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Investigation of biochemical and histopathological effects of tarantula cubensis D6 on lung tissue in cecal ligation and puncture-induced polymicrobial sepsis model in rats

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Abstract
To investigate the protective effect of Tarantula Cubensis D6 (TCD) on inflammation and oxidative stress in rat cecal ligation and puncture (CLP) in modified polymicrobial sepsis model. Wistar albino rats were randomly divided into 4 groups: sham control group, sepsis (CLP) group, low dose TCD + CLP group, and high dose TCD + CLP group. Lung tissue samples of rats were prepared to determine levels of some cytokines related to inflammation, oxidant/antioxidant parameters apoptosis markers. In addition, lung, liver, renal, and heart tissue samples of rats were prepared to determine colony counts of E. coli. We showed that Tarantula cubensis D6 significantly reduced the increasing TNF-α, IL-1β, IL-6, TOS, OSI levels in the CLP group compared to the sham control group, and causes an increase in the decreasing TAS value and significantly reduces caspase-3 and NF-KB expressions. We determined that while E. coli colony counts increased in organs such as lung, heart, liver, and kidney in the CLP group, it was decreased in TCD groups. TCD reduces polymicrobial sepsis-induced lung injury through antioxidant, antiapoptotic, and antioxidant effects.

Keywords: Inflammation, oxidative stress, apoptosis, tarantula cubensis D6, polymicrobial sepsis, E.coli

Introduction

Sepsis is a systemic response to life-threatening infection, and despite intensive treatment strategies, it is still one of the leading causes of morbidity and mortality in intensive care units. [1]. Pathogen-induced uncontrolled inflammation followed by immunodeficiency or immunosuppression is the underlying mechanism of sepsis [2]. Cecal ligation-puncture (CLP) with induced polymicrobial sepsis is the most commonly used model among researchers because it is very similar to sepsis in humans [3]. The lung is the first organ suffering from sepsis, and the inflammatory response plays a central role in the pathogenesis of acute lung injury [4]. Sepsis-induced lung injury results from the imbalances among proinflammatory cytokines released from necrotic tissues activated immunocytes and oxidant-antioxidant mechanisms [5]. Nuclear factor kappa B (NF-κB) activation during sepsis leads to increased gene expression and proinflammatory cytokines biosyntheses [6] such as tumor necrosis factor (TNF-α), interleukin-1β (IL-1β) and interleukin-6 (IL-6), contribute to the development of acute lung injury (ALI) [7]. Experimental and clinical studies have shown that anti-inflammatory and antioxidant agents may contribute to better treatment for sepsis [8]. Because, besides inflammation, production of free oxygen radicals (ROS) causes lung injury by DNA damage and protein denaturation. ROS can also initiate a systemic inflammatory response and induce severe ALI by activating various signaling pathways and inflammatory mediators [9,10]. Excessive ROS production during sepsis leads to the depletion of the antioxidant system [11,12]. For this reason, the inhibition of pathological events such as microbial invasion, systemic inflammation, and oxidative stress is the main target of sepsis treatment. We assessed whether treatment with Tarantula Cubensis D6 (TCD) could reduce ALI in CLP-induced polymicrobial sepsis-induced rats since inflammation and oxidative stress in sepsis-induced lung injury is a promising target for sepsis treatment.

Tarantula Cubensis is a member of the Mygale genus, which consists of large, mouse-shaped, hairy tarantulas [13]. TCD was obtained by processing the whole spider according to the rules of "Pharmacopeia

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Germanica" and diluting it with 96% alcohol (Anonymous Richter Pharma, Theranekron D6. http://www.richter-pharma.at/product-theranekron-d6_301.html.) In some studies, it has been determined that Tarantula Cubensis extract (TCE) has demercatif, regenerative, anti-inflammatory, and resorptive effects [14,15].

