was used to open the epitopes (pH: 6.0; UK, AP-9003-500, Thermo Fischer Scientific). The slides were then kept in a 3% hydrogen peroxide solution in methanol to prevent the activities of endogenous peroxidases. Ultra V block solution (Thermo Fischer Scientific, UK, TA-125-UB) was applied for the prevention nonspecific staining. Later, it was incubated with primary antibodies (NEK7 1:100, TLR2 1:100, and NLRP3 1:400 dilution ratios) at 4°C overnight. Then, it was incubated with biotinylated goat anti-polyvalent secondary antibody (Thermo Fischer Scientific, UK, TP-125-BN) at 37°C for 40 minutes in an oven. Sequentially, after several washes with PBS, it was incubated with streptavidin peroxidase (Thermo Fischer Scientific, UK, TS-125-HR) at 37°C for 30 minutes in an oven. Visualization of the antibody complexes was carried out by incubation with diaminobenzidine (DAB) chromogen (Thermo Fischer Scientific, UK, TA-125-HD). Therefore, counterstaining of the sections was done with Gill III Hematoxylin (Merck, Germany, 1.05174.1000). Dehydration of the sections was done by passing through a series of successively increasing alcohol dilutions and sealing with entellan. Examination of the sections was carried out with an Olympus BX53 light microscope. Immunoreactivity levels were analyzed using Image J Version 1.46 (NIH, Bethesda, Maryland).

2.5. ELISA method

For biochemical analyses, tissue samples were taken from experimental animals. MDA levels were measured in lung tissue. The protocol using the kit provided by the manufacturer was performed to determine rat MDA levels. Results were obtained using an ELISA reader device at 450 nm and were given in nmol/mL for MDA.

2.6. Micro CT method

Lung samples obtained from various groups were fixed within a slender paraffin block and meticulously oriented in a uniform manner within plastic tubes for micro-CT scanning. Achieving an isotropic voxel size of 20 µm within a 35 mm field of view was realized through adjustments in resolution parameters. Subsequently, images were captured for each sample using a Bruker Skyscan 1275 apparatus located in Kontich, Belgium. For imaging through bone tissue, an isotropic voxel size of 10 µm was attained employing settings of 40 kV, 250 µA, exposure time of 49 ms, rotation step of 0.2, and a 360-degree rotation within a high-resolution 23 mm field of view. Axial, coronal, and sagittal images for each sample were scrutinized utilizing Dataviewer software from Skyscan, Kontich, Belgium. For three-dimensional (3D) volumetric visualization and the subsequent analysis of area/volume measurements through Micro CT, CTAn software from Skyscan, Aartselaar, Belgium, was utilized.

2.7. Statistical analysis

Numerical values of the scored histopathological findings were compared by two-way analysis of variance using the GraphPad Prism 8 Version 8.4.3 program, and quantitative data showing immunohistological staining intensity (%) were compared by one-way analysis of variance. Differences between groups were determined by applying Tukey's multiple comparison test. $p<0.05$ was considered statistically significant. One-way ANOVA was used for multiple comparisons between groups.

3. Results

3.1. Histological analysis results

Bronchioles, alveolar, and pulmonary arteries were observed in normal histological structure in the lung tissues of the control and melatonin groups. Hemorrhage, cellular infiltration, and thickening of the alveolar wall were common in the lungs of the LPS group. A decrease in these symptoms was observed in the MEL + LPS group (Table 1, Figure 1A).

3.2. Immunohistochemical analysis

EK7, TLR2, and NLRP3 expressions were observed to increase in the LPS and LPS +MEL groups compared to the control group. It was determined that NEK7 and TLR2

Table 1. Damage rates observed in lung tissues.

Groups	Bleeding	Cellular Infiltration	Alveolar wall thickening
Control	0.22±0.09	0.22±0.09	0.11±0.09
MEL	0.22±0.19	0.33±0.16	0.5±0.16
LPS	1.22±0.38^{ab}	1.05±0.09^{ab}	1.83±0.28^{ab}
MEL + LPS	0.66±0.28^{c}	0.72±0.09^{a}	0.66±0.28^{ac}

$^{a}p<0.05$ indicates a difference compared to the control group, $^{b}p<0.05$ indicates a difference compared to the MEL group, $^{c}p<0.05$ indicates a difference compared to the LPS group (MEL, Melatonin group; LPS, Lipopolysaccharide group).

