ORIGINAL ARTICLE

DOI: 10.4274/nkmj.galenos.2024.77598
Nam Kem Med J 2024;12(1):8-16

Effects of Nintedanib on the Lungs via NLRP3 in a Model of Lipopolysaccharide-induced Acute Lung Injury in Rats

Sıçanlarda Lipopolisakkarid Kaynaklı Akut Akciğer Hasarı Modelinde Nintedanibin NLRP3 Yoluyla Akciğerler Üzerindeki Etkileri

Gulchin TANRİVERDİYEVA[1], Pelin AYDIN[1,2], Erdem TOKTAY[3], Elif ÇADIRCI[1], Zekai HALICI[1]

[1]*Atatürk University Faculty of Medicine, Department of Medical Pharmacology, Erzurum, Turkey*
[2]*Erzurum City Hospital, Clinic of Anesthesiology and Reanimation, Erzurum, Turkey*
[3]*Kafkas University Faculty of Medicine, Department of Histology and Embryology, Erzurum, Turkey*

ABSTRACT

Aim: To demonstrate the possible protective efficacy of nintedanib, a tyrosine kinase inhibitor with demonstrated antifibrotic and antitumor activity, in a model of acute lung injury (ALI), a severe lung disease, through NLR family pyrin domain containing 3 (NLRP3) and nuclear factor kappa B (NF-κB) pathways.

Materials and Methods: In this study, 40 male Wistar albino rats were used. These rats were divided into 5 groups of equal sizes. Before the experiment began, nintedanib was administered orally to selected groups at doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg. At 24 hours, 12 hours, or 1 hour after nintedanib administration, rats selected for the lung injury model were administered intratracheal LPS. Interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) amounts were measured by ELISA method and NLRP3, caspase-1, IL-1β and NF-kB gene expressions were measured by reverse-transcriptase polymerase chain reaction.

Results: It was observed that administration of nintedanib lowered the elevated NLRP3, caspase-1, IL-1β, and NF-κB expressions and the IL-1β and TNF-α cytokine levels in the tissues of rats with LPS-induced ALI. The findings obtained for the rats included in the lung injury group that received 50 mg/kg nintedanib were most similar to those of the healthy control group.

Conclusion: In rats modeled with ALI, nintedanib was shown to modulate the NLRP3/NF-κB signaling pathway and reduce the effects of ALI.

Keywords: ALI, NF-κB, nintedanib, NLRP3, tyrosine kinase inhibitor

ÖZ

Amaç: Antifibrotik ve antitümör etkinliği gösterilmiş bir tirozin kinaz inhibitörü olan nintedanibin ciddi bir akciğer hastalığı olan akut akciğer hasarı (ALI) modelinde etkileri ve bu hastalığa karşı olası koruyucu etkinliğinin ortaya konulması ve NLRP3 / nükleer faktör kappa B (NF-κB) yolağı üzerine olası etkisinin incelenmesidir.

Gereç ve Yöntem: Bu çalışmada 40 adet erkek Wistar albino sıçan kullanıldı. Bu sıçanlar eşit büyüklükte 5 gruba ayrıldı. Deney başlamadan önce, nintedanib seçilen gruplara 25 mg/kg, 50 mg/kg ve 100 mg/kg dozlarında oral olarak uygulandı. Nintedanib uygulamasından 24 saat, 12 saat veya 1 saat sonra, ALI için seçilen sıçanlara intratrakeal LPS uygulandı. İnterlökin (IL)-1β ve tümör nekroz faktörü-α (TNF-α) miktarları ELISA yöntemiyle, NLRP3, kaspaz-1, IL-1β ve NF-kB gen ekspresyonları ise revers-transkriptaz polimeraz zincir reaksiyonu yöntemiyle ölçüldü.

Bulgular: Nintedanib uygulamasının, LPS ile indüklenen ALI'li sıçanların dokularında yüksek NLRP3, kaspaz-1, IL-1β ve NF-κB ekspresyonlarını ve IL-1β ve TNF-α sitokin düzeylerini düşürdüğü gözlendi. 50 mg/kg nintedanib alan akciğer hasarı grubunda yer alan sıçanlardan elde edilen bulgular, sağlıklı kontrol grubuna en çok benzeyen bulgulardı.

Sonuç: ALI ile modellenen sıçanlarda nintedanibin NLRP3/NF-κB sinyal yolunu modüle ettiği ve ALI'nin etkilerini azalttığı gösterilmiştir.

Anahtar Kelimeler: ALI, NF-κ, B, nintedanib, NLRP3, tirozin kinaz inhibitörü

Address for Correspondence: Zekai HALICI MD, Atatürk University Faculty of Medicine, Department of Medical Pharmacology, Erzurum, Turkey
Phone: +90 532 386 88 84 **E-mail:** hzekai@atauni.edu.tr **ORCID ID:** orcid.org/0000-0001-6854-6059
Received: 21.11.2023 **Accepted**: 10.01.2024

INTRODUCTION

Acute lung injury (ALI) is a type of lung inflammation with high mortality rates and it is characterized by the disruption of the alveolar capillary barrier and gas exchange dysfunction[1,2]. It is also characterized by decreased lung compliance, persistent hypoxemia, and respiratory failure. Cases of ALI are commonly observed in intensive care units. Various factors may cause ALI, but the most common causes are sepsis, pneumonia, disseminated intravascular coagulation, burns, acute pancreatitis, massive blood transfusions, aspiration of gastric contents, shock, emergency blood transfusions, and trauma[3]. The pathogenesis of this acute and severe disease is unclear. The current consensus suggests that ALI occurs as a result of damage caused by uncontrolled cytokine production due to an inflammatory process[4]. ALI has no specific treatment. Hence, new strategies, biomarkers, and treatments are needed to reduce ALI-related morbidity and mortality rates[5].

Prior studies have examined the use of different mechanical ventilation techniques and different pharmacological agents in the treatment of ALI[6-8]. However, despite the large number of studies, satisfactory results have not yet been achieved. Tyrosine kinases and their related pathways were more recently discovered, and it has been shown that tyrosine kinases and their related pathways may be involved in the physiopathology of many diseases. Accordingly, they have become new targets for therapeutic approaches.

