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Effect of Colchicine on Secondary Acute Lung Injury in an Experimental Sepsis Model in Rats

Deneysel Bir Sıçan Sepsis Modelinde Kolşisinin Sekonder Akut Akciğer Hasarı Üzerindeki Etkisi

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Abstract

Introduction: An irregular systemic inflammatory response in sepsis can induce acute lung injury (ALI), which is characterized by the exaggerated inflammation of the lung tissue. Colchicine inhibits several inflammatory pathways which may prevent sepsis-induced secondary lung injury. We aimed to examine the histopathological effects of colchicine on lung tissue in a lipopolysaccharide (LPS)-induced sepsis model in rats.
Materials and Methods: Twenty-eight rats were randomly divided into the following four groups: "sham" (n=6), "colchicine only" (n=6), "sepsis only" (n=8) and "sepsis+colchicine" (n=8). In the "sham" group, 0.9% NaCl was administered intraperitoneally (IP) and intragastrically (IG). In the "Colchicine only" group, 0.9% NaCl was administered IP and colchicine was administered IG. Sepsis was induced in the "sepsis only" and "sepsis+colchicine" groups by administering of 1 mg/kg of LPS IP at the 0-time point. In the "sepsis+colchicine" group, colchicine was also administered IG at the 90th minute. An observational sepsis scoring system was used to evaluate the signs of sepsis. Subsequently, the rats were sacrificed at the 24th hour. The lung tissues were examined according to the American Thorax Association's assessment report on ALI in animals.
Results: The histopathological lung injury score in the "sepsis+colchicine" group was significantly higher than in the "sham" group (0.23±0.18 vs. 0±0; p<0.05) and was significantly lower than in the "sepsis" only group (0.23±0.18 vs. 0.57±0.14; p<0.001). Evaluation of the lung damage score revealed that colchicine suppressed the increase in alveolar and interstitial neutrophil counts and limited the increase in hyaline membrane neutrophil counts and alveolar wall thickness in the "sepsis+colchicine" group when compared with the "sepsis only" group.
Conclusion: In our experimental sepsis rat model, administration of colchicine for sepsis limited the secondary lung damage.
Keywords: Sepsis, acute lung injury, colchicine, rat model

Öz

Giriş: Sepsiste düzensiz sistemik enflamatuvar yanıt, sekonder akut akciğer hasarına (ALI) neden olabilir. Bu akciğer dokusunun abartılı enflamasyonu ile karakterizedir. Kolşisin bazı enflamatuvar yolları inhibe ederek sepsis kaynaklı akciğer hasarını önleyebilir. Bu çalışma, lipopolisakarit (LPS) ile oluşturulan deneysel sıçan sepsis modelinde kolşisinin akciğer dokusu üzerindeki histopatolojik etkilerini incelemek için tasarlanmıştır.
Gereç ve Yöntem: Yirmi sekiz sıçan rastgele dört gruba ayrıldı; altışar sıçan 'sham' (grup 1) ve 'sadece kolşisin' (grup 2) gruplarına, sekizer sıçan 'sadece sepsis' (grup 3) ve 'sepsis+kolşisin' (grup 4) gruplarına ayrıldı. Grup 1'e (sham) hem intraperitoneal (IP) hem de intragastrik (IG) olarak %0,9 NaCl verildi. Grup 2'ye (sadece kolşisin grubu) IP NaCl ve IG kolşisin verildi. Grup 3 ve 4'te sepsis, 0 zaman noktasında 1 mg/kg LPS IP uygulanmasıyla

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indüklendi. Grup 4'e deneyin 90. dakikasında, ek olarak IG olarak kolşisin verildi. Sepsis belirtilerini gözlemlemek için gözlemsel sepsis skorlama sistemi kullanıldı ve sıçanlar 24. saatte sakrifiye edildi. Akciğer dokuları Amerikan Toraks Derneği'nin hayvanlarda ALI'ye ilişkin değerlendirme raporuna göre incelendi.

