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RESEARCH ARTICLE

Dexpanthenol Inhibits Inflammation and Apoptosis in LPS-Induced Acute Lung Injury by Reducing Increased VCAM-1 and Caspase-3 Expressions in Rats

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ABSTRACT

This study aims to investigate the effects of Dexpanthenol (Dex), a stable alcoholic analogue of D-pantothenic acid which has anti-oxidant, antiapoptotic, and antiinflammatory properties, on lipopolysaccharide (LPS)-induced lung damage via caspase-3 (cas-3) and vascular cell adhesion molecule-1 (VCAM-1) levels. According to the experimental plan of study, thirty-two Wistar Albino rats were distributed randomly into four groups as control, LPS (5 mg/kg, intraperitoneally (i.p), single dose), LPS (30 minutes before last Dex treatment) + Dex (500 mg/kg, i.p, for 3 days) and Dex. After six hours of LPS application, lung tissues of the rats were taken for histopathological, immunohistochemical and biochemical examinations. According to results of the study, LPS caused hyperemia, neutrophil leukocyte chemotaxis and thickened septal tissue on lung. Inducing inflammation by increasing VCAM-1 expression and triggered apoptosis by increasing cas-3 expression in lung tissue. In addition, LPS decreased total antioxidant status levels, which is a marker of anti-oxidant capacity, and increased oxidative stress index and total oxidant status values, which are indicators of oxidative stress. Dex has shown its effect by reversing all these alterations and normalizing the values. These results suggest that Dex can be used as a preservative to reduce LPS-induced acute toxicity in the lung.

Keywords: Cas-3, Dexpanthenol, Inflammation, Lung, VCAM-1

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Dekspantenol, Sıçanlarda LPS'nin Neden Olduğu Akut Akciğer Hasarında Artan VCAM-1 ve Kaspaz-3 Ekspresyonlarını Azaltarak İnflamasyonu ve Apoptozu İnhibe Eder

ÖZ

Bu çalışma, D-pantotenik asidin antioksidan, antiapoptotik ve antiinflamatuar özelliklere sahip stabil bir alkolik analoğu olan Dekspantenol'ün (Dex), lipopolisakkarit (LPS) kaynaklı akciğer hasarı üzerindeki etkilerini vasküler hücre adezyon molekülü-1 (VCAM-1) ve kaspaz-3 (cas-3) seviyeleri üzerinden incelemeyi amaçlamaktadır. Çalışmanın deneysel planına göre otuz iki adet Wistar Albino sıçan rastgele dört farklı gruba ayrıldı: Kontrol, LPS (5 mg/kg, intraperitoneal (ip), tek doz), LPS (son Dex uygulamasından 30 dakika önce) + Dex (500 mg/kg, ip, 3 gün boyunca) ve Dex. LPS uygulamasından 6 saat sonra sıçanların akciğer dokuları histopatolojik, immünohistokimyasal ve biyokimyasal incelemeler için alındı. Çalışmanın sonuçlarına göre LPS, akciğerde hiperemi, nötrofil lökosit kemotaksisi ve kalınlaşmış septal dokuya neden oldu. Akciğer dokusunda VCAM-1 düzeylerini artırarak inflamasyonu indükledi ve cas-3 düzeylerini artırarak apoptozu tetikledi. Ayrıca LPS, antioksidan kapasitenin bir belirteci olan total antioksidan status düzeylerini düşürürken, oksidatif stresin göstergeleri olan total oksidant status ve oksidatif stres indeks değerlerini artırdı. Dex, tüm bu değişiklikleri tersine çevirerek ve değerleri normalleştirerek etkisini gösterdi. Bu sonuçlar, Dex'in LPS kaynaklı akut akciğer hasarında toksisiteyi azaltmak için bir koruyucu olarak kullanılabileceğini düşündürdü.

Anahtar Kelimeler: Akciğer, Cas-3, Dekspantenol, İnflamasyon, VCAM-1

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INTRODUCTION

Acute lung injury (ALI) is one of the main causes of mortality, especially in intensive care patients (Bellani et al. 2016). It is seen clinically due to blood transfusions and it can be experimentally induced by acid aspiration, pulmonary ischemia-reperfusion, and sepsis (Matute-Bello et al. 2011).