Tyrosine kinase is an enzyme that plays an important role in intracellular signal transduction and catalyzes protein phosphorylation. Tyrosine kinases modulate numerous signaling pathways for survival and other various cellular functions via the phosphorylation of amino acid residues. On the other hand, tyrosine kinase inhibitors also interfere with the binding of ATP and its substrate, preventing phosphorylation[9,10].

Various tyrosine kinases and inhibitors are associated with these effects. Moreover, new tyrosine kinases and inhibitors continue to be discovered as time goes on. The different types of tyrosine kinase inhibitors are characterized by the receptors they inhibit and their mechanisms of action on different pathways. Inhibitors of the HER family (EGFR-1 and EGFR-2) and first-generation and second-generation multi-target tyrosine kinase inhibitors (VEGFR and PDGFR) are some examples of these compounds[11].

Among these inhibitors, nintedanib, which had recently begun being investigated experimentally in cases of inflammation and related diseases and which has been licensed for the treatment of pulmonary hypertension, is the subject of the present study. It was previously found that nintedanib mitigates the expression of mesenchymal markers, inhibits phosphorylation, and improves hemodynamics[12].

Nintedanib is a molecule designed as a multi-target first-generation tyrosine kinase inhibitor that inhibits both receptor (FGFR-1, VEGFR-2, and PDGFR) and non-receptor (Src, Lyn, and Lck) tyrosine kinases[13]. There are ongoing studies on the efficacy of nintedanib against various types of cancer, including ovarian cancer, colorectal cancer, and non-small-cell lung cancer. When prior studies are examined, it is seen that nintedanib has been used in the treatment of idiopathic pulmonary fibrosis due to its effects via PDGFR[14]. It has also been shown that this drug inhibits VEGF receptor expression and angiogenesis in prostate cancer cells *in vitro*, and it decreases microvessel density[15]. VEGF-C/VEGFR-3 signaling was shown to ameliorate the effects of experimental lung injury in macrophages, which suggests that this mechanism could also play a role in the treatment of ALI and acute respiratory distress syndrome (ARDS)[16].

NLRP3 is an inflammasome belonging to the NLR family. Inflammasomes are important functional members of the innate immune system and the most characteristic inflammasome is NLRP3. It is known as the main inflammasome associated with inflammation and it plays a pivotal role in cases of ALI[17].

Prior studies have explored the relationship between nintedanib and NLRP3. In the literature, it has been shown that nintedanib treatment reduces the number of inflammatory cells, effectively alleviating the lung damage caused by polyhexamethylene guanidine (PHMG). It was also determined that nintedanib significantly reduced the expression of inflammatory cytokines and fibrotic factors and the activation NLRP3 in lung tissues. These results suggest that nintedanib may reduce the inflammatory response and improve pulmonary fibrosis in the lungs of mice administered PHMG[18].

ALI-induced mortality is known to be associated with the loss of epithelial barrier function[19]. NLRP3 was shown to have a regulatory role in TGF-β signaling in tubular epithelial cells and it was also observed that, in addition to affecting IL-1β and IL-18 maturation and pyroptosis induction, NLRP3 affects immunity and tissue damage[20].

In light of these previous findings, it is possible that nintedanib could alleviate ALI by modulating the NLRP3/NF-κB signaling pathway. Thus, the aim of the present study is to examine the effects of nintedanib, a tyrosine kinase inhibitor shown to have antifibrotic and antitumor efficacy, in a model of ALI and to demonstrate its possible protective efficacy against this disease and the connection of its possible efficacy with the NLRP3 and NF-κB pathways.

MATERIALS AND METHODS

1. Experimental Animals

In this study, 40 Wistar albino male rats weighing 240-280 g were used. All rats were obtained from the Experimental

Animal Laboratory of the Medical Experimental Application and Research Center of Atatürk University. All treatments and procedures were carried out according to the national guidelines accepted by the local ethics committee. The study was approved by the Local Ethics Council of Animal Experiments of Atatürk University (date: 26.04.2022, no: E-42190979-000-2200127868). Rats were housed in standard polypropylene cages in an environment with controlled temperature (22±1 °C) and humidity (50-60%) and a 12-hour light/dark photoperiod. They were provided standard food and tap water ad libitum.

2. Chemicals

Nintedanib (Ofev, Boehringer Ingelheim, Germany), LPS (*E. coli* 055:B5, Sigma-Aldrich Chemie GmbH, Germany), ketamine (Ketalar 500 mg/10 mL, Pfizer, Turkey), and xylazine (Basilazine 2%, Biotek, Turkey) were used in this study.

3. Experimental Groups

The rats were randomly divided into 5 groups with 8 rats in each group as follows:

Group 1: Healthy control group,

Group 2: ALI group (5 mg/kg LPS),

Group 3: ALI (5 mg/kg LPS) + nintedanib (NDN) at 25 mg/kg,

Group 4: ALI (5 mg/kg LPS) + NDN at 50 mg/kg,

Group 5: ALI (5 mg/kg LPS) + NDN at 100 mg/kg.

4. Experimental Design and Preparation of the Utilized Drugs

The 40 male Wistar albino rats used in this study were divided randomly into 5 groups of equal size. Nintedanib was administered via oral gavage to selected groups at doses of 25 mg/kg, 50 mg/kg, and 100 mg/kg. At 24 hours, 12 hours, or 1 hour after nintedanib administration, selected rats were administered LPS. The half-life of nintedanib was considered while selecting these 3 dosages[21,22].

The rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) 1 hour after the last dose was given[23]. After sterilization, a midline neck incision was made using a surgical scalpel. After the incision was made, 5 mg/kg LPS[24] (Sigma-Aldrich) was dissolved in 0.2 mL of 0.09% NaCl and administered intratracheally to all groups except the healthy control group. In order to ensure homogeneous infiltration of the lungs, the rats were rotated repeatedly on 3 axes. The incision was then surgically sutured and sodium fusidate (2%) was applied topically[25]. Rats were euthanized with thiopental at a high dose of 50 mg/kg after 24 hours and then blood and lung tissue samples were collected.

5. Molecular Assays

This study was conducted *in vivo*. Within the scope of the study, gene expressions were examined molecularly. Reverse-transcriptase polymerase chain reaction (RT-PCR) was used to examine lung NLRP3, caspase-1, NF-κB, and IL-1β mRNA expressions (Table 1). For this purpose, lung tissue samples were homogenized, their RNAs were isolated, their cDNAs were synthesized, and mRNA expressions were quantitatively examined.