Bulgular: Histopatolojik akciğer hasarı skorlama sistemi değerlendirmesine göre; 'sepsis+kolşisin' grubunda skor 'sham' grubuna göre anlamlı olarak yüksek (0,23±0,18'e karşı 0±0, p<0,05), ancak 'sadece sepsis' grubuna göre anlamlı olarak düşük bulundu (0,23±0,18'e karşı 0,57±0,14, p<0,001). Akciğer hasar skorunun analizinde: kolşisin, 'sepsis+kolşisin' grubunda 'sadece sepsis' grubuna göre alveolar ve interstisyel nötrofil sayısındaki artışı baskılamış, hiyalin membran sayısındaki ve alveolar duvar kalınlığındaki artışı da sınırlamış olarak gözlendi.

Sonuç: Çalışmamızda oluşturduğumuz deneysel sepsis sıçan modelinde sepsis oluştuktan sonar verilen kolşisinin sepsise bağlı sekonder akciğer hasarını sınırladığı görülmüştür.

Anahtar Kelimeler: Sepsis, akut akciğer hasarı, kolşisin, sıçan deney modeli

Introduction

Sepsis is a common clinical syndrome caused by an irregular systemic inflammatory response to an infection. Sepsis and inflammation lead to multiple organ failure and often lung injury. The severity of this clinical entity ranges from a subclinical disease to an acute lung injury (ALI) and acute respiratory distress syndrome (ARDS)[1].

ALI/ARDS may occur due to direct (e.g., pneumonia and gastric aspiration) or indirect (e.g., non-pulmonary sepsis and multiple traumas) causes. Direct lung injury is caused by an insult to the alveolar epithelium, whereas indirect lung injury is caused by an insult to the systemic and capillary endothelium[2]. The main pathogenesis in ALI/ARDS is alveolar injury and activation of the alveolar macrophages, followed by migration of circulating macrophages to the injury site and inflammation. Proinflammatory cytokines and toxic mediators that are released by the activities of these cells damage the lung capillary and alveolar epithelial cells, causing widespread lung damage[3]. The severity of lung damage is directly related to the intensity of neutrophil migration to the lungs[4-6].

Colchicine is an antimitotic, antiinflammatory, and antifibrotic drug. It accumulates heavily in neutrophils and affects neutrophil functions even at low doses. It produces its effects mainly by disrupting tubulin polymerization and microtubule formation, which suppresses the mobilization and chemotaxis of inflammatory cells. It also suppresses leukocyte adhesion, aggregation, and activation by disrupting the E-selectin distribution on the endothelial surface and intracellular transport of endosomes/exosomes. Furthermore, it suppresses the production and secretion of free radicals and superoxide in neutrophils[7]. Colchicine reduces IL-1β and IL-18 secretion by suppressing inflammasome (in particular NLRP3) activity[8]. Considering the role of neutrophil migration to the lungs in the pathogenesis of ALI/ARDS, the inhibitory effects of colchicine on neutrophil migration and inflammation may effectively reduce lung damage secondary to sepsis.

The potential role of colchicine in preventing and treating secondary lung damage due to sepsis has yet to be fully investigated. Herein, we aimed to examine the histopathologic effects of colchicine on secondary lung injury in an experimental sepsis model induced by lipopolysaccharides (LPS) in rats[9,10].

Materials and Methods

Animal Model

We divided 28 Wistar male rats weighing 300-350 g into the following four groups: "sham" (n=6), "colchicine only" (n=6), "sepsis only" (n=8), and "sepsis+colchicine" (n=8). The rats in the "sham" group were administered 0.9% NaCl intraperitoneally (IP) as well as intragastrically. The "sham" group was the control group to all the intervention groups. The rats in the "colchicine only" group were administered colchicine but not LPS, and it was created to control for the possible pulmonary effects of colchicine alone, if any. The rats in the "sepsis only" group were administered LPS but not colchicine, and it was the control group for the actual treatment. The "sepsis+colchicine" group was the treatment group. The experimental groups and timings of all the interventions are summarized in Table 1.

Induction of Sepsis

We prepared an LPS solution (*Escherichia coli* serotype O55:B5, cat. no. L2880; Sigma-Aldrich) just before performing the experiment at a concentration of 0.36 mg/ml. To induce sepsis, we IP administered 1 ml of the solution (1-1.2 mg/kg) to the rats in the "sepsis only" and "sepsis+colchicine" groups at the 0-time point. We IP administered an equal volume of 0.9% NaCl to the rats in the "sham" and "colchicine only" groups.