There is a complex inflammatory process in the pathogenesis of ALI, and recent studies have focused on basement membrane destruction in the development of ALI. Endothelial cells, intrapulmonary leukocytes and their inflammatory products cause ALI by interacting with lung parenchyma cells. (Ashbaugh et al. 2005, Matute-Bello et al. 2008, Butt et al. 2016).

Lipopolysaccharide (LPS), a glycopeptide taking part in the external membrane of gram-negative bacteria, induces inflammation in rat models by acting Toll-like receptor-4 on monocytes, macrophages and other cells (Raetz and Whitfield 2002). This receptor signaling triggers many intracellular post receptor mechanisms and causes apoptosis with an increase of caspase-3 (cas-3) expression and inflammation with an enhancement of vascular cell adhesion molecule-1 (VCAM-1) expression (Wang et al. 2019). It also causes oxidative stress by triggering the formation of reactive oxygen species (Park et al. 2015).

Dexpanthenol (Dex) is a stable alcoholic analogue of D-pantothenic acid (Vitamin B5), which is used especially in wounds, irritations and wrinkles, is easily accessible due to its cheapness, and is frequently found in local pharmacies (Heise et al. 2012, Stettler et al. 2017). In previous studies; it has been proven to be effective against oxidative stress on heart tissue (Kose et al. 2020), against nephrotoxicity (Pınar et al. 2020), hair strengthening (Shin et al. 2021), and prevention of hepatotoxicity (Ucar et al. 2018).

In this study, it was aimed to prove the preservative effect of Dex in the case of ALI in rats.

MATERIAL AND METHODS

Ethical Approval

All experiments were performed in accordance with the ARRIVE (Animal Research: Reporting in Vivo Experiments) guidelines in 2.0, and study approved by the Local Ethical Committee on Animal Research of Suleyman Demirel University (No:2022-02/29).

Study animals and design of experiment

A total of 32 adult female, Wistar Albino rats weighing 310–360 g were placed in a temperature controlled room (about 22°C) with specific humidity (60%[±5]) conditions and 12 hour dark/12 hour light cycle was maintained. Each rat was fed a standard commercial diet (Korkuteli Yem, Antalya, Türkiye).

Experimental design of this study was shown in Figure 1.

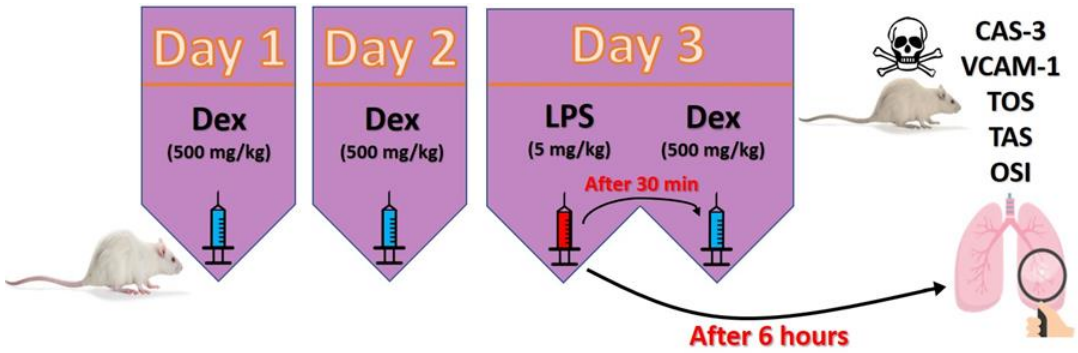

Figure 1: Experimental design of this study

The rats were divided into the following four groups eight rats each. Groups as;

1-Control Group (n = 8); 1 ml of isotonic saline solution given to the rats, intraperitoneally (i.p.) once a day for three days from the left inguinal area and one dose of 1 ml isotonic saline solution i.p. from the right inguinal region of the rats on the third day, 30 minutes before the last saline application.

2- LPS Group (n = 8); 1 ml of saline given to the rats by i.p. injections, once a day for three days from the left inguinal region and a single dose of 5 mg/kg, 0.5-1 ml LPS (048K4126, Sigma Aldrich, USA) i.p. was applied from the right inguinal area of the rats on the third day, 30 minutes before the last saline application.