6. RT-PCR

6.1. Extraction of RNA from Lung Tissues

Lung samples were weighed individually and stored at 4 °C for up to 4 weeks with RNAlater RNA Stabilization Reagent (QIAGEN, Germany). Tissues were homogenized with TissueLyser II (QIAGEN) and RNA extraction was performed in a QIAcube RNA isolation device (QIAGEN). Tissue samples were weighed individually and total RNA isolation was carried out using the QIAcube in combination with the RNeasy Mini Kit (QIAGEN) following the instructions provided by the manufacturer. Total mRNA was measured at 260 nm by nanodrop spectrophotometry (EPOCH, Biotek).

Table 1. RT-PCR gene sequencing

GAPDH	NM_017008.4	F: GCA AGT TCA ACG GCA CAG R: CTC AAC AGT ATA AAG AGC
NF-κB	NM_001276711.1	F: GAG ATT GTG CCA AGA GTG AC R: CTT GTC TTC CAT GGT GGA TG
NLRP3	NM_001191642.1	F: GTG GAG ATC CTA GGT TTC TCT G R: CAG GAT CTC ATT CTC TTG GAT C
IL-1β	NM_031512.2	F: TGC TGT CTG ACC CAT GTG AG R: GTC GTT GCT TGT CTC TCC TTG
Caspase-1	NM_012762.3	F: GAG CTG ATG TTG ACC TCA GAG R: CTG TCA GAA GTC TTG TGC TCT G

RT-PCR: Reverse-transcriptase polymerase chain reaction, NF-κB: Nuclear factor kappa B

6.2. Obtaining cDNA from RNA

cDNA synthesis was performed from total RNA using a cDNA reverse transcription kit. Each reaction was performed with the amount of RNA specified in the kit's instructions. The amount of cDNA obtained was measured by nanodrop spectrophotometry (EPOCH Plate, Biotek) and the cDNA was stored at -20 °C.

6.3. Quantitative Examination of mRNA Expressions by Real-time PCR

Lung mRNA expressions were examined using the SYBR GREEN Gene Expression Master Mix Kit. GAPDH was used as the reference gene. The recommended amount of cDNA was pipetted for the amplification and quantification processes, which were carried out in the recommended cycles.

6.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Lung samples stored at -80 °C were subjected to physical homogenization in liquid nitrogen using the TissueLyser II device (QIAGEN). Each sample was weighed to 100 mg. The weighed tissues were then homogenized in 1 mL of PBS homogenate buffer in an Eppendorf tube using the TissueLyser II device. Subsequently, the tissues were centrifuged for ELISA as recommended by the manufacturer of the kits. The interleukin (IL)-1β and tumor necrosis factor-α (TNF-α) cytokine levels of the obtained supernatants were measured. For IL-1β measurements, a rat IL-1β ELISA kit (Cat. No. E0119RA-96T, BTLAB, UK) was used, while a rat TNF-α ELISA kit (Cat. No. E0764RA-96T) was used for TNF-α measurements. Protein amounts were measured using the Lowry method and the obtained data were normalized according to cell protein concentrations.

7. Histopathological Method

Lung tissues obtained via necropsy were placed in 3.7% neutral formalin solution. Routine alcohol-xylol follow-up procedures were performed for the tissues and then the tissues were embedded in paraffin blocks. Sections of 5 µm in thickness were transferred to poly-L-lysine slides and stained with Masson's trichrome. The histopathological findings observed in the lung tissues, including perivascular edema, submucosal edema, alveolar septal thickening, and inflammation, were scored semi-quantitatively as being absent (0), mild (1), moderate (2), or severe (3) compared to the healthy control group.

Statistical Analysis

Ct values were automatically converted to ΔCt values with the device and the obtained results were statistically evaluated with IBM Statistical Package for the Social Sciences statistics 25.0 (IBM Corp., USA). Data with non-homogeneous variance were subjected to one-way ANOVA and the Games-Howell test as a post-hoc multiple comparison test. Data with homogeneous variance were subjected to one-way ANOVA and the Tukey test as a post-hoc multiple comparison test. Values of $p<0.05$ were considered significant.

RESULTS

1. Molecular Findings

1.1. Caspase-1 Expression in Lung Tissues

When the caspase-1 expressions of the groups were examined, it was determined that the healthy control group had the lowest levels of caspase-1 expression. It was also observed that the ALI group had very significantly higher caspase-1 expression levels compared to the healthy control group ($p<0.001$). When the caspase-1 expressions of the ALI + NDN groups were examined, it was found that the groups that received all 3 dosages of nintedanib had very significantly lower caspase-1 expressions compared to the ALI group, the lowest being the caspase-1 expression of the ALI + 50 NDN group ($p<0.001$).

1.2. IL-1β Expression in Lung Tissues

When the IL-1β expressions of the groups were evaluated, it was determined that the healthy control group had the lowest levels of IL-1β expression. It was also observed that the ALI group had very significantly higher IL-1β expression levels compared to the healthy control group ($p<0.001$). When the IL-1β expressions of the ALI + NDN groups were examined, it was found that the groups that received all 3 dosages of nintedanib had very significantly lower IL-1β expressions compared to the ALI group, the lowest being the IL-1β expression of the ALI + 50 NDN group ($p<0.001$) (Figure 1).

1.3. NF-κB Expression in Lung Tissues

When the NF-κB expressions of the groups were examined, it was determined that the healthy control group had the lowest NF-κB expression. It was also observed that the ALI group had very significantly higher NF-κB expression levels compared to the healthy control group ($p<0.001$). When the NF-κB expressions of the ALI + NDN groups were evaluated, it was found that the groups that received all 3 dosages of nintedanib had very significantly lower NF-κB expressions compared to the ALI group, the lowest being the NF-κB expression of the ALI + 50 NDN group ($p<0.001$) (Figure 1).