Colchicine Administration

We prepared a colchicine solution (Ibrahim Ethem Ulugay, İstanbul, Turkey) at a concentration of 0.4 mg/ml. We administered 0.6-0.7 mg/kg of colchicine by oral gavage at the 90th minute of the experiment to the rats in "colchicine only" and "sepsis+colchicine" groups. We administered an equal volume of 0.9% NaCl to the rats in the "sham" and "sepsis only" groups.

Monitoring of the Rats

To validate the sepsis model and monitor the disease course, we used an observational scoring system that allows the

standardized evaluation of symptoms such as possible postsepsis changes in consciousness, ptosis, piloerection, respiratory distress, and decreased response to stimuli (Supplementary Table 1)[11]. We applied the scoring system at the -24th, 0, 2nd, 4th, 6th, 12th, and 24th hour of the experiment. Additionally, we recorded the amount of feed and water consumption. We weighed the rats at the 0, 12th, and 24th hour. The experimental groups and the timings of the procedures are shown in Table 2.

Blood and Tissue Collection

The colchicine concentration peaks in the serum 0.5-2 h after oral administration and in neutrophils after 12-48 hours[12]. Therefore, we administered ether anesthesia at the 24th hour of the experiment and sacrificed the rats by drawing all the blood from the vena cava caudalis. Additionally, we collected tissue specimens from the lungs.

Histopathology

The lung tissues were fixed in 10% formalin in ten times the volume for 24 h. After the tissue was processed, it was embedded in paraffin blocks and stained with hematoxylin and eosin. Masson trichrome histochemical staining was used for the evaluation of fibrosis. The lung tissues were examined under a light microscope (≥5 fields per sample) according to the American Thorax Association's Assessment Report on ALI in animals by a pathologist who was blinded to the study groups (Supplementary Table 2) (Figure 1)[13]. The lung injury scoring system was applied to hematoxylin and eosin-stained sections, and fibrosis was evaluated using the Masson trichrome-stained slides.

The tumor necrosis factor-α (TNF-α) concentration was measured using the collected blood sample.

Statistical Analysis

Statistical analyses were performed using Statistical Package for the Social Sciences (version 15.0) and R Studio (version 1.1.383). The continuous data were expressed as mean and standard deviation, and the categorical data were expressed as percentages. The Shapiro-Wilk test was used to evaluate the normal distribution of the data. The chi-square and Fisher's exact tests were to analyze categorical data. The Student's t-test and

Table 1. Experimental groups, initiatives, and the timings of the interventions

	Sham (n=6)	**Colchicine only (n=6)**	**Sepsis only (n=8)**	**Sepsis+colchicine (n=8)**
-24 hours	Weight Fever Marking			
-1 hour	Weight Fever Observational sepsis scoring			
0 The point of time	**Intraperitoneal administration of 0.9% NaCl (1 ml)**		**Intraperitoneal administration of 1-1.2 mg/kg of LPS (1 ml)**	
90th minute	**0.9% NaCl 0.5 ml**	**Colchicine 0.6-0.7 mg/kg (0.5 ml)**	**0.9% NaCl 0.5 ml**	**Colchicine 0.6-0.7 mg/kg (0.5 ml)**
2nd, 4th, and 6th hour	Observational sepsis scoring Fever			
12th and 23rd hour	Observational sepsis scoring Fever Weight			
24th hour	Rats sacrificed and tissues/blood samples collected			

Table 2. Comparison of the mean observational sepsis scores among the rats in the different groups

	Sham (n=5)	**Colchicine only (n=6)**	**Sepsis only (n=8)**	**Sepsis+colchicine (n=8)**	**p value**
2nd hour (mean±SD)	0±0	0±0	6±3.59*	7±3.78*	<0.001**
4th hour (mean±SD)	0.80±1.30	0.50±0.55	11.50±1.69*	10.75±3.01*	<0.001**
6th hour (mean±SD)	0.80±0.84	0.83±0.41	13.50±1.78*	12.75±1.91*	<0.001**
12th hour (mean±SD)	2.80±1.79	0.17±0.41	6.13±1.73*	8.13±1.81*	<0.001**
24th hour (mean±SD)	0±0	0.33±0.82	2.88±1.81*	5±3.55*	<0.001**

*Statistically significant difference in the scores in the post-hoc analyses. There was no significant difference in the scores between the sepsis groups ("sepsis only" vs. "sepsis+colchicine").