3- LPS + Dex Group (n = 8); 1 ml, 500 mg/kg Dex (Bepanthene, Bayer Türk Kimya, Türkiye) given to the rats by i.p. once a day for three days from the left inguinal region and a single dose of 5 mg/kg, 0.5-1 ml LPS i.p. was applied from the right inguinal area of the rats on third day 30 minutes before the last Dex application.

4- Dex Group (n = 8); 1 ml, 500 mg/kg Dex given to the rats by i.p. once a day for three days from the left inguinal region and one dose of 1 ml saline solution i.p. from the right inguinal area of the rats on third day 30 minutes before the last Dex application.
Six hours after LPS application, 85-105 mg/kg Ketamine (Keta-Control, Doğa İlaç, Türkiye) and 9-11 mg/kg Xylazine (Rompun, Bayer, Germany) were administered to all rats. Blood was taken from the vena cava inferior for euthanasia from the rats who underwent abdominal incision following anesthesia. One-half of the lung specimens were placed in liquid nitrogen and stored at -25 °C until biochemical analysis. The remaining lung specimens were fixed with 10% neutral buffered formalin for immunohistochemical and histopathological analysis.

Histopathological examination
Lung tissues of rats were taken and fixed in 10% neutral buffered formalin. After fixation, tissues were regularly processed by a fully automated tissue processing equipment (Leica ASP300S; Leica Microsystem, Nussloch, Germany) and embedded with paraffin. Then, 5μm sections were taken from paraffin blocks with a Leica RM 2155 RT microtome (Leica Microsystem, Nussloch, Germany). After 1 day of drying, the slides were passed through xylol and alcohol series. Then stained with Hematoxylin–Eosin (HE) and analyzed through a light microscope.
Lung damage was evaluated in 10 different randomly selected fields under 20x objective for each rat. For injury scores, hyperemia, edema, increase in septal tissue thickness and necrosis were evaluated between 0-4. Accordingly, 0, no damage; 1, damage 1-25% of the field; 2, damage on 26-50% of the field; 3, damage 51-75% of the field; and 4 were assessed as damage above 76%. The score of each animal was found by dividing the total score by the number of fields examined and rounding up this number. Statistical analysis was performed on these data and the difference between the groups was found.

Immunohistochemical examination
Further, two series of sections taken from all blocks drawn on poly-L-lysine coated slides and were stained immunohistochemically for cas-3 (Anti-cas-3 Antibody (E8): sc7272, 1/100 dilution), and VCAM-1 (VCAM-1 (M/K-2):sc-18864, 1/100 dilution) (Santa cruz,Texas, USA) expressions by streptavidin biotin method according to manufacturer's directive. Antibody Diluent (ab64211) (Abcam Cambridge, UK) was used for dilution of the primary antibodies. For antigen retrieval, sections were boiled twice in citrate buffer solution (pH 6.00) using 700 MW irradiation. Sections were incubated with primary antibodies for 12 hours and immunohistochemical analysis was performed. Streptavidin-biotin immunoenzymatic antigen detection system [EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (ab80436), (Abcam, Cambridge, UK)] was used for detection. All evaluations were performed on blind specimens by, pathologists from different centers.
Sections were examined one by one for each antibody for immunohistochemical analysis. Ten different areas in each segment were scanned for evaluation under 40X objective magnification.
All slides were analyzed for immunopositivity, and a semiquantitative analysis was carried out. Samples were analyzed by examining five different sections for each sample and each section was then scored from 0 to 3 according to the intensity of staining (0, absence of staining; 1, slight; 2, medium; and 3, marked staining). Rats included in this study were evaluated randomly in a blinded fashion by the researcher without knowing which rat was included in which group. For morphometric examinations of sections, an Olympus CX41 light microscope was used. Morphometric evaluations were made by using the Database Manual CellSens Life Science Imaging Software System (Olympus Corporation, Tokyo, Japan).