1.4. NLRP3 Expression in Lung Tissues

When the NLRP3 expressions of the groups were examined, it was determined that the healthy control group had the lowest levels of NLRP3 expression. It was also observed that the ALI group had very significantly higher NLRP3 expression compared to the healthy control group ($p<0.001$). When the

NLRP3 expressions of the ALI + NDN groups were examined, it was found that the groups that received all 3 dosages of nintedanib had very significantly lower NLRP3 expressions compared to the ALI group, the lowest being the NLRP3 expression of the ALI + 50 NDN group (p<0.001) (Figure 1).

2. ELISA Findings

2.1. Effect of Nintedanib on TNF-α Cytokine Levels in Lung Tissues

When the TNF-α levels of the groups were examined, it was determined that the healthy control group had the lowest TNF-α levels. It was also observed that the ALI group had very significantly higher TNF-α levels compared to the healthy control group (p<0.001). The ALI + NDN groups had very significantly lower TNF-α levels compared to the ALI group. Among the ALI + NDN groups, the ALI + 50 NDN group was found to have TNF-α levels most significantly similar to those of the healthy control group (Figure 2).

2.2. Effect of Nintedanib on IL-1β Cytokine Levels in Lung Tissues

Considering IL-1β levels of the groups, it was revealed that the healthy control group had the lowest IL-1β levels. It was also observed that the ALI group had very significantly higher IL-1β levels compared to the healthy control group (p<0.001). When the IL-1β levels of the ALI + NDN groups were examined, it was found that the groups that received all 3 dosages of nintedanib had very significantly lower IL-1β levels compared to the ALI group, the lowest being the IL-1β levels of the ALI + 25 NDN and ALI + 50 NDN groups (p<0.001). Among the ALI + NDN groups, the ALI + 50 NDN group had IL-1β levels most significantly similar to those of the healthy control group (Figure 3).

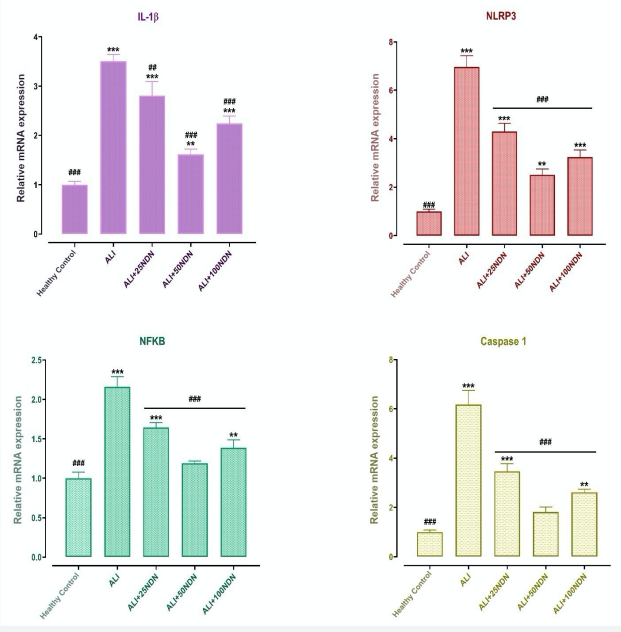

Figure 1. IL-1β, NLRP3, NF-κB, and caspase-1 expressions in lung tissues

****Signifies p<0.001 compared to the healthy group. ###Signifies p<0.001 compared to the ALI group*

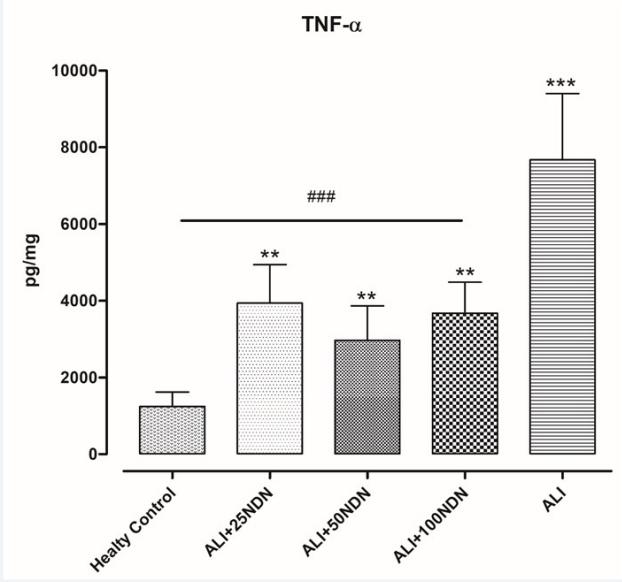

Figure 2. Lung tissue TNF-α ELISA results

****Signifies p<0.001 compared to the healthy group. ###Signifies p<0.001 compared to the ALI group*

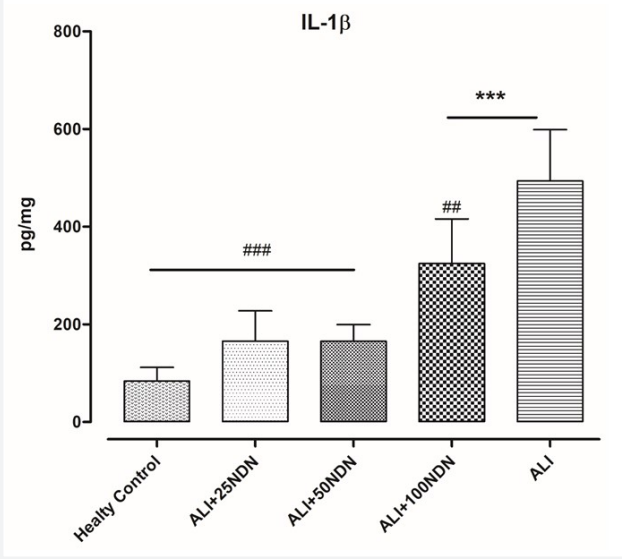

Figure 3. Lung tissue IL-1β ELISA results

****Signifies p<0.001 compared to the healthy group. ###Signifies p<0.001 compared to the ALI group*

3. Histopathological Findings

In the histopathological examination of the healthy control group, the bronchi, terminal bronchioles, respiratory bronchioles, alveolar sacs, alveolar walls, arteries, veins, and capillary vessel structures in the lung tissues were examined in detail. No pathological findings were found (Figures 4A, 4B).