**p<0.05 was considered statistically significant.

SD: Standard deviation

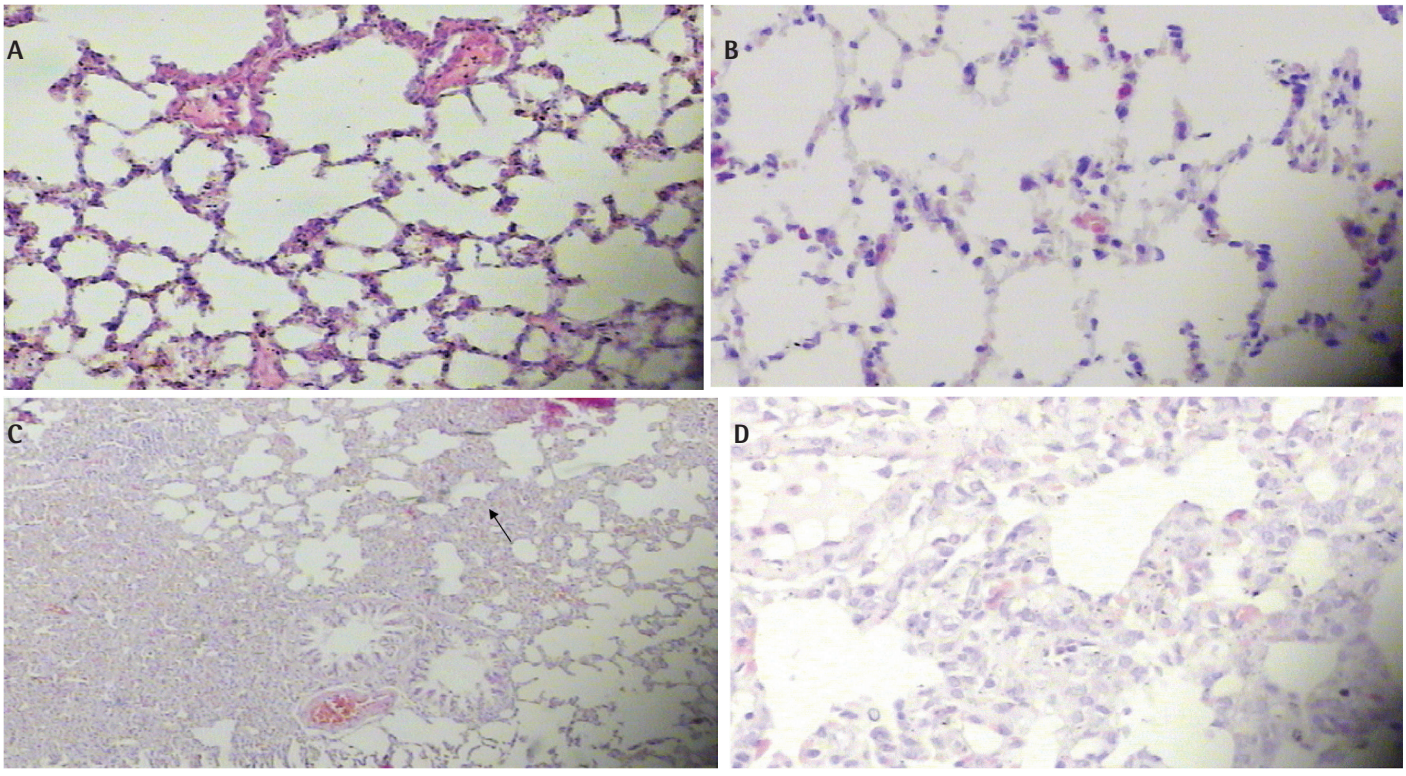


Figure 1. Hematoxylin-eosin-stained images of the lung tissue in the (A) Sham group (x100): Normal lung tissue, (B) Colchicine only group (x400): Normal lung tissue, (C) Sepsis only group (x40): Increase in the interstitial neutrophil count and thickness of the alveolar walls (black arrow), and (D) Sepsis+colchicine group (x400): Apparent decrease in the neutrophil accumulation and alveolar wall thickening when compared to the sepsis only group