Biochemical Analysis
Oxidative stress in lung tissue was analyzed by the following steps: Lung tissues were diluted with 5x (w/v) phosphate buffered saline (10 mM pH 7,4) and homogenized using Janke&Kunkel IKA Ultra-Turrax T25 (Germany) tissue homogenizator. After the homogenization process, the samples were centrifuged at +4°C at 2000 rpm for 20 minutes (Nüve NF 1200 R, Turkey). From the supernatants, tissue total antioxidant status (TAS) and total oxidant status (TOS) levels were measured on Beckman Coulter AU 5800 biochemistry autoanalyzer (USA), and also oxidative stress index (OSI) levels of the samples were calculated (Erel 2004a).
TAS levels were measured using an automated colorimetric measurement method developed by Erel (Erel 2004b). The tissue TAS levels were expressed as μmol trolox equiv/l.
TOS levels of lung tissue were measured using an automated colorimetric method and the results were expressed as μmol H2O2 equiv/l (Erel 2005). OSI was calculated using the formula: OSI = TOS/TAS.

Statistical Analysis
The immunohistochemical findings of the groups were compared between the groups for statistical analysis. For this comparison, One-way ANOVA, Duncan, LSD tests (posthoc tests) were used with SPSS-22.00 package program. Significance level was accepted as $P < 0.05$.

RESULTS

Histopathological Findings
At the histopathological examination, marked hyperemia and increased septal tissue thickness in LPS group was observed. Emphysema was also a

common finding in lungs in LPS group. Dex treatment decreased pathological findings. Normal tissue structure was observed in control and Dex groups. Histopathological appearances are shown in Figure 2.

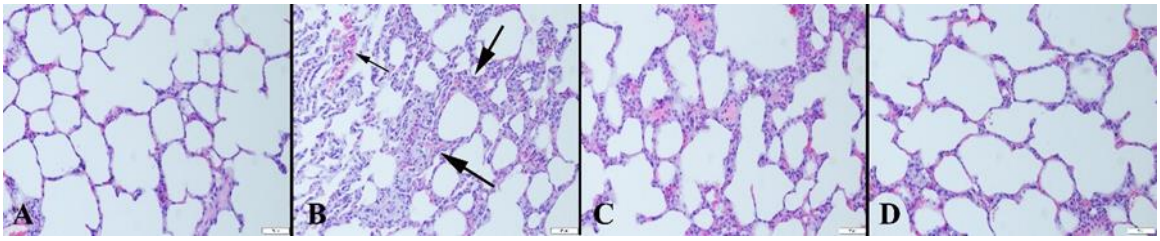

Figure 2: Histopathological view of groups. (A) Normal tissue structure in control group, (B) neutrophil leukocyte chemotaxis and hyperemia (thin arrow) and thickened septal tissue (thick arrows) in LPS group, (C) Decreased hyperemia and septal tissue thickness in LPS+Dex group, (D) Normal lung tissue histology in Dex group, HE, scale bars=0.05mm.

Immunohistochemical Findings

Immunohistochemical examination revealed significantly increased expressions for both cas-3 and VCAM-1 in LPS group (1,50±0,53, 1,12±0,64; respectively) compared to control (0,12±0,35; for both markers) ($p<0.001$; for both markers). Dex treatment (LPS+Dex group) decreased immunohistochemical expressions of both markers (0,37±0,51, 0,25±0,46; respectively) compared to LPS group (Figures 3-4) ($p<0.001$; for both markers). No or very slight expressions noticed in control group and Dex group (0,12±0,35, 0,25±0,46; respectively). Statistical analysis results shown in Figure 5.

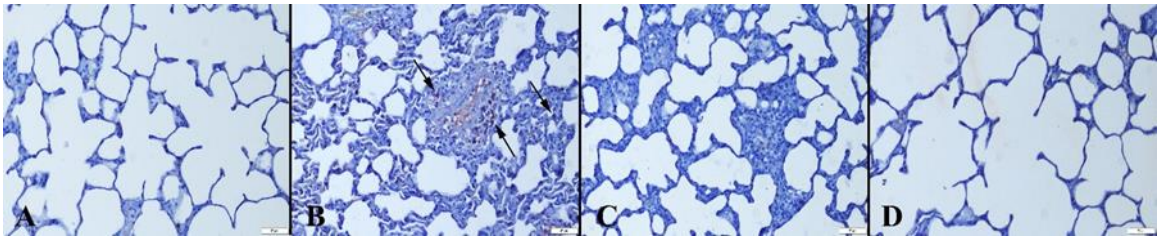

Figure 3: Cas-3 immunohistochemistry findings between the groups. (A) Negative expression in control group, (B) increased expressions (black arrows) in LPS group, (C) decreased expression in LPS+Dex group, (D) no expression in Dex group, Streptavidin-biotin peroxidase method, scale bars=0.05mm.