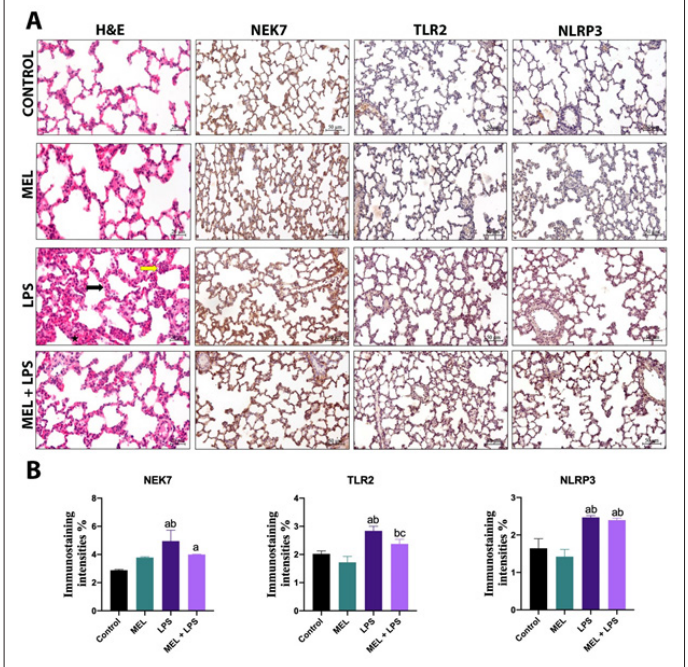

Fig. 1. Hematoxylin Eosin (H&E) images and immunohistochemical staining images of lung tissues of the experimental groups (A). Bar graphs showing NEK7, TLR2, and NLRP3 immunostaining intensities (B). The magnification of H&E images is 40X and the scale bar is 20 m. The star represents bleeding, the yellow arrow represents cell infiltration, and the black arrow represents alveolar wall thickening. The magnification of immunohistochemistry staining images is 20X and the scale bar is 50 m. Data shown in bar graphs are expressed as mean±SD. [a]$p<0.05$ shows that there is a difference compared to the control group, [b]$p<0.05$ shows that there is a difference compared to the MEL group, [c]$p<0.05$ shows that there is a difference compared to the LPS+MEL group.

expressions were decreased in the MEL + LPS group compared to the LPS group. When we compared it with the control group data, we found that NEK7 expression increased 1.71-fold in the LPS group and 1.38-fold in the MEL + LPS group. Findings suggested that the expression of TLR2 was noticed to be increased 1.4 times in the LPS group and 1.17 times in the MEL + LPS group as compared to the control group. We did not find statistically significant difference between the LPS and MEL + LPS groups in terms of NLRP3 expression. Finally, NLRP3 expression was observed to increase 1.5-fold in the LPS group and 1.45-fold in the MEL + LPS group compared to the control group (Figures 1A and 1B).

3.3. ELISA method results

There was a significant increment in MDA levels in the LPS group as compared to the other experimental groups. However, these levels were found to be reduced in the tissues in the MEL + LPS group (Figure 2).

3.4. Micro CT method results

Figure 3A shows Micro-CT images of lung tissues. When the micro-CT results were examined, the total volume value was significantly lower in the LPS group compared to the other groups. Object volume and Object surface values were significantly higher in the CONTROL and MEL groups as compared to groups exposed to LPS (Figure 3B).

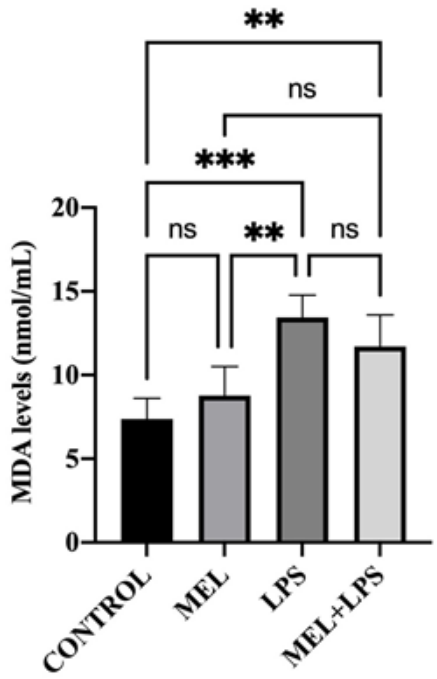

Fig. 2. MDA levels of lung tissues. Data shown in bar graphs are expressed as mean±SD. $p<0.05$ indicates a statistically significant difference (ns: p>0.05; **p<0.01; ***p<0.001).