Table 3. Comparison of the 24-hour feed and water consumption among the rats in the different groups

	Sham (n=5)	Colchicine only (n=6)	Sepsis only (n=8)	Sepsis+colchicine (n=8)	p value
Feed (g) (mean±SD)	31.4±1.04	30.7±2.08	25.3±0.69*	18.5±0.64 *	<0.001**
Water (ml) (mean±SD)	73±1.86	75±1.80	50.6±2.03*	43.8±4.00*	<0.001**

*Group(s) with a statistically significant difference in the post-hoc analyses.

**Statistically significant difference was found between the groups.

SD: Standard deviation

Mann-Whitney U test were used to analyze continuous data. The ANOVA and Kruskal-Wallis test were used to compare the means in the presence of more than two groups. A p value of <0.05 was considered statistically significant.

Results

The study included the following four experimental groups: "sham" (n=6), "colchicine only" (n=6), "sepsis only" (n=8), and "sepsis+colchicine" (n=8). Histopathological examination of the lung tissue from one rat in the "sham" group revealed an organized pneumonia. Thus, this rat was excluded from the study because we believed it was sick prior to the experiment.

During the 1st hour of the experiment, piloerection, ptosis, and lethargy were observed in all of the rats who were administered LPS. The symptoms of sepsis developed within the first two hours of the experiment, became most evident between the 4th and 6th hours, and regressed at the 24th hour. The observational sepsis score was higher in the sepsis groups than in the "sham" and "colchicine only" groups. This indicates that we were able to create a successful experimental sepsis model in rats by IP injecting 1 mg/kg of LPS. The difference between the groups decreased at the 24th hour. The average 24-hour feed and water consumption were also significantly lower in the sepsis groups than in the "sham" and "colchicine only" groups (Table 3).

Evaluation of the lung damage score revealed that colchicine suppressed the increase in alveolar and interstitial neutrophil counts and limited the increase in hyaline membrane neutrophil counts and alveolar wall thickness in the "sepsis+colchicine" group when compared to the "sepsis only" group. The mean total lung histopathology scores were significantly different

Table 4. American Thoracic Society's lung injury score among the rats in the different groups

	Sham (n=5)	Colchicine only (n=6)	Sepsis only (n=8)	Sepsis+colchicine (n=8)	p value
Alveolar neutrophil (mean±SD)	0±0	0±0	1.13±0.64*	0.25±0.46	<0.001**
Interstitial neutrophil count (mean±SD)	0±0	0±0	1.13±0.64*	0.25±0.46*	<0.001**
Hyaline membrane (mean±SD)	0±0	0±0	0.88±0.84	0.25±0.71	0.038**
Intraalveolar fibrin (mean±SD)	0±0	0±0	0±0	0±0	-
Thickening of the alveolar wall (mean±SD)	0±0	0.83±0.41	1.25±0.46*	0.63±0.75	0.003**
Total lung histopathology score (mean±SD)	0±0*	0.09±0.08*	0.57±0.14*	0.23±0.18*	<0.001**

*Group(s) with a statistically significant difference in the post-hoc analyses

**Statistically significant difference was found between the groups.

SD: Standard deviation

Table 5. TNF-α values at the 24th hour among the rats in the different groups

	Sham	Colchicine only	Sepsis only	Sepsis+colchicine	p value
TNF-α (mean±SD)	22.53±0.85	22.57±2.11	22.03±2.15	22.93±2.61	0,874*

*Statistically insignificant.

TNF-α: Tumor necrosis factor-a, SD: Standard deviation

between the groups (p<0.001) (Table 4). The post-hoc analyses revealed the following:

1. The lung histopathology score in the "colchicine only" group was not significantly higher than that in the "sham" group (p=1)

2. The lung histopathology score was significantly higher in the "sepsis only" group than in the other groups (p<0.001)

3. The lung histopathology score in the "sepsis+colchicine" group was significantly higher than in the "sham" group (0.23±0.18 vs. 0±0; p<0.05) and significantly lower than in the "sepsis only" group (0.23±0.18 vs. 0.57±0.14; p<0.001) (Supplementary Table 3, Table 4).