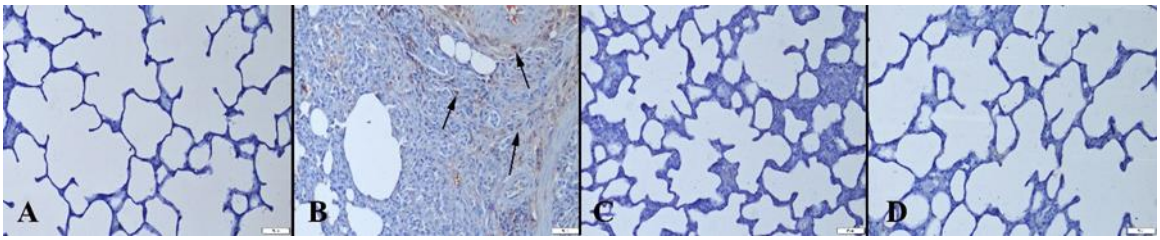

Figure 4: VCAM-1 immunohistochemistry results among the groups. (A) Negative immunoreaction in control group, (B) marked expressions (black arrows) in LPS group, (C) decreased immunoexpression in LPS+Dex group, (D) no expression in Dex group, Streptavidin-biotin peroxidase technique, scale bars=0.05mm.

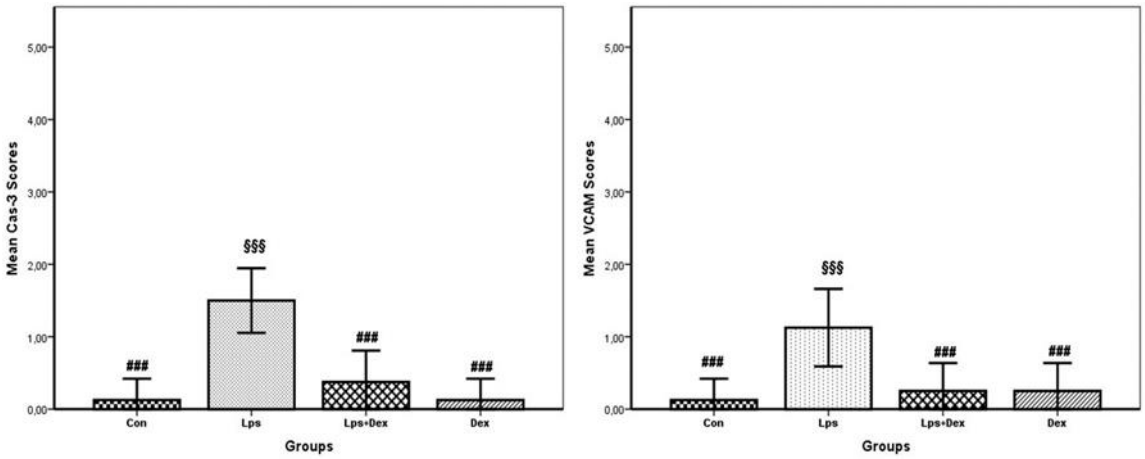

Figure 5: Statistical analysis of immunohistochemical scores. The dissimilarity between the means of groups carrying different signs between the groups are statistically significant, P<0.001. Data standard deviation (SD). Differences between groups and results of immunohistochemical scores are assessed by One way ANOVA test (post hoc Duncan test).

Biochemical Results

As shown in Table 1, the levels of TOS and OSI were significantly higher compared with control group (p=0.023 and p=0.002; respectively) and the grades of TAS were significantly lower in LPS group compared with control group (p=0,043). In the LPS+Dex group, TOS and OSI grades were lower while the TAS grades were higher than LPS group but not significant (p=0,214 for TOS; p=0,358 for OSI; p=0,508 for TAS). On the other hand, TOS and OSI grades were higher in the LPS+Dex group compared to the control group, and TAS grades were lower, but not significant (p=0,264 for TOS; p=0,105 for OSI; p=0,159 for TAS). In the Dex group, TAS grades were significantly higher compared to the LPS group (p=0,024), TOS and OSI grades were significantly lower compared to LPS group (p≤0.001 for both) and LPS+Dex group (p=0.017 and p=0.005; respectively).