Areas of advanced edema in the vascular adventitia and a large number of inflammatory cells (circles in images) were found in the ALI group. Alveolar septal thickening (stars) due to advanced edema was observed in the alveolar walls of the alveolar tree, encompassing the respiratory parenchyma of the lungs. Erythrocyte clusters infiltrating the alveoli from place to place in this tissue was another important finding (Figure 4C).

Similar to the ALI group, submucosal thickening due to edema and inflammatory cells were also observed in the ALI + 25 NDN group. However, the observed findings were not as severe in the ALI + 25 NDN group compared to the ALI group (Figure 4D). The alveolar septal thickening observed in the ALI + 25 NDN group was also not as severe compared to the ALI group (Figure 4D).

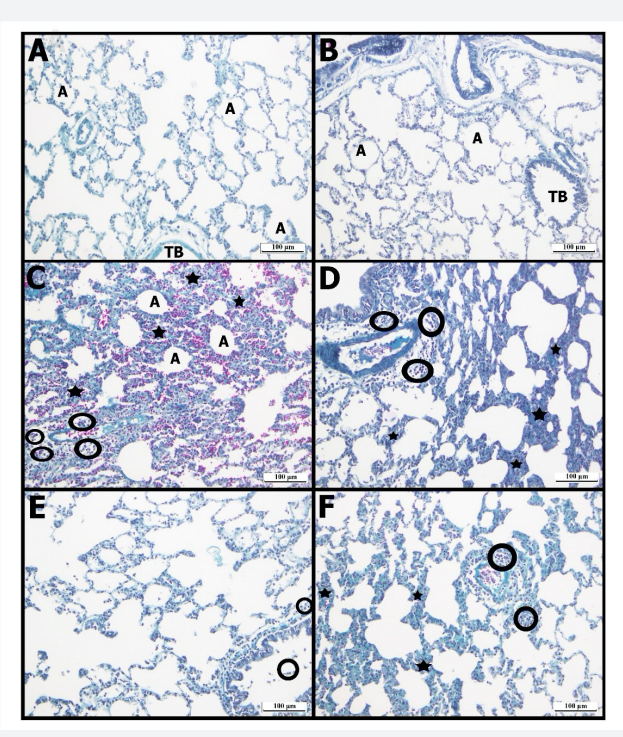

Figure 4. Lung histopathology findings with Masson's trichrome staining. A, B: Healthy control group, C: ALI group, D: ALI + NDN 25 group, E: ALI + NDN 50 group, F: ALI + NDN 100 group (A: alveoli, TB: terminal bronchiole, AD: alveolar wall, Circle: alveolar wall thickening and perivascular or submucosal edema, Star: alveolar septal thickening)

It was observed that the edematous areas around the submucosa surrounding the vessels and around the terminal bronchioles were improved significantly in the ALI + 50 NDN group. The alveolar septal thickening in the ALI + 50 NDN group was insignificant enough to be considered healthy. Inflammatory cells were also rarely observed in these tissues. In general, this group was observed to have a histological appearance similar to that of the healthy control group (Figure 4E).

Submucosal edematous areas around the vessels were also observed in the ALI + 100 NDN group. However, the observed findings were not as severe in this group as in the ALI group. In addition, mild alveolar septal thickening was observed in this group (Figure 4F).

DISCUSSION

In this study, the effects of nintedanib on LPS-induced ALI via NLRP3/NF-κB were examined. It was observed that the elevated expressions of IL-1β, caspase-1, NLRP3, and NF-κB and the elevated levels of IL-1β and TNF-α cytokines due to LPS administration decreased with the administration of all 3 selected dosages of nintedanib. The administration of nintedanib at 50 mg particularly improved inflammation and pulmonary edema and ultimately alleviated ALI, nearly reaching the same status as that seen in the healthy control tissues.

LPS is a glycolipid, and the LPS found on the outer membranes of Gram-negative bacteria binds to a specific lipopolysaccharide-binding protein (LBP), especially in serum[26]. LPS binding to LBP triggers the production of inflammatory mediators by activating the CD14/TLR-4 receptor complex in immune cells. LPS, which is an important mediator of Gram-negative bacterial sepsis, constitutes a very useful tool for studying the effects of Gram-negative bacterial infections in humans and animals. This approach allows for the examination of the effects of inflammatory responses that occur in cases of bacterial infections[27]. LPS is also one of the most suitable models for studying ALI caused by immune responses[28]. The lung consists of two fundamental anatomical structures, namely the vascular and airway structures. The lung epithelium protects the lung, especially from harmful toxins. Epithelial tissues that cover both airway and vascular structures play major roles in ALI[29]. Activation of the NF-κB pathway and the inflammation of the airway epithelium are the most important local and systemic responses to lung injury[30]. Mitogen-activated protein kinase (MAPK) regulates LPS-induced NF-κB activation[31], and NF-κB stimulation activates a large number of intracellular signaling pathways[32,33]. NF-κB also activates NLRP3, which is known as the main regulator of inflammation. NF-κB regulates the inflammatory response by directly converting pro-IL-

1β to active IL-1β, as well as activating NLRP3 to convert procaspase-1 to active caspase-1[34]. Inhibition of the PDGFRβ/Akt/NF-κB/NLRP3 pathway and modulation of NF-κB activity have been shown to be effective mechanisms for alleviating ALI[35,36]. NLRP3 is mainly activated in two ways. The first type of activation, as noted above, occurs through NF-κB and IL-1β. The second type of activation occurs via important signaling receptors in innate immune cells that can be activated by various pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs)[37]. NLRP3 is the primary protein that causes inflammation by forming a complex with pro-caspase-1 via ASC. The activation of the NLRP3 inflammasome leads to the transformation of procaspase-1 to caspase-1. This transformation also triggers the production and secretion of mature IL-1β and IL-18[38]. IL-1β, IL-18, and TNF-α are important proinflammatory cytokines produced by macrophages in response to PRR activation. TNF production is mainly regulated at the transcription level. It has also been shown that NLRP3 plays pivotal roles in the physiopathology of infectious agents such as *Staphylococcus aureus*[39], *Pseudomonas aeruginosa*[40], *Mycobacterium tuberculosis*[41], and influenza A virus[42], which cause ALI.

In a prior study, it was found that reduction of elevated NF-κB and NLRP3 transcription due to LPS via various treatment methods was effective in alleviating lung damage by reducing the amounts of proinflammatory TNF-α and IL-1β cytokines[43].