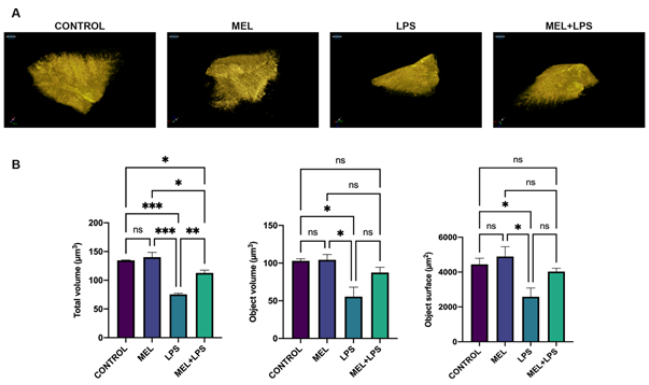

Fig. 3. Micro CT images of lung tissues (A), Volume measurements results with micro CT images (B). Data shown in bar graphs are expressed as mean±SD. $p<0.05$ indicates a statistically significant difference (ns: $p>0.05$; *$p<0.05$; **$p<0.01$; ***$p<0.001$).

4. Discussion

In this study, the immunoreactivity of melatonin to NLRP3/NEK7/TLR-2 molecules in the lung in endotoxic shock induced by the LPS agent was evaluated. While bleeding, cellular infiltration, and thickening of the alveolar wall were observed in the lung of the LPS group; A significant decrease in these symptoms was reported in the LPS + MEL group. NEK7, TLR2, and NLRP3 expressions increased significantly in the LPS and LPS+MEL groups compared to the control group; It was determined that NEK7, TLR2, and MDA levels decreased significantly in the LPS+MEL group compared to the LPS group. In this context, melatonin, an anti-oxidant molecule in sepsis-related acute lung injury, may have a positive effect on molecules in the inflammatory pathway.

LPS is responsible for initiating endotoxicity by inducing uncontrolled release of pro-inflammatory mediators from immune cells, especially monocytes and macrophages [33]. LPS interacts with TLR-4 and is transported

to the cell generating the host response [34]. Following stimulation of TLRs, various interleukins and pro-inflammatory mediators such as TNF-α are released by monocytes, macrophages, and lymphocytes and enter the systematic circulation [35]. In this context, micro-thrombi formation and pathology resulting in vascular occlusion, inadequate oxygen delivery, ischemia, and endothelial cell damage are observed [36]. In this context, various molecules such as melatonin may contribute to the reduction of organ damage in sepsis [37].

In similar studies in the literature, it has been shown by various analyses that LPS application causes damage to lung tissue and also increases TLR2 and TLR4 expression [38, 39]. Following ligand binding, TLR2 heterodimerization triggers intracellular transduction that results in activation of NF-κB, inflammatory cascade, and consequent production of important inflammatory cytokines [40]. Therefore, blocking TLR2 activation may help inhibit an active immune response and prevent damaging immunopathology. Therapeutic effects were determined in a study in which sepsis treatment was performed with an anti-TLR2 monoclonal antibody. It was reported that pretreatment with anti-TLR2 alone showed significant protection against sepsis-related death in a CLP-induced sepsis model in mice [41]. Therefore, anti-TLR2 therapy in sepsis patients can be suggested as a powerful therapeutic approach. In a study in 2022, TLR2 and TLR4 polymorphisms and susceptibility to sepsis were examined. By examining DNA samples with RT-PCR, it has been reported that there is a significant relationship between TLR2 Arg753Gln polymorphisms and sepsis [42]. Accordingly, TLR2 genotype may be a risk factor for sepsis. We have shown that TLR2 expression increased in the LPS group, similar to the literature, and melatonin treatment exerted inhibitory effects.