Table 1. Oxidative stress markers of lung tissues in rats

	TOS (mmol H2O2Equivalents/L)		**TAS (mmol TroloxEquivalents/L)**		**OSI (TOS/[TAS*10])**	
GROUPS	**Mean ± SD**	***p* value**	**Mean ± SD**	***p* value**	**Mean ± SD**	***p* value**
CONTROL	53,11 ± 9,48		1,14 ± 0,15		4,76 ± 1,32	
LPS	62,78 ± 2,66[a]	**a:**p=0.023	0,99 ± 0,11[a]	**a:**p=0.043	6,36 ± 0,70[a]	**a:**p=0.002
LPS+Dex	57,68 ± 8,08		1,04 ± 0,16		5,56 ± 0,59	
Dex	47,50 ± 9,76[b, c]	**b:**p≤0.001 **c:**p=0.017	1,16 ± 0,12[b]	**b:**p=0.024	4,12 ± 1,02[b, c]	**b:**p≤0.001 **c:**p=0.005

Values are presented as means±SD. The relationships between groups and results of biochemical markers are assessed by one-way ANOVA (posthoc LSD). 'a' represents comparison with control group, 'b' represents comparison with LPS group, 'c' represents comparison with LPS+Dex group LPS – Lipopolysaccharide; Dex – Dexpanthenol; SD – Standard Deviation.

DISCUSSION

Lungs are one of the vital organs that are at the top of the list of organs necessary for life. Lungs are frequently exposed to infectious factors due to their close relationship with the external environment or their intense blood supply. Neutrophil leukocyte infiltrates cause increase in inflammatory cytokines and protein leakage in acute pulmonary inflammation, are common findings in ALI. In the acute phase of ALI, neutrophils are the most abundant inflammatory cells at the site of injury and this situation is vital to host defense. However, excessive activation of neutrophils triggers tissue damage by releasing various inflammatory mediators. Overall, in treatment studies, reduced neutrophil counts were associated with a favorable prognosis. Because cytokines not only cause recruitment and activation of neutrophils

at the site of inflammation, but also cause severe inflammatory damage. Neutrophil infiltration and cytokine release particularly cause endothelial and epithelial cell damage (Li-Mei et al. 2016). In this study, intraperitoneal LPS application caused a significant inflammatory reaction in the lungs and Dex treatment improved both histopathological and immunohistochemical findings.

Sepsis-related lung injury is frequently observed in the community (Varisco 2011). In this case, the lung can be heavily affected due to the high vascularization of systemic inflammation. Oxidative and pro-inflammatory cytokines circulating in the blood bind to their surface receptors in every tissue they encounter, triggering some intracellular signaling mechanisms. As a result, a cumulative damage picture may occur as some cytokines synthesized in that cell affect other nearby cells (Si-Cong et al. 2021). Neutrophil leukocytes are the first defense cells to come to the damaged area to tissue repair. In order to invade the damaged tissue, these leukocytes must adhere to some adhesion molecules while circulating in the vessel, approach the vessel endothelium and be extravasated by taking advantage of the increase in permeability. As Qureshi et al. mentioned in their study, VCAM-1, which increases in a TNF-α-dependent manner and interacts with neutrophils, is one of these adhesion molecules (Qureshi et al. 2003). There are many studies, such as the studies of Vogel et al., Alapati et al., on reducing the expression of VCAM-1 in inflammatory or pathologic conditions (Alapati et al. 2015, Vogel et al. 2017).

Detection of these neutrophil leucocytes in the damage area with various analysis methods shows that the event is acute. The scene of neutrophilic leukocytosis detected in the LPS group in this study, also supports this view. The fact that Dex causes a decrease in cell density also creates the impression that the intraperitoneally administered drug can be used in acute events. In addition, as demonstrated immunohistochemically, it can be said that the reduction of VCAM-1 expressions, which was increased in the LPS group, by Dex is effective in reducing neutrophilic leukocytosis.