In this study, we examined the levels of TNF-α, IL-1β, IL-6, NF-κB, caspase 3, TAS, TOS, and OSI. The downregulated levels of these parameters in septic rats suggest that TCD has a beneficial effect on CLP-induced ALI. At the same time, E. coli colony counts, which increased in lung, heart, kidney, and liver tissues of the CLP group were decreased by TCD administration, and microbiologically suppressed the inflammation pathway.

Material and Methods

Ethics Committee Approvals and Centers where the Research Conducted

Ethical permission related to the study was obtained from the Atatürk University Experimental Animals Local Ethics Committee with decision number 69 on 06.30.2017. All the interventions in the study were carried out at Atatürk University Experimental Animal Production and Research Center in accordance with the protocol of the board. The rats were kept in a temperature range of 20-22 ºC, 55% +/- 5% humidity, 12 hours light / dark period. The rats were fed ad libitum with regular tap water and standard pellet feed.

Experimental Animals and Experimental Design

In this study, 32 healthy male rats (220-250 gr) of the genus Wistar Albino were used. The rats were randomly divided into 4 groups. The formation of groups and the applications are as follows.

Group 1 (Sham control group, n=8): We reached the peritoneum with a 2 cm incision from the abdominal area of the rats, and they were closed with a suture without any procedure.

Group 2 (CLP group, n=8): The cecum was isolated by reaching the peritoneum with a 2 cm incision from the abdominal area of the rat, and the ileocecal valve was ligated up to 2 cm distal, then it was pierced by 18-gauge needle (4 holes), the cecum was put back the abdomen and abdomen was closed with 3.0 silk suture.

Group 3 (TCD30+CLP group, n=8): TCD was administered intraperitoneally in low dose (30 mcg) 30 minutes before the CLP model were administered the same procedures as used in group 2.

Group 4 (TCD60+CLP group, n=8): TCD was administered intraperitoneally in high dose (60 mcg) 30 minutes before the CLP model were administered the same procedures as used in group 2.

In the CLP groups (group 2, 3, 4), the abdominal region was washed with povidone-iodine after being shaved. Analgesic lidocaine solution was applied to the suture areas of the rats to remove the error margin that might be caused by pain stress. The rats were deprived of food postoperatively but had free access to water for 18 hours until they were sacrificed.

Collection and Storage of Tissue Samples

After 18 hours of CLP model, the rats were sacrificed by general overdose anesthesia, xylazine hydrochloride 10 mg / kg (Rompun®, Bayer, Istanbul, intraperitoneally), ketamine 60 mg/kg (Ketalar®, Pfizer, Istanbul, intraperitoneally) and their heart, kidney, lung and liver tissues were removed. The tissues were cleaned with 0.9% saline. The drying was then gently performed with sterile sponges, and one of the lung tissue specimens was maintained in a 10% formaldehyde solution for histopathological treatment and the other at -80°C for biochemical analyses. Histopathological and biochemical examinations were performed in lung tissue, and microbiological examinations were performed in lung, heart, kidney, and liver tissues. E. coli counts were evaluated for microbiological examination.

Tissue homogenization (Determination of biochemical parameters)

Lung tissue was weighed at a weight of 100 mg and homogenized with 2 ml of phosphate buffer. Homogenized lung tissues were centrifuged at 5000 rpm and +4 [deg.] C. for 20 minutes, and the top located supernatants were carefully transferred to Eppendorf. Levels of TAS (Elabscience), TOS (Elabscience), OSI, and TNF-α (Catalog No: E-EL-R0019, Elabscience) were measured from supernatants using rat-specific ELISA kits. OSI; calculated as shown in the formula: OSI = ([TOS, mmol H2O2 equivalent/L] / [TAS, mmol Trolox equivalent/L] x 10) [16]. Measurements were made according to their protocols.

Histopathological examination

The inflammatory and apoptotic properties of the groups were investigated immunohistopathological using antibodies of caspase-3 (Abcam), NF-κB (Abcam), IL-1β (Abcam) and IL-6 (Abcam). Tissue injury grades between the groups were determined by Hematoxylin-Eosin Dyes.