A comparison of the histopathological evaluations of the lung tissues are depicted in Figure 1A-1D.

The serum TNF-α levels of the rats in the "sham," "colchicine only," "sepsis only" and "sepsis+colchicine" groups are shown in Table 5. There was no statistically significant difference in the TNF-α levels between the groups (p=0.874).

Discussion

In this study, we examined the effects of colchicine on indirect ALI due to LPS-induced sepsis. We were able to create a successful experimental sepsis model in rats by IP injecting 1 mg/kg of LPS. Colchicine did not cause significant lung injury and its administration limited the secondary lung injury due to LPS-induced sepsis.

There are several experimental models of ALI. However, no model can completely mimic all the features of indirect sepsis associated with ALI/ARDS in humans. The IP LPS injection-induced sepsis and ALI/ARDS model is one of the most widely used models. Administration of the LPS injection causes the accumulation of polymorphonuclear leukocytes in the alveolar capillaries. In this model, the pulmonary capillary endothelium is damaged first, and pulmonary interstitial edema is the most prominent pathological change. In the second hour of the experiment, signs of systemic inflammation and lung damage began to appear, which peaked within 24 hours depending on the dose. LPS-induced lung injury is similar to the neutrophilic inflammatory response observed in the lungs of patients with ARDS[14-16]. We preferred the sepsis model induced by the IP administration of LPS, which is characterized by alveolar neutrophilia, because we based our hypothesis on colchicine's ability to inhibit neutrophil migration.

The susceptibility of experimental animals to the bacterial endotoxin LPS varies. Additionally, susceptibility may differ in different populations of the same species[17]. Furthermore, the potencies of different LPS preparations are different. Different doses have been used in IP LPS injection-induced models in rats. In a previous study conducted in the same institution with the same rat population and LPS preparation, mortality was observed at LPS doses of 1.5 mg/kg and 3 mg/kg[18]. In this study, we used 1 mg/kg so that the number of included rats would not fall below the statistically significant level till the end of the experiment. We calculated the colchicine dose by using an animal equivalent dose calculation chart, which was adapted and modified from the FDA draft guidelines. The calculated colchicine dose for a rat weighing 300 mg was 0.9-1 mg/kg. Although 0.8 mg/kg colchicine is associated with severe toxic

effects, low-dose colchicine (0.02 mg/kg) produces sufficient antiinflammatory effects in different organs. Therefore, we used a moderate dose of 0.6 mg/kg[19,20].

Higher observational sepsis scores, lower feed and water consumption, and higher lung injury scores in the sepsis groups than in the other groups indicated that we successfully induced a sepsis model. The differences in the observational sepsis score between the groups had disappeared by the 24th hour. This indicates that the dose used was probably low; thus, the rats recovered within 24 hours. Our study findings were consistent with those of previous similar experiments[15]. Signs of sepsis and lung injury appear two hours after the parenteral administration of LPS. Additionally, the plasma concentration of colchicine peaks approximately one hour after its oral administration. Therefore, we believed that the administration of colchicine 90 minutes after the administration of LPS would be appropriate to evaluate the effectiveness of the treatment of the induced sepsis.

Although our study does not have data on how colchicine limits lung damage at the cellular level, we believe that colchicine inhibits the neutrophilic invasion of the lung. In a model of oleic acid-induced lung injury, colchicine did not reduce the number of circulating neutrophils; however, it significantly reduced neutrophil migration to the lungs[21]. In another study where colchicine was administered to LPS-treated rat cells, the IL-1β, IL-6, and TNF-α levels decreased or completely disappeared depending on the dose. In the same study, colchicine attenuated the infiltration of leucocytes and neutrophils, secretion of inflammatory cytokines, and down-regulation of superoxide dismutase activity via the inhibition of JNK, Erk ½, and p-38 signaling pathways[22].

Similar to our study, the study by Ozdemir et al.[23] found that colchicine administration to newborn Wistar rats with hypoxia-induced lung damage reduced alveolarization, lung inflammation, and oxidative stress and decreased the TNF-α and IL-1β levels in bronchoalveolar lavage fluid. In another study by Ghio et al.[24] on male Sprague Dawley rats, colchicine significantly reduced neutrophil migration, lung injury, and mortality, even when administered 30 minutes after phosgene exposure.