Previous studies also showed that NLRP3 played important roles in ALI and that experimental treatment with NLRP3 antagonists provided very significant benefits against ALI[17,44].

Similar to those studies, the findings of the present study revealed that the NLRP3/NF-κB signaling pathway was activated due to LPS administration. It was found that elevated NLRP3, NF-κB, caspase-1, and IL-1β gene expressions and TNF-α and IL-1β cytokine levels were alleviated with nintedanib treatment. These findings suggest that the inflammatory cascade is induced due to increased receptor activation following LPS administration, and lung damage occurs due to increased cytokine release activated as a result of NLRP3 activating the NF-κB signaling pathway[45]. Moreover, it was observed that this inflammatory cascade was blocked quite effectively in the groups that received nintedanib. In prior clinical studies, it was found that high levels of IL-1 and IL-18 were produced by the alveolar macrophages of patients with ALI, and it was shown that this increase in IL-1 and IL-18 production was directly associated with mortality and morbidity[46,47]. It is known that IL-1 and IL-18 cytokines are directly associated with the NLRP3 pathway. In this context, prior experimental studies of ALI achieved very positive results by antagonizing IL-1 and IL-18 cytokines[48,49].

Nintedanib is a first-generation multi-target tyrosine kinase inhibitor. It is used in the treatment of idiopathic pulmonary fibrosis because it reduces lung function disruption and inhibits the proliferation of vascular cells[50]. In addition to idiopathic pulmonary fibrosis, it is approved for use in cases of fibrosing interstitial lung diseases. Nintedanib can thus be applied against common health problems that occur around the world and its use has become a focus of attention. It has also been shown that nintedanib can be used in the treatment of pulmonary arterial hypertension since it blocks the phosphorylation of PDGF and FGF receptors, improving neointimal lesions and medial wall thickening in pulmonary arteries[12]. Nintedanib inhibits both receptor and non-receptor tyrosine kinases. Prior studies showed that the inhibition of Src tyrosine kinase alleviates ALI[51]. PDGF, a growth factor whose receptors are targeted by tyrosine kinase inhibitors, plays a key role in the pathogenesis of lung diseases such as pulmonary fibrosis, ALI, and ARDS. It is a chemotactic factor for monocytes and granulocytes during inflammation, and overexpression of PDGF can induce inflammatory damage[52]. In addition to PDGFR, the receptors of VEGF, which play important roles in many organs by directly regulating vascular permeability to water and proteins, are also targeted by nintedanib. VEGF increases mRNA expression, activates inflammation, and causes capillary leakage and pulmonary edema in cases of ALI[53]. Moreover, it was shown that VEGF inhibition reduced pulmonary edema due to ALI[54]. A prior study revealed that nintedanib inhibited angiogenesis by reducing the increased VEGF receptors in prostate cancer cells. In the same study, it was also observed that microvascular density decreased as a result of nintedanib administration[15]. When the results of the present study are considered, it can be said that nintedanib suppresses inflammation and alleviates ALI both through growth factors and directly via the innate immune system.

The possible effects of nintedanib observed in the present study can also be explained by its direct effects on NLRP3. Nintedanib, which is mainly used to treat idiopathic pulmonary fibrosis, has been the focus of many studies in the literature due to its effects on various signaling pathways. To date, however, only one study has examined the relationship between nintedanib and NLRP3. In that study, it was shown that nintedanib could alleviate lung fibrosis by reducing the activation of the NLRP3 inflammatory response in the lungs and improving pulmonary fibrosis, and it was furthermore shown that nintedanib reduced IL-1 and TNF-α levels[18]. In another study that examined the anti-inflammatory effects of nintedanib, it was found to reduce IL-1 levels[55]. In the present study, the effects of nintedanib on NLRP3 in ALI were elucidated for the first time in the literature.

This study also showed the improvement in lung tissues histopathologically. When the histopathologic findings of

the ALI group were examined, it was observed that there was diffuse perivascular and submucosal edema. At the same time, alveolar septal thickening and severe inflammation were detected. It was observed that the severity of these findings decreased and ALI was attenuated in the groups given all three doses of nintedanib. In the ALI + 50 NDN group, these findings were found to be very mild and close to the healthy group.

Study Limitations

The limitation of our study is that Western blot analysis, which is used to identify specific proteins, could not be performed in lung tissue.

CONCLUSION

Through the ALI model designed for this study, it was shown for the first time that nintedanib reduced LPS-induced ALI via the NLRP3/NF-kB signaling pathway. It was molecularly, biochemically, and histopathologically confirmed that nintedanib reduced the gene expressions of NLRP3, IL-1β, caspase-1, and NF-κB and the levels of IL-1β and TNF-α cytokines in cases of LPS-induced ALI. These results support the possibility of nintedanib providing beneficial results by protecting against ALI and suggest that the use of this drug may be effective.

Acknowledgement

This study is a part of Gülçin Tanriverdiyeva's master's thesis.

Ethics

Ethics Committee Approval: The study was approved by the Local Ethics Council of Animal Experiments of Atatürk University (date: 26.04.2022, no: E-42190979-000-2200127868).

Informed Consent: Animal experiment.

Authorship Contributions

Surgical and Medical Practices: Z.H., G.T., P.A., E.T., E.Ç., Concept: Z.H., G.T., Design: Z.H., G.T., Data Collection or Processing: P.A., E.T., E.Ç., Analysis or Interpretation: P.A., E.T., E.Ç., Literature Search: Z.H., G.T., Writing: Z.H., G.T.