Hematoxylin-Eosin Staining Procedure

Lung tissue specimens left in formalin fixation for 72 hours were manually blocked by passage through alcohol, xylol, and paraffin series in a semi-automated tissue monitoring device. Following the blockage procedure, 5-micron sections were taken in the microtome device, and after a few preliminary treatments, hematoxylin and eosin staining were carried out for some time, and the preparations were closed with entellan. Prepared specimens were photographed under the microscope system of the Olympus brand that has photographic attachment [17].

Immunohistochemically Staining Procedure

Following the follow-up and blocking procedures, 5-micron sections were taken in positively charged slides, and they were stained in the fully automated Ventana BENCHMARK GX model immunohistochemically staining machine. Prepared specimens were photographed under the microscope system of the Olympus brand that has photographic attachment [17].

Microbiological Examination

For biopsy specimens taken under aseptic conditions, each tissue was homogenized in 2 ml BHI (Brain Heart Infusion) medium in sterile glass homogenizers. Homogenized tissue samples (lung, heart, kidney, and liver tissues) were standardized to 100 mg/ml. 0.1 ml of each sample was inoculated into the broth agar, Columbia blood agar, BHI blood agar, MacConcay agar, Chocolate agar and Sabouraud dextrose agar (at 30 ° C). All media were left at 37 ° C for 24-48 hours of incubation. Identification of bacteria in breeding cultures was made by standard microbiological methods and gram staining. [18].

Statistical Analysis

TAS, TOS, OSI, TNF-α results were analyzed using the IBM SPSS 20.0 package program. For statistical measurements, one-way ANOVA test followed by Tukey HSD test for multiple comparisons of groups. Data are presented as mean ± standard deviation (SD). A value of $p < 0.05$ was considered statistically significant.

GraphPad 5.0 Prism (La Jolla, CA) software was used for microbiological data analysis and graphical drawing. Data are presented as mean ± SD. Intergroup comparisons were analyzed by One-way ANOVA, followed by Tukey's post-hoc test. A value of $p < 0.001$ was considered statistically significant.

Results

Biochemical Analysis

The effects of TCD on TNF-α, TOS, TAS, and OSI levels in the CLP-induced lung injury model are shown in Figures 1 and 2 (a-c).

TNF-α Level Results

TNF-α levels were significantly increased in the CLP group statistically compared to the sham control group ($p<0.05$), and TNF-α levels were decreased in TCD treated groups ($p<0.05$, Figure1).

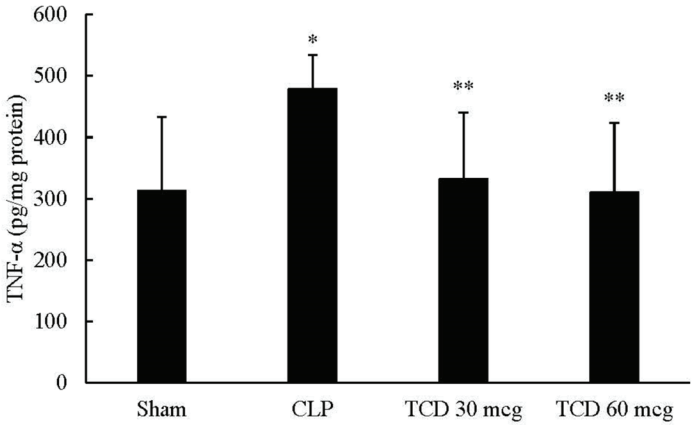

Figure 1. CLP group compared to the Sham group ($p < 0.05$), TCD treated groups against CLP group ($p<0.05$)

Statistical analyses were performed with One-way ANOVA, and Tukey HSD test was used for multiple comparisons. Data were presented as mean ± Standard Deviation (SD) (n = 8).

Oxidant-antioxidant parameter results

In Figure 2 (a-c), TOS, TAS, and OSI levels are shown for each group. TOS and OSI levels increased statistically in the CLP group compared to the sham control group but decreased in the TCD group compared to the CLP group ($p < 0.05$). TAS levels were statistically decreased in the CLP group compared to the sham control group, whereas it was increased in the TCD treated groups compared to the CLP group ($p<0.05$).