The life-threatening characteristics of severe coronavirus disease-19 are a cytokine storm and hyper-inflammatory state which often lead to multiorgan dysfunction and lung injury[9]. Considering the common inflammatory pathways, colchicine may demonstrate the same favorable effects on the course of COVID-19[25]. Although small randomized studies and retrospective studies have demonstrated the significant benefits of colchicine, the same benefit has not been observed in some large-scale randomized controlled studies[10,26-28]. Colchicine has been evaluated as a treatment option for other viral diseases, and a study demonstrated that it suppresses respiratory syncytial virus replication *in vitro* in cells and *in vivo* in neonatal rats by regulating the production of antioxidant factors[29].

In most previous studies, colchicine is administered first, followed by the agent that causes sepsis/systemic inflammation[21,23,24]. This practice is incompatible with the clinical situation. In our study, we first administered LPS, and subsequently administered colchicine after the signs of sepsis were observed. Considering that colchicine cannot be administered before the development of sepsis and lung injury in humans, we believe that our experimental model is more compatible with a possible clinical scenario. We aimed to demonstrate that the administration of colchicine after the development of sepsis could also be effective. Colchicine can potentially be used in humans to limit systemic inflammation and lung injury during the course of the disease. However, despite a clear relationship between ARDS and neutrophil recruitment, it has been difficult to establish a direct causal relationship between neutrophils and endothelial epithelial barrier deterioration or clinical outcomes in ARDS. For example, ARDS may develop in patients with neutropenia[30]. This may explain why colchicine does not completely cause regression of the lung injury.

In our study, the mortality and histopathological lung injury score in the "colchicine only" group was not significantly higher than those in the "sham" group, indicating that colchicine is safe when administered at optimum doses. In a study by Wiesenfeld et al.[31], administration of 10-30 mg/kg of oral colchicine increased the LPS-induced mortality rate. Similarly, in a study by Igarashi et al.[32] where 1, 3, or 10 mg/kg of colchicine was administered subcutaneously with or without 1 mg/kg of LPS, all the rats that received 33 mg/kg of colchicine died within 72 h. Additionally, all the rats who were administered 1 mg/kg of colchicine only survived for 72 h. However, all the rats who were administered a combination of LPS (1 mg/kg) and colchicine (1 mg/kg) died within 24 h, suggesting a synergistic effect on mortality. We believe that using high-dose colchicine or an administration route other than the oral route might have increased the mortality rate. Further studies are needed to determine the optimum colchicine dose for LPS-induced sepsis.

In our study, there was no significant difference in the TNF-α values in the blood samples obtained after sacrificing the rats at the 24th hour between the groups. In a study by Jiang et al.[33], the TNF-α levels in male Wistar rats peaked in the 2nd hour in the group that was administered 10 mg/kg of LPS (O55:B5), which decreased to normal after the 6th hour. In a study conducted in male Sprague Dawley rats, the TNF-α level peaked at the 90th minute and decreased after the 4th hour after the administration of 5 mg/kg of LPS (O55:B5)[34]. However, due to technical difficulties we were unable to draw blood at all time

points (2, 4, 6, 12 hours). Additionally, we could not observe the peak TNF-α levels which was expected to be observed in the sepsis groups. Similarly, the absence of a significant difference in the serum TNF-α values at the 24th hour indicates the onset of sepsis recovery in the sepsis groups.

Study Limitations

Although our results demonstrated that colchicine limits the histopathological lung injury, we could not determine the underlying mechanisms. Additionally, the study results cannot be generalized to patients with ARDS. Furthermore, they support the conduction of larger randomized controlled trials.

Conclusion

In our study, administration of colchicine to rats with lung injury secondary due to LPS-induced sepsis limited the extent of the injury based on the histopathological examination results. Further animal studies are required to clarify the dosage, appropriate administration time, and benefits/harms of colchicine for LPS-induced sepsis.

Ethics

Ethics Committee Approval: We obtained approval from Local Ethics Committee for Animal Experiments of the Dokuz Eylül University before the experiment (date: 27.03.2019, no: 16/2019).