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

Financial Disclosure: Financial support of the study was provided by Atatürk University Scientific Research Projects Coordination Unit (TYL-2023-11601)

REFERENCES

1. Zhang Z, Wang X, Ma C, Li Z, Chen H, Zhang Z, et al. Genipin protects rats against lipopolysaccharide-induced acute lung injury by reinforcing autophagy. Int Immunopharmacol. 2019;72:21-30.
2. Wu P, Yan H, Qi J, Jia W, Zhang W, Yao D, et al. L6H9 attenuates LPS-induced acute lung injury in rats through targeting MD2. Drug Dev Res. 2020;81:85-92.
3. Tai W, Xu Y, Ding J, Wu H, Du M, Qu X, et al. Fibrocytes Ameliorate Acute Lung Injury by Decreasing Inflammatory Cytokine and Chemokine Levels and Reducing Neutrophil Accumulation in the Lung. Cell Physiol Biochem. 2017;44:1526-36.
4. Liang Y, Yang N, Pan G, Jin B, Wang S, Ji W. Elevated IL-33 promotes expression of MMP2 and MMP9 via activating STAT3 in alveolar macrophages during LPS-induced acute lung injury. Cell Mol Biol Lett. 2018;31;23:52.
5. Mokrá, D. Acute lung injury - from pathophysiology to treatment. Physiol Res. 2020;31;69:S353-66.
6. Santa Cruz R, Villarejo F, Irrazabal C, Ciapponi A. High versus low positive end-expiratory pressure (PEEP) levels for mechanically ventilated adult patients with acute lung injury and acute respiratory distress syndrome. Cochrane Database Syst Rev. 2021;30;3:CD009098.
7. Mokra D, Mikolka P, Kosutova P, Mokry J. Corticosteroids in Acute Lung Injury: The Dilemma Continues. Int J Mol Sci. 2019;25;20:4765.
8. Patel VJ, Biswas Roy S, Mehta HJ, Joo M, Sadikot RT. Alternative and Natural Therapies for Acute Lung Injury and Acute Respiratory Distress Syndrome. Biomed Res Int. 2018;16;2018:2476824.
9. Krause DS, Van Etten RA. Tyrosine kinases as targets for cancer therapy. N Engl J Med. 2005;14;353:172-87.
10. Thompson AM, Rewcastle GW, Tercel M, Dobrusin EM, Fry DW, Kraker AJ, et al. Tyrosine kinase inhibitors. 1. Structure-activity relationships for inhibition of epidermal growth factor receptor tyrosine kinase activity by 2,3-dihydro-2-thioxo-1H-indole-3-alkanoic acids and 2,2'-dithiobis(1H-indole-3-alkanoic acids). J Med Chem. 1993;20;36:2459-69.
11. Natoli C, Perrucci B, Perrotti F, Falchi L, Iacobelli S; Consorzio Interuniversitario Nazionale per Bio-Oncologia (CINBO). Tyrosine kinase inhibitors. Curr Cancer Drug Targets. 2010;10:462-83.
12. Tsutsumi T, Nagaoka T, Yoshida T, Wang L, Kuriyama S, Suzuki Y, et al. Nintedanib ameliorates experimental pulmonary arterial hypertension via inhibition of endothelial mesenchymal transition and smooth muscle cell proliferation. PLoS One. 2019;24;14:e0214697.
13. Varone F, Sgalla G, Iovene B, Bruni T, Richeldi L. Nintedanib for the treatment of idiopathic pulmonary fibrosis. Expert Opin Pharmacother. 2018;19:167-75.
14. Richeldi L, du Bois RM, Raghu G, Azuma A, Brown KK, Costabel U, et al. Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. N Engl J Med. 2014;29;370:2071-82.
15. da Silva RF, Nogueira-Pangrazi E, Kido LA, Montico F, Arana S, Kumar D, et al. Nintedanib antiangiogenic inhibitor effectiveness in delaying adenocarcinoma progression in Transgenic Adenocarcinoma of the Mouse Prostate (TRAMP). J Biomed Sci. 2017;12;24:31.
16. Yamashita M, Niisato M, Kawasaki Y, Karaman S, Robciuc MR, Shibata Y, et al. VEGF-C/VEGFR-3 signalling in macrophages ameliorates acute lung injury. Eur Respir J. 2022;14;59:2100880.
17. Li D, Ren W, Jiang Z, Zhu L. Regulation of the NLRP3 inflammasome and macrophage pyroptosis by the p38 MAPK signaling pathway in a mouse model of acute lung injury. Mol Med Rep. 2018;18:4399-409.
18. Kim HY, Kim MS, Kim SH, Joen D, Lee K. Protective Effects of Nintedanib against Polyhexamethylene Guanidine Phosphate-Induced Lung Fibrosis in Mice. Molecules. 2018;7;23:1974.
19. Pittet JF, Griffiths MJ, Geiser T, Kaminski N, Dalton SL, Huang X, et al. TGF-beta is a critical mediator of acute lung injury. J Clin Invest. 2001;107:1537-44.
20. Lorenz G, Darisipudi MN, Anders HJ. Canonical and non-canonical effects of the NLRP3 inflammasome in kidney inflammation and fibrosis. Nephrol Dial Transplant. 2014;29:41-8.
21. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015;21:677-87.

22. Ma HJ, Huang XL, Liu Y, Fan YM. Sulfur dioxide attenuates LPS-induced acute lung injury via enhancing polymorphonuclear neutrophil apoptosis. Acta Pharmacol Sin. 2012;33:983-90.

23. Chimenti L, Camprubí-Rimblas M, Guillamat-Prats R, Gomez MN, Tijero J, Blanch L, et al. Nebulized Heparin Attenuates Pulmonary Coagulopathy and Inflammation through Alveolar Macrophages in a Rat Model of Acute Lung Injury. Thromb Haemost. 2017;117:2125-34.

24. Aydin P, Magden ZBA, Uzuncakmak SK, Halici H, Akgun N, Mendil AS, et al. Avanafil as a Novel Therapeutic Agent Against LPS-Induced Acute Lung Injury via Increasing CGMP to Downregulate the TLR4-NF-κB-NLRP3 Inflammasome Signaling Pathway. Lung. 2022;200:561-72.

25. Liu Y, Zhou J, Luo Y, Li J, Shang L, Zhou F, et al. Honokiol alleviates LPS-induced acute lung injury by inhibiting NLRP3 inflammasome-mediated pyroptosis via Nrf2 activation in vitro and in vivo. Chin Med. 2021;29;16:127.

26. Matute-Bello G, Frevert CW, Martin TR. Animal models of acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2008;295:L379-99.

27. Yılmaz Sipahi E, Atalay F. Experimental Models of Acute Lung Injury. Eurasian J Pulmonol. 2014;16:69-77.

28. Chen H, Bai C, Wang X. The value of the lipopolysaccharide-induced acute lung injury model in respiratory medicine. Expert Rev Respir Med. 2010;4:773-83.