TCD administration significantly reduced CLP-induced lung injury dose-dependently with antioxidant and anti-inflammatory properties.

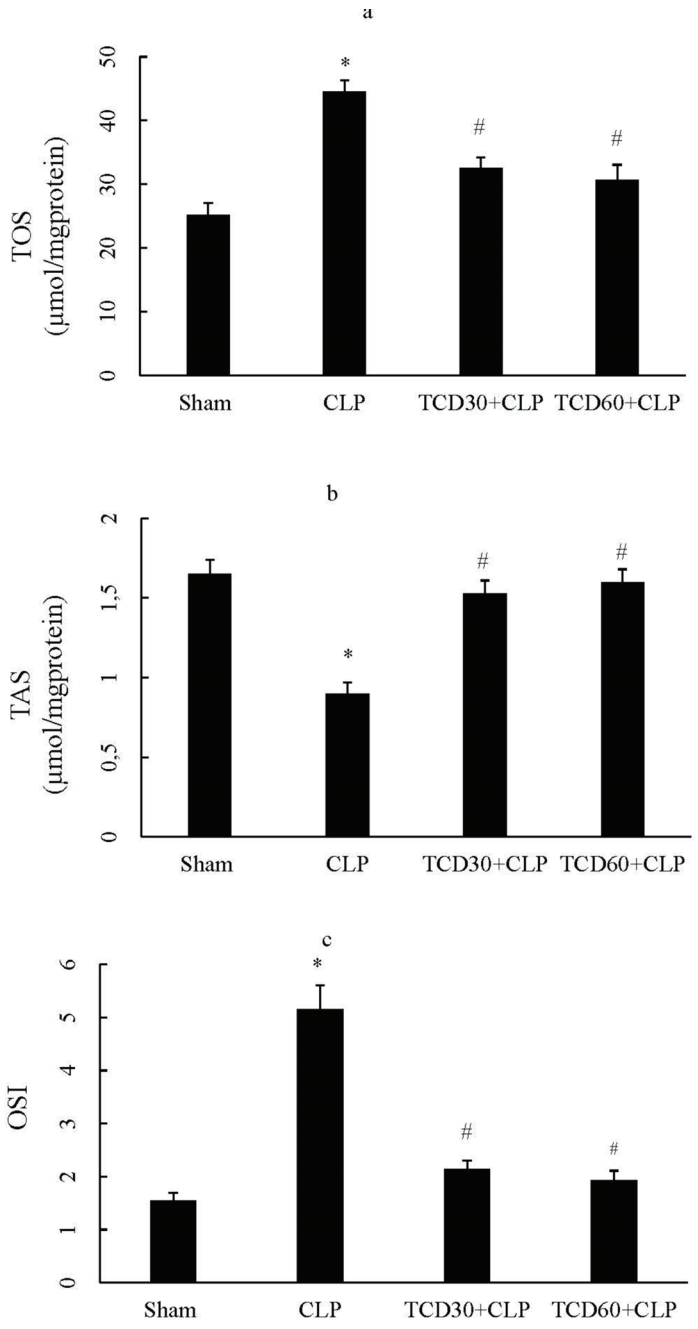

Figure 2 Shows TOS (a), TAS (b), OSI (c) levels. The * symbol represents a statistically significant difference compared to the sham control group, whereas the # symbol represents a statistically significant difference compared to the CLP group. A value of $p < 0.05$ was considered statistically significant

Histopathological Examination

Figure 3 shows the staining of the groups by the hematoxylin-eosin method. Figure 3 and table 1 show immunohistochemical staining and evaluation of caspase-3, NF-κB, IL-1β, and IL-6 in the study groups.