The anti-inflammatory properties of Dex were proven on nephrotoxicity in the Pınar et al research and on cardiotoxicity in the Kose et al research (Kose et al. 2020, Pınar et al. 2022). Cytokine-mediated increase in permeability in vascular structures in the area of inflammation and, accordingly, edematous scene and hyperemia in that tissue can be observed. These developing forms of damage cause septal thickening and dyspnea in the thin and expandable lung tissue. The hyperemia and septal thickening detected in the injury group of this study were reduced by Dex, indicating that it prevented the progression of the damage in the lung tissue.

Although apoptosis is generally described as programmed cell death, some external factors can lead healthy cells to apoptosis (Naim et al. 2005). Caspases are enzymes that play an important role in apoptosis. These enzymes, which are primarily synthesized as inactive proteins, are activated in various ways. A lot of cellular and morphological transformations that consist during cell death, develop as a result of a number of processes in which these enzymes play a role (Berger.et al., 2006). In the study of Miao et al., LPS was shown to cause embryonic damage due to apoptosis via cas-3 (Miao and Cui 2022).

As it is known, inflammation also contributes to the formation of oxidant molecules circulating in the blood and oxidative stress that occurs in the tissue. It is known that these two important damage mechanisms trigger apoptosis resulting in tissue cell death. Activation/inhibition of the aforementioned intracellular pathways mediate cell to undergo apoptosis. The increased cas-3 levels detected in the lung tissues of the injury group in this study, indicate that apoptosis due to this LPS-induced systemic inflammatory condition also occurs in the lung. Dex, which is normally used as an epithelial reparative, has a reducing effect on cas-3 levels, which can be considered as an important finding.

The significant increment in OSI and TOS levels in the injury group indicates that oxidative stress is stimulated in the lung tissue. In addition to this, the significant decrease in TAS is interpreted as the tissue uses the endogenous antioxidant system in the fight against oxidant substances and therefore shows a decrease. The fact that there was no significant change in the Dex applied group compared to the control, indicates that the drug is not harmful to the lung tissue. Moreover, the fact that the amount of antioxidant enzymes in the treatment groups are higher than in the damage group may indicate that the drug reduces oxidative stress secondary to the suppression of inflammatory conditions, or it can be interpreted that the drug administration time-dose requires a change, since the experimental model is an acute model. For this situation, the ideal use of the drug should be determined by studying other models that include different durations and use different doses.

As it was demonstrated by Li-Mei et al. (2016), Dex is improving histological structure of lungs by arranging the changes in tumor necrosis factor alpha, interleukin-6, malondialdehyde, superoxide dismutase, and glutathione. In this study, we obtained results that supports the findings of Li-Mei et al. showed that Dex is providing a protective effect through oxidative stress markers, cas-3 and VCAM-1 levels and improve the histological structure similar to the previous study (Li-Mei et al. 2016). Moreover, the innovative aspects of the study are that Dex, which is used in another indication and is easy to obtain, regresses the serious inflammatory response in the lung, by reducing the levels of VCAM-1, an adhesion molecule, and by reducing the apoptotic process that causes cell death, by reducing the levels of caspase-3,

where various apoptotic pathways intersect in the cell. Thanks to this feature, it is predicted that it can be used in many pathological processes using the same mechanisms and can regress the damage scene.

CONCLUSION

As a result, with Dex application, inflammation and apoptosis were prevented by reducing VCAM-1 and cas-3 expressions in lung injury secondary to systemic inflammation. It is obvious that the intracellular pathways of this active substance should be investigated in animal models in which different duration and dose applications are made to solve the mechanism of action. Unraveling these mechanisms may shed light on many new areas for the indications for use of this drug.
The results of this study showed that Dex may be a potential drug choice for the treatment of the LPS induced lung damage. Further studies are needed on this subject.

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Ethics Committee Information: This study was authorized by the Animal Experiments Local Ethics Committee of Suleyman Demirel University, Isparta, Turkey (Ethic No: 2022-02/29).

Conflict of Interest: There is no financial conflict of interest with any organization, instution, person related to our article and there is no conflict of interest between the authors.

Authors Contribution Rate: The authors declared that they contributed equally to the article.

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