29. Wang X, Adler KB, Erjefalt J, Bai C. Airway epithelial dysfunction in the development of acute lung injury and acute respiratory distress syndrome. Expert Rev Respir Med. 2007;1:149-55.

30. Cheng DS, Han W, Chen SM, Sherrill TP, Chont M, Park GY, et al. Airway epithelium controls lung inflammation and injury through the NF-kappa B pathway. J Immunol. 2007;15;178:6504-13.

31. Kim HJ, Lee HS, Chong YH, Kang JL. p38 Mitogen-activated protein kinase up-regulates LPS-induced NF-kappaB activation in the development of lung injury and RAW 264.7 macrophages. Toxicology. 2006;225:36-47.

32. Tang J, Xu L, Zeng Y, Gong F. Effect of gut microbiota on LPS-induced acute lung injury by regulating the TLR4/NF-kB signaling pathway. Int Immunopharmacol. 2021;91:107272.

33. Shaukat A, Guo YF, Jiang K, Zhao G, Wu H, Zhang T, et al. Ginsenoside Rb1 ameliorates Staphylococcus aureus-induced Acute Lung Injury through attenuating NF-κB and MAPK activation. Microb Pathog. 2019;132:302-12.

34. Afonina IS, Zhong Z, Karin M, Beyaert R. Limiting inflammation-the negative regulation of NF-κB and the NLRP3 inflammasome. Nat Immunol. 2017;9;18:861-9.

35. Li J, Ma J, Li M, Tao J, Chen J, Yao C, et al. GYY4137 alleviates sepsis-induced acute lung injury in mice by inhibiting the PDGFRβ/Akt/NF-κB/NLRP3 pathway. Life Sci. 2021;15;271:119192.

36. Fan Z, Yao J, Li Y, Hu X, Shao H, Tian X. Anti-inflammatory and antioxidant effects of curcumin on acute lung injury in a rodent model of intestinal ischemia reperfusion by inhibiting the pathway of NF-Kb. Int J Clin Exp Pathol. 2015;1;8:3451-9.

37. Jo EK, Kim JK, Shin DM, Sasakawa C. Molecular mechanisms regulating NLRP3 inflammasome activation. Cell Mol Immunol. 2016;13:148-59.

38. Kim EH, Park MJ, Park S, Lee ES. Increased expression of the NLRP3 inflammasome components in patients with Behçet's disease. J Inflamm (Lond). 2015;2;12:41.

39. Miller LS, Pietras EM, Uricchio LH, Hirano K, Rao S, Lin H, et al. Inflammasome-mediated production of IL-1beta is required for neutrophil recruitment against Staphylococcus aureus in vivo. J Immunol. 2007;179:6933-42.

40. Cohen TS, Prince AS. Activation of inflammasome signaling mediates pathology of acute P. aeruginosa pneumonia. J Clin Invest. 2013;123:1630-7.

41. Mishra BB, Moura-Alves P, Sonawane A, Hacohen N, Griffiths G, Moita LF, et al. Mycobacterium tuberculosis protein ESAT-6 is a potent activator of the NLRP3/ASC inflammasome. Cell Microbiol. 2010;12:1046-63.

42. Allen IC, Scull MA, Moore CB, Holl EK, McElvania-TeKippe E, Taxman DJ, et al. The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. Immunity. 2009;30:556-65.

43. Du ZA, Sun MN, Hu ZS. Saikosaponin a Ameliorates LPS-Induced Acute Lung Injury in Mice. Inflammation. 2018;41:193-8.

44. Wang L, Lei W, Zhang S, Yao L. MCC950, a NLRP3 inhibitor, ameliorates lipopolysaccharide-induced lung inflammation in mice. Bioorg Med Chem. 2021;30:115954.

45. Afonina IS, Zhong Z, Karin M, Beyaert R. Limiting inflammation-the negative regulation of NF-κB and the NLRP3 inflammasome. Nat Immunol. 2017;18:861-9.

46. Jordan JA, Guo RF, Yun EC, Sarma V, Warner RL, Crouch LD, et al. Role of IL-18 in acute lung inflammation. J Immunol. 2001;167:7060-8.

47. Kuipers MT, Aslami H, Janczy JR, van der Sluijs KF, Vlaar AP, Wolthuis EK, et al. Ventilator-induced lung injury is mediated by the NLRP3 inflammasome. Anesthesiology. 2012;116:1104-15.

48. Wu J, Yan Z, Schwartz DE, Yu J, Malik AB, Hu G. Activation of NLRP3 inflammasome in alveolar macrophages contributes to mechanical stretch-induced lung inflammation and injury. J Immunol. 2013;190:3590-9.

49. Mulligan MS, Ward PA. Immune complex-induced lung and dermal vascular injury. Differing requirements for tumor necrosis factor-alpha and IL-1. J Immunol. 1992;149:331-9.

50. Wollin L, Distler JHW, Redente EF, Riches DWH, Stowasser S, Schlenker-Herceg R, et al. Potential of nintedanib in treatment of progressive fibrosing interstitial lung diseases. Eur Respir J. 2019;54:1900161.

51. Oyaizu T, Fung SY, Shiozaki A, Guan Z, Zhang Q, dos Santos CC, et al. Src tyrosine kinase inhibition prevents pulmonary ischemia-reperfusion-induced acute lung injury. Intensive Care Med. 2012;38:894-905.

52. El-Agamy DS. Nilotinib ameliorates lipopolysaccharide-induced acute lung injury in rats. Toxicol Appl Pharmacol. 2011;253:153-60.

53. Mura M, dos Santos CC, Stewart D, Liu M. Vascular endothelial growth factor and related molecules in acute lung injury. J Appl Physiol (1985). 2004;97:1605-17.

54. Jo YY, Kim DW, Choi JY, Kim SG. 4-Hexylresorcinol and silk sericin increase the expression of vascular endothelial growth factor via different pathways. Sci Rep. 2019;9:3448.

55. Wollin L, Maillet I, Quesniaux V, Holweg A, Ryffel B. Antifibrotic and anti-inflammatory activity of the tyrosine kinase inhibitor nintedanib in experimental models of lung fibrosis. J Pharmacol Exp Ther. 2014;349:209-20.