Hematoxylin-Eosin Staining Results

Histopathological examination of the sham group revealed that lung tissue was in normal healthy appearance, terminal and respiratory bronchioles, alveolar sacs and walls, lung parenchyma cells were healthy, and no pathological findings were found. Advanced edema areas and leukocyte infiltration in the group of sepsis (CLP) attract attention. Examination of the TCD30 + CLP group showed that the thickness of the alveolar walls decreased, but leukocyte infiltration

and edema areas appeared to be mildly present in the connective tissue areas surrounding some bronchioles. In the TCD60 + CLP group, no pathological condition similar to the sham group was observed (Figure 3).

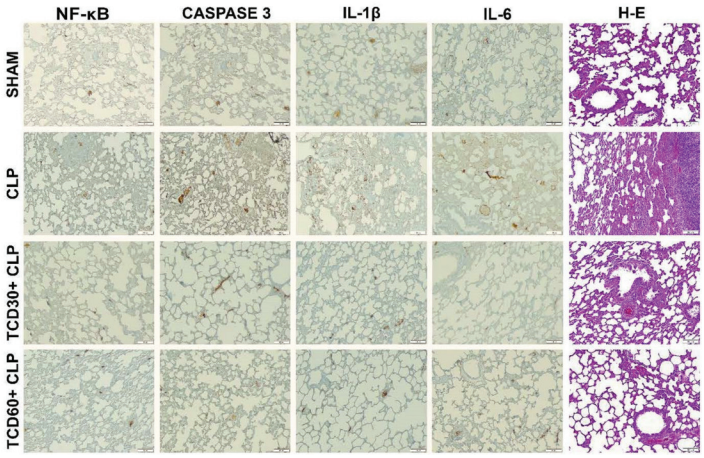

Figure 3. NF-κB, caspase-3, IL-1β, and IL-6 immunoprecipitations and hematoxylin-eosin staining results of Sham, CLP, TCD30 + CL, and TCD60 + CLP groups were shown

Immunohistochemistry results

In order to better understand the immunohistochemically evaluation results, NF-κB and Caspase-3, IL-1β, IL-6 immunopositivity were scored as; - (no), + (mild), ++ (moderate), +++ (severe) (Table 1).

Immunohistochemically staining with NF-κB IL-1β and IL-6 antibodies showed negative results in the Sham, TCD30 + CLP and TCD60 + CLP groups, while mild immunopositivity was observed in the CLP group. Immunohistochemically staining with Caspase-3 antibody showed negativity in Sham and TCD60 + CLP groups while mild immunopositivity was observed in TCD30 + CLP group and moderate in CLP group. (Table 1, Figure 3).

Microbiological Results

The breeding microorganism was identified as Escherichia coli (E. coli). E. coli levels were shown in all groups in lung, heart, kidney, and liver tissues, as shown in Figure 4.

Colony numbers according to groups in different tissues

Figure 4 shows E. Coli colony counts in heart, liver, kidney, and lung tissues. Heart tissue: Increased E. coli colony counts in the sepsis group were found to decrease in the high dose TCD treated group ($p<0.001$). Very high level of statistical significance was found between high dose and low dose ($p<0.001$). Liver tissue: Increased E. coli colony counts in the sepsis group were found to decrease in low and high dose TCD treated groups ($p<0.001$). There was no statistically significant difference between high dose and low dose ($p>0.05$). Kidney tissue: Increased E. coli colony counts in the sepsis group were found to decrease in low and high dose TCD treated groups ($p<0.001$). A very high level of statistical significance was found between high dose and low dose ($p<0.001$). Lung tissue: Increased E. coli colony counts in the sepsis group were found to decrease in low and high dose TCD treated groups ($p<0.001$). A very high level of statistical significance was found between high dose and low dose ($p<0.001$).