Informed Consent: Consent form was filled out by all participants.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: O.Y., M.C., H.O.Y., S.A., Concept: M.C., O.Y., Design: M.C., O.Y., Data Collection or Processing: M.C., H.O.Y., Analysis or Interpretation: M.C., H.O.Y., S.A., Ş.K., O.Y., Literature Search: M.C., H.O.Y., Writing: M.C., H.O.Y.

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Click for Supplementary Tables 1, 2, 3.

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Supplementary Table 1. Murine sepsis score (Shrum et al. 2014)[11]

Parameters	Scoring	
Appearance	0	The fur is soft
	1	A pinch of hair is piloerected
	2	Most of the back is piloerected
	3	Piloerection is present or absent, the rat is fluffy-looking
	4	Piloerection is present or absent, the rat looks weakened
The level of consciousness	0	Active
	1	It is active, but avoids standing upright
	2	The rat's activity have noticeably slowed down, but it is still moving
	3	The activity is affected, the rat moves only when provoked, tremor is observed in its movements
	4	The activity is visibly affected, the rat remains stationary even when provoked, tremor can be observed
Activity	0	A normal amount of activity is observed. The rat eats, drinks water, climbs, runs, fights, etc.
	1	Slightly reduced activity, the rat wanders on the cage floor
	2	Activity is suppressed, the rat is motionless except for intermittent investigative movements
	3	There is no activity, the rat stands still
	4	There is no activity, tremor is observed in the rat, especially in the hind limbs
Response to a stimulation	0	The rat responds immediately to an audible warning or touch
	1	Reacts slowly to voice or does not react at all, reacts quickly to touch (tries to escape)
	2	No response to voice, moderately responsive to touch (takes a few steps)
	3	No response to voice, reacts slightly to touch (no movement)
	4	No response to voice. There is little or no reaction to touch. Can't fix himself when pushed
Eyes	0	Open
	1	The eyes are not fully open, secretion accumulation is possible
	2	Eyes are half shut, secretion accumulation is possible
	3	Eyes are more than half shut, secretion accumulation is possible
	4	Eyes closed and/or with abundant secretion
Respiratory rate	0	Normal rapid rat breathing
	1	Slightly slowed breathing (breathing rate cannot be counted by eye)
	2	Moderately reduced breathing (in the upper range of visible breathing)
	3	Severely decreased breathing (respiratory rate can be easily counted, 0.5 sec between breaths)
	4	Very severely decreased breathing (more than 1 second between breaths)
The quality of breathing	0	Normal
	1	Short periods of hard breathing
	2	Breathing is difficult, no gasping
	3	Breathing is difficult, intermittent gasping is present
	4	Gasping

Supplementary Table 2. American Thoracic Society's lung injury scoring system in animals

	Score		
*Parameters	0	1	2
The number of neutrophils in the alveolar space	None	1-5	>5
The number of neutrophils in the interstitial space	None	1-5	>5
Number of hyaline membranes	None	1	>1
The amount of proteinous debris	None	1	>1
Thickening of the alveolar septum	<2 times	2-4 times	>4 times

*≥5 fields were examined

Supplementary Table 3. Post-hoc comparison of the total lung histopathology scores between the groups

Dependent variable	(I) Group	(J) Group	Mean difference (I-J)	Std. error	Sig.	95% Confidence interval	
						Lower limit	Upper limit
Lung histopathology score	1	2	-0.08667	0.07813	1.000	-0.3122	0.1388
		3	-0.57375*	0.07355	0.000	-0.7860	-0.3615
		4	-0.23750*	0.07355	0.022	-0.4498	-0.0252
	2	1	0.08667	0.07813	1.000	-0.1388	0.3122
		3	-0.48708*	0.06968	0.000	-0.6882	-0.2860
		4	-0.15083	0.06968	0.246	-0.3519	0.0503
	3	1	0.57375*	0.07355	0.000	0.3615	0.7860
		2	0.48708*	0.06968	0.000	0.2860	0.6882
		4	0.33625*	0.06451	0.000	0.1501	0.5224
	4	1	0.23750*	0.07355	0.022	0.0252	0.4498
		2	0.15083	0.06968	0.246	-0.0503	0.3519
		3	-0.33625*	0.06451	0.000	-0.5224	-0.1501

*The mean difference is significant at the 0.05 level.

Standart error; Sig.