Selective inhibition of NLRP3 inflammasome activation may be a promising approach in the treatment of acute lung injury caused by inflammatory diseases such as sepsis [43]. In the literature, it has been shown that animals with deletion of the NLRP3 gene are protected against sepsis-induced organ damage and shock [19, 44]. Suppression of the NLRP3 inflammasome also reduces sepsis-induced platelet activation [45]. NLRP3 inflammasome activation can accelerate caspase-1 maturation, leading to acute lung injury. In the CLP-induced sepsis model in C57BL6 mice, an increase in NLRP3 was reported with acute lung injury [46]. In this regard, therapeutic approaches that limit the inflammatory response in sepsis, such as NLRP3 inhibition, may have a positive effect. About NLRP3, NEK7 can activate this molecule by binding to the leucine-rich repeat region [47, 48]. In addition, in sepsis-related acute lung injury, the NEK7 molecule is involved in NLRP3 activation [20]. In our study, we observed significant increase in the levels of NEK7 and NLRP3 in the lung in the LPS group as compared to the control group. A study in 2023 showed that NEK7 expression increased with LPS application and Total tanshinones (TTN) treatment suppressed this increase. It has been shown that the increase in NLRP3 and NEK7 production is abolished by TTN treatment or inhibition of NEK7 in LPS-induced lung injury mice [49]. Considering the interaction of NEK and NLRP3 proteins, the decrease in NLRP3 expression can be revealed statistically with different sepsis experimental models and numbers. In our findings, NEK7 resulted in a significant decrease in the lungs of septic mice given melatonin agents. Based on these data, therapeutic approaches such as melatonin may reduce the severity of sepsis-induced acute lung injury. In the literature, it was reported that IL-1β, TNF-α, NF-κB, matrix metalloproteinase-9 (MMP-9), and TGF-β decreased with the application of melatonin in lung injury due to sepsis model [50, 51]. Melatonin can reduce acute lung injury in endotoxemic rats. Our findings are consistent with the literature, bleeding, cellular infiltration, and alveolar wall thickening were commonly observed in the lung tissues of LPS application compared to the control and melatonin groups. Administration of melatonin along with LPS reduced these symptoms. The therapeutic application of melatonin, a powerful antioxidant substance, may have protective properties against tissue damage caused by LPS [7]. The positive properties of melatonin in sepsis have been attributed to reasons such as anti-oxidant, anti-inflammatory, and inhibition of nitric oxide production [52–54]. In previous studies, it was shown that with the application of a melatonin protective agent to an experimental sepsis model, VEGF decreased in the lung [13] and the SIRT1/SGK1/Nedd4–2 pathway was activated [55]. Our study reveals that melatonin administration in the experimental endotoxemia model lung may help reduce lung damage by inhibiting NEK7 and TLR2 expressions.

The widespread use and development of in vivo imaging in small animal disease models is very important in elucidating the etiology of various diseases and in developing effective diagnostic/treatment methods [56]. Bell et al., measured mouse lung tissue volume in an interstitial lung disease model using the micro-CT method. They reported that the results were compatible with histopathology and that the micro-CT method was a reliable non-invasive method [57]. In another study, micro-CT method was used in the endoxin-induced acute rat lung injury model. In another study, micro-CT method was used in the endoxin-induced acute rat lung injury model. In the study, it was reported that the total air volume of the lung decreased in rats exposed to endotoxin. They reported that the micro-CT method was suitable for the structural evaluation of the lung ultrastructure and its changes during endotoxin-induced lung injury [58]. In another similar study, acute lung injury was caused by LPS. In the analysis using the Micro-CT method, it was reported that LPS caused a decrease in total lung volüme [59]. In our study, we have used micro-CT methodology for the evaluation of melatonin-mediated effects on lung tissues in the endotoxic shock caused by LPS agent in rats and found results that were correlated with the results obtained in histopathological and immunohistochemical analyses. In addition, the results we obtained and the information in the literature support each other.

5. Conclusion

Our research showed that NLRP3/TLR2/NEK7 immunoreactivity increased in the LPS-induced experimental endotoxemia model and the positive effects of melatonin on acute lung injury. Additionally, micro CT results showed that lung volume decreased in LPS group and treatment groups approached control group. However, performing only MDA results and immunohistochemical analyses using the Eliza method can be considered a limitation of our research. Future research works should be

focused on a detailed mapping of protein networks related to inflammation in acute lung injury.

Conflict of interests
The author has no conflicts with any step of the article preparation.

Consent for publications
The author read and approved the final manuscript for publication.

Ethics approval and consent to participate
The experimental protocol of this study was approved by the Kayseri Erciyes University Animal Ethics Committee (Ethical committee decision number: 22/277).

Informed consent
The authors declare that no patients were used in this study.

Availability of data and material
The data that support the findings of this study are available from the corresponding author upon reasonable request

Authors' contributions
Arif Osman TOKAT, Osman ÖZTÜRK: Conceived and designed the study. Aslı OKAN, Sümeyye UÇAR, Ece EROĞLU: Collected and analyzed the data, and wrote the manuscript. Züleyha DOĞANYİĞİT, Mert OCAK, Şükrü ATEŞ: Contributed to the study design, data analysis, and interpretation of results. Seher YILMAZ: Contributed to the study design, reviewing &editing of manuscript, and answering reviewers.

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