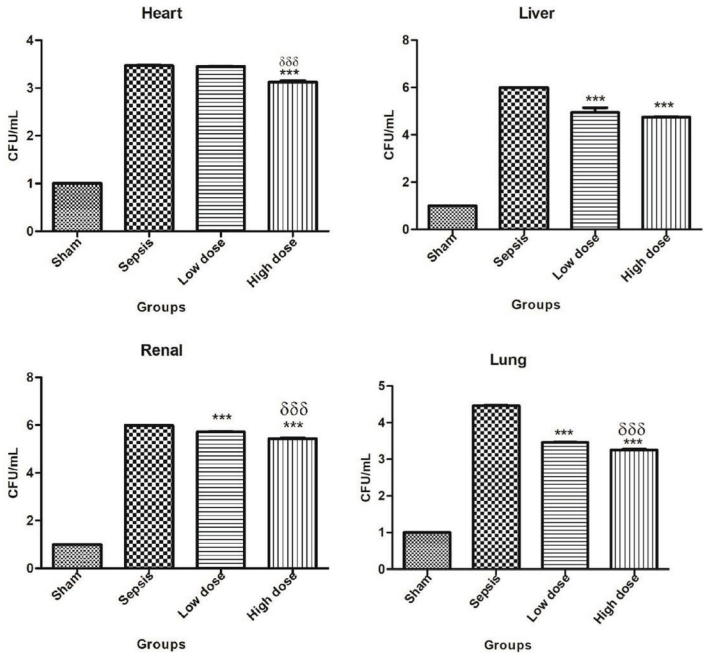

Figure 4. Number of E. coli colonies in Heart, Liver, Kidney, and Lung tissues. ***: according to the Sepsis group, *** $p<0,001$, $^{\delta\delta\delta}$: according to the low dose group, $^{\delta\delta\delta}p<0,001$

Table 1. Immunohistochemistry scoring table

Groups/ Parameters	NF-κB	Caspase-3	IL-1β	IL-6
Sham	-	-	-	-
CLP	+	++	+	+
TCD 30mcg	-	+	-	-
TCD 60mcg	-	-	-	-

NF-κB, Caspase-3, IL-1β, IL-6 immunopositive; -; No imunopositivity; +: Mild immunopositivity; ++: Moderate immunopositivity; +++: Severe immunopositivity

Discussion

Sepsis (the systemic inflammatory response to infection) is a significant public health threat to life in the 21st century, with increasing incidence and high mortality rate in intensive care units worldwide [19,20]. Excessive tissue damage or death is seen in approximately 30-50% of patients with sepsis [21]. The highest organ damage in sepsis is seen in the lungs, liver, kidney, heart, and intestine, although lungs are the most affected organs [22,23]. 50% of all sepsis cases begin with an infection in the lungs [24]. Acute lung injury, primarily adult respiratory distress syndrome, is a serious life-threatening medical condition characterized by extensive inflammation in the lungs, and it has a mortality rate as high as 30% [25]. The pathogenesis of ALI includes factors such as excessive and uncontrolled inflammatory response, oxidation/ anti-oxidation imbalance [26].

Inflammation is the base of many acute inflammatory conditions, such as sepsis [27]. Sepsis is a severe systemic inflammation that results in inflammatory and immunological responses such as NF-κB pathway activation [28] and overproduction of pro-inflammatory cytokines such as IL-1, IL-6, and TNF-α [29]. It is the critical transcription regulator of inflammatory genes such as NF-κB, TNF-α, IL-1β, and IL-6. Activation of NF-κB may promote transcription of these pro-inflammatory genes,

may trigger the inflammation cascade and play an essential role in various inflammatory diseases [30-33]. Cytokines such as TNF-a, IL-1, and IL-6 lead to the activation of leukocytes and subsequently, to organ damage [34]. The inhibition of microbial invasion, systemic inflammation, and pathological events induced by oxidative stress is the primary goal of sepsis treatment [35]. ALI is a clinical issue that causes acute and excessive pulmonary inflammation and continues to cause high morbidity and mortality rates despite modern clinical practice [36,37]. For this reason, sepsis studies have focused on expanding anti-inflammatory strategies. In a study of cows with mastitis, it was determined that TCE improved the mastitis when the Staphylococcus aureus and E. coli were isolated at high doses [38]. Standard treatment was 5 days in hand-foot-mouth disease in cattle, while TCE lesions were corrected in 2 days. [39]. Colonel MK et al. showed that in cattle with blue tongue disease, TCE administration reduced leukocyte levels, oral lesions, and rectal body temperature after 24 hours, and all cattle were healed on the 10th day of the treatment [40]. In our study, reduction of TNF-α, IL-1β, IL-6 levels, and NF-κB immunopositivity in septic rats by TCD, suggesting that TCD alleviated CLP-induced ALI.

