

Original Article

The role of pomegranate seed oil on kidney and lung tissues in the treatment of sepsis: animal pre-clinical research

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Abstract

Objectives: Sepsis is a common disease with a high mortality. Decreasing the speed is possible with early and intensive therapy. However, most medicines have been tested, but none has proven effective. Therefore, the study aimed to discover the protective and therapeutic effects of pomegranate seed oil (PSO).

Methods: The cecal ligation puncture (CLP) method was used to induce sepsis. The experimental procedure was started with the animals divided haphazardly into four groups: control (C), sepsis (CLP), CLP + low dose PSO (CLP + LD), and CLP + high dose PSO (CLP + HD). First, the cecum was filled with feces. The full cecum was tied under the ileocecal valve for ligation and punctured. At 1 hour after CLP, 0.32 mg/kg and 0.64 mg/kg of PSO were administered. 24 hours after, lung and kidney specimens were collected.

Results: Neutrophil infiltration/aggregation and alveolar wall thickness decreased in lung with PSO groups compared with the CLP. The findings for overall lung injury were similar. In renal, all parameters were increased in the CLP compared with C, except for vascular vacuolization and hypertrophy. According to the CLP, all parameters were significantly lower in CLP + HD. Furthermore, glomerular vacuolization, degeneration, and necrosis of tubular cell, dilatation of bowman space, and tubular hyaline cylinders reduced CLP + LD versus CLP. Thiobarbituric acid-reactive substances decreased in lung, with the PSO groups. In addition, superoxide dismutase increased in PSO groups versus CLP.

Conclusions: We conclude that the high-dose PSO is especially effective in treating sepsis.

Key words: cecum; ligation; multiple organ failure; perforation, pomegranate; sepsis.

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Introduction

Sepsis is a complex syndrome defined as a typical response to infection. Acute and reversible dysfunction of two or more systems with uncontrolled inflammation is defined as multiple organ dysfunction syndromes (MODS). The incidence of organ dysfunction in the intensive care unit (ICU) is 91% for the respiratory system, 62% for the cardiovascular system, 53% for the renal system, 48% for the neurologic system, and 24% for the liver. Dysfunction of these organs can be prevented by supportive care [1]. Researchers have recently focused on antioxidants and anti-inflammatory agents as innovative therapeutics [2]. Pomegranate (*Punica granatum* L., *Punicaceae*) is an anti-inflammatory and antioxidant medicinal plant that grows in the Mediterranean region [3]. About one-fifth

of the total seed weight is pomegranate seed oil. Conjugated octadecatrienoic acids, especially punicic acid, are the major constituents of PSO [4]. PSO has antioxidant [5], anti-inflammatory [6], antimicrobial [7], antiproliferative, and anticancer [6] effects. Herein, we demonstrated PSO's curative effects on lung and kidney tissue in sepsis.

Methods

Experimental Group

The experimental study was arranged with the approval of the Gazi University Experimental Animal Ethics Committee (Ethics Number: 20.10.2020-E.112213). In addition, a Guide for the Care and Use of Laboratory Animals was carried out concerning this experiment. The study was done and accomplished at

the Gazi University Life Sciences Application and Research Center. Rats were kept under standard conditions.

The thirty Wistar albino, weighing 225–300 g, male rats used in the experiment were obtained from the Gazi University Experimental Animals Research Center. Rats were randomly separated into four categories. The category C included six rats; the other eight were in CLP, CLP + LD, and CLP + HD.

Sepsis model

The CLP method was used for inducing sepsis. 50 mg/kg ketamine hydrochloride (Ketalar® Flask, Parke-Davis, USA) additionally 10 mg/kg dose of xylazine hydrochloride was (Alfazyne, 2%, Ege Vet) administered. After the abdomens were shaved, the cecum was exposed by an abdominal incision. After the colon was stroked for filling feces, the ileocecal valve was ligated. Two punctures were made in the front cecum, then the abdomen was sutured. In CLP, no further procedures were received. In C, only colon patting was performed after the abdominal incision. The peritoneal cavity was filled with prewarmed saline after the entire experimental procedure.

Pomegranate Seed Oil

Pomegranate seed oil was purchased from Troils Oil Industry and Trade Joint Stock Company (Turkiye, Antalya). It has 3-4% palmitic and 2-3% stearic, 6-7% oleic, 6-7% linoleic, 0-1% arachidic, 0-3% 11-eicosenoic, and 79-84% punicic, which consists of acid components. The dose of CLP + LD is 0.32 mg/kg, and the CLP + HD is 0.64 mg/kg. After 1 hour of perforation, doses were injected intraperitoneally. Based on oxidative stress and the anti-inflammatory response according to ischemia-reperfusion studies, doses were chosen [8].

Histopathology

Tissue samples were taken from the rats 24 hours later, and the rats were sacrificed by intracardiac blood collection under deep anesthesia. Tissues were processed with paraffin for 24 hours after being submerged in 10% buffered formalin. After 4 µm paraffin tissue sections were taken, they were dyed with hematoxylin-eosin (H&E). The scores from ten different areas were collected, and the total score was named in each tissue sample score.

Alveolar thickness and neutrophil infiltration/aggregation were adjudged, and lung damage was assessed. Parameters were appraised as points; none: 0, mild: 1, medium: 2, and severe: 3. Total

lung injury: sum of scores [9]. For renal injury, we assessed that vacuolization of glomerul (GV), vacuolization and hypertrophy of vascular (VVH), tubule dilation (TD), tubular cell degeneration and necrosis (THDN), dilatation of bowman space (BSD), tubular hyaline cylinders (THC), infiltration of lymphocyte (LI), and shedding of tubular cell (TCS). Parameters were appraised as 0: none; + 1: mild; + 2: moderate; + 3: severe [10].

Biochemistry

Malondialdehyde (MDA) and superoxide dismutase (SOD) were investigated. After that, the level of lipid peroxidation and oxidative stress were defined in both lung and kidney tissue. MDA interacted with thiobarbituric acid (TBA), and the product of this reaction, chromogranin, was analyzed at a 532 nm spectrophotometric wavelength [11]. Superoxide is produced as a result of the inhibition of nitroblue tetrazolium reduction (NBT) by xanthine-xanthine oxidase. SOD was determined by measuring the NBT reduction sample formazan precipitate (NBTH2) at 560 nm [12].

Statistical Analysis

A Statistical Package for the Social Sciences v.20.0 (SPSS Inc., Chicago, IL, USA) was implemented statistically. The significant values had to be $p < 0.05$ and indicated with a mean \pm standard error (Mean \pm SE). Variables were researched with the Kolmogorov–Smirnov test. Depending on the distribution, one-way ANOVA followed by the Tukey post hoc test was used for multiple groups comparison histopathological and biochemical parameters.

Results

Histopathology

Lung damage was evaluated by determining infiltration and aggregation of neutrophils, also alveolar wall thickness. According to the CLP group, infiltration and aggregation of neutrophil was significantly elevated in CLP versus C ($p = 0.001$) but declined, especially in the CLP + HD (Table 1, Figure 1).

The alveolar wall thickness value was higher in CLP versus C at a significant rate ($p = 0.001$). However, following treatment, especially with a high dose, alveolar wall thickness indicated a lower value versus CLP (Table 1, Figure 1).

Total lung injury was significantly higher in the CLP and CLP + LD groups compared to the control ($p < 0.001$ and $p = 0.028$, in order of writing). However,

in the treated group, it was diminished significantly, especially with a high dose (Table 1, Figure 1).

GV, TD, VVH, THDN, BSD, THC, LI, and TCS were assessed, and renal injury was evaluated. In light microscopy, GV was defined as disparate between the groups at a significant rate ($p= 0.041$). CLP group GV was higher than C ($p = 0.031$). PSO treatment, especially at high doses, remarkably reduced GV compared to CLP (Table 2, Figure 2). TD was different between the groups in a significant way ($p = 0.042$). CLP group TD increased more according to C ($p = 0.046$). TD was reduced with high dose PSO contrasted to CLP in significant ($p = 0.037$) (Table 2, Figure 2). All groups had different THDN ratios in significance (p

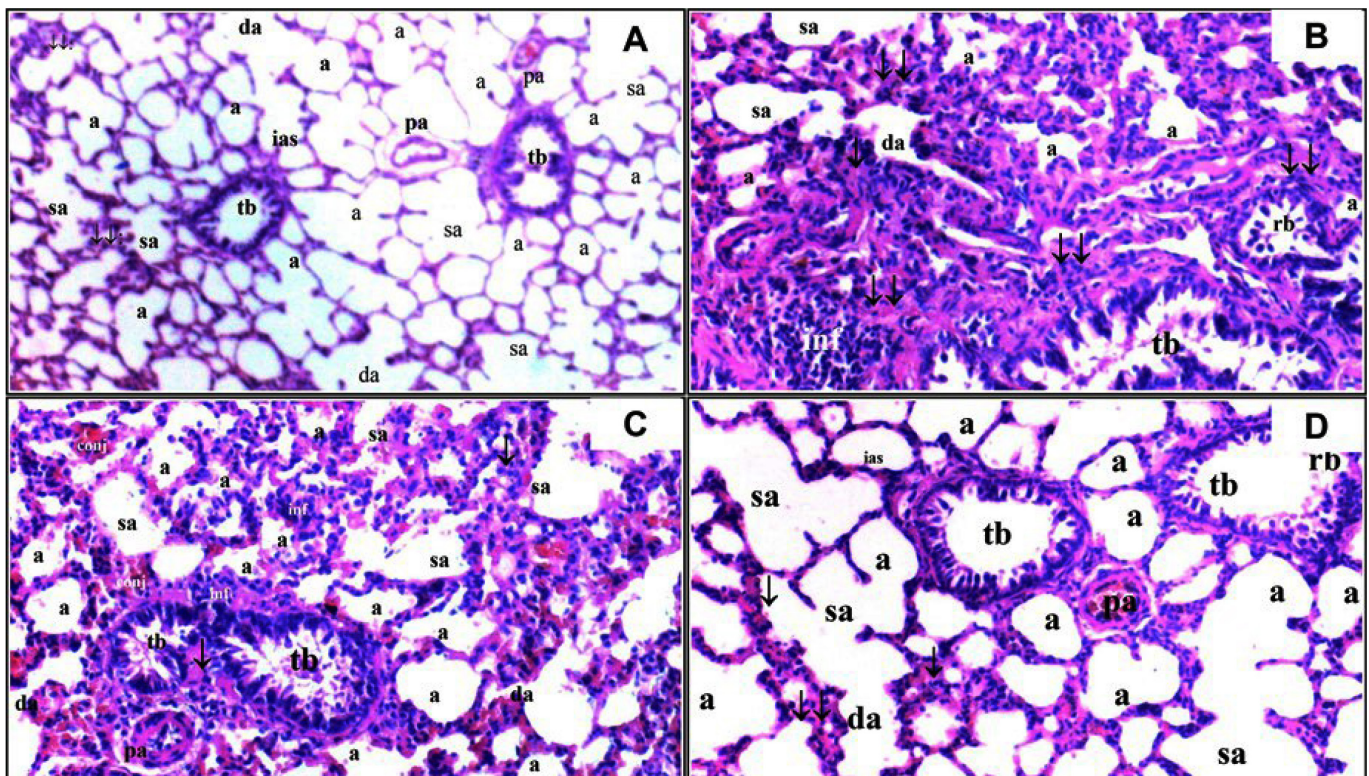
$= 0.026$). The THDN rate of the CLP group was higher than the C group ($p = 0.007$) (Table 2, Figure 2). The different BSD ratios were detected in all groups and were significant ($p=0.017$). BSD level was statistically significant ($p = 0.017$) compared to C in the CLP group. PSO, which is high, had a better effect on the BSD level than CLP (Table 2, Figure 2). All groups had different THC levels in a significant way ($p = 0.006$). CLP group THC had increased levels as opposed to C ($p = 0.005$). In particular, the group that used high-dose PSO reduced THC levels versus CLP ($p = 0.035, p = 0.003$, in order of writing) (Table 2, Figure 2). All groups had distinctive LI levels, which were statistically significant ($p = 0.034$). The CLP group LI level was high versus C

Table 1. Lung histopathological analysis (Mean ± SE).

	Group C (n = 6)	Group CLP (n = 8)	Group CLP + LD (n = 8)	Group CLP + HD (n = 8)	<i>p</i> **
Neutrophil Infiltration /aggregation	0.33 ± 0.21	1.50 ± 0.19*	0.88 ± 0.23 [#]	0.50 ± 0.19 [#]	0.007
Alveolar wall thickness	0.17 ± 0.17	1.75 ± 0.31*	0.88 ± 0.23 [#]	0.75 ± 0.16 [#]	0.002
Total score	0.50 ± 0.22	3.25 ± 0.37*	1.75 ± 0.41* [#]	1.25 ± 0.25 [#]	< 0.001

* $p < 0.05$: compared with group C, [#] $p < 0.05$: compared with group CLP.

Figure 1. Lung histopathology. **A.** Control group (a: alveoli, tb: terminal bronchiole, sa: saccus alveolaris, ↓↓:(septum) thickening, da: ductus alveolaris, ias: nter alveolar septum), (H&EX40); **B.** Cecal ligation perforation group (a: alveoli, sa: saccus alveolaris, da: ductus alveolaris, ↓↓:(septum) thickening, tb: terminal bronchiole, rb: respiratory bronchiole, inf: inflammation, ↓:pulmonary vessel thickening), (H&EX40); **C.** Cecal ligation perforation + low dose pomegranate seed oil group (a: alveoli, sa: saccus alveolaris, da: ductus alveolaris, ↓:pulmonary vessel thickening, pa: pulmonary artery conjuncture: capillary congestion, inf: inflammation), (H&EX40); **D.** Cecal ligation perforation + high dose pomegranate seed oil group (a: alveoli, sa: saccus alveolaris, ↓↓: (septum) thickening, ↓: pulmonary vessel thickening, rb: respiratory bronchiole, pa: pulmonary artery branches, conj: capillary congestion, da: ductus alveolaris, ias: inter alveolar septum), (H&EX40).



($p = 0.049$). In the PSO group, especially at high doses, the LI level was reduced versus CLP ($p = 0.010$) (Table 2, Figure 2). TCS was different between the groups at a significant rate ($p = 0.042$). VVH and TCS levels were similar between the groups ($p = 0.162$, $p = 0.062$, in order of writing) (Table 2, Figure 2).

Biochemistry

All groups had different lung tissue thiobarbituric acid-reactive substances (TBARS) levels that were