Increased ROS production leads to lipid peroxidation, DNA oxidative damage, lung damage with protein denaturation. ROS can also initiate systemic inflammatory responses, and activate various signaling pathways and inflammatory mediators, play an essential role in the pathogenesis of sepsis and induce severe ALI [9,10,41,42]. Oxidative damage is vital in the pathogenesis of sepsis and is thought to play a protective role against sepsis and complications of antioxidant therapy [43]. Increased TOS and OSI, which are markers of oxidative stress in the lung tissues of septic rats, have been reported [44]. It has been shown that TCE may reduce the damage by activating the antioxidant system in aflatoxin-induced liver injury in rats. [45]. In another study conducted on cows with Papillomatosis, application of TCE revealed that total antioxidant level increased and total oxidant level decreased at the end of the 15th day. [46]. We assessed oxidative stress in the lung tissue to investigate the possible mechanisms of the protective effect of TCD against CLP-induced ALI and observed oxidative stress decreased with TCD.

ROS plays a vital role as an inducer of cell death pathways involving apoptosis, anoikis, and autophagy [47]. Among these cell death pathways, apoptosis plays an essential role in the elimination of unnecessary cells and is induced by the activity of caspase family proteins [48]. Apoptosis is a form of programmed cell death, resulting in several interconnected intracellular caspase proteins [49,50]. Caspase-3 is the major protease of cascade reactions during apoptosis and plays a critical role in cell apoptosis [51]. Increased caspase-3 levels in septic rats have been demonstrated [52]. In a study on breast cancer cell culture, TCE induces death of cancer cells by inducing apoptosis [53]. It has been shown that apoptotic index increases with TCE in dogs with breast adenocarcinoma [54]. Although TCE kills cancer cells by increasing apoptosis to prevent the proliferation of cancer cells, in our study, TCE reduced the level of caspase-3 and reduced anti-apoptotic activity to the least extent of CLP-induced lung injury.

The most common microorganisms in the community are Escherichia coli, Streptococcus pneumonia, and Staphylococcus aureus, although the microorganisms are causing sepsis are variable depending on whether they originate from the hospital or out of the hospital [55]. The cecum is colonized by microorganisms. Usually, a large number of Gram-negative and Gram-positive bacteria, and cecum puncture causes the fecal material in the cecum to infiltrate into the peritoneal cavity and cause an excessive immune response formation by the microbe [56]. Since CLP, a murine model of bacterial peritonitis, is accepted as the "gold standard" animal model of sepsis [57-60], we used this model in the present study. In the present study, it was observed that E. coli was the microorganism that was cultured in lung, heart, kidney and liver tissues in the microbiological examination of the CLP sepsis model and E. coli levels were decreased in a dose-dependent manner in the low and high dose TCD groups compared to the CLP group.

To make effective changes in the clinical management of sepsis, the pathogenesis of septic organ damage should be better understood for the development of therapeutic strategies. Clearly observed in sepsis studies is that inflammation, oxidative stress, and apoptosis suppression can provide significant contributions to the treatment of sepsis. In our study, inflammation, oxidative stress, and apoptosis pathways are suppressed by TCD, and this promise hopes in the treatment of sepsis.

Conclusion

CLP-induced sepsis TCD provides protection against lung damage with its antioxidants, anti-inflammatory, and anti-apoptotic properties.

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Conflict of interest
The authors declare that there are no conflicts of interest.

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Ethical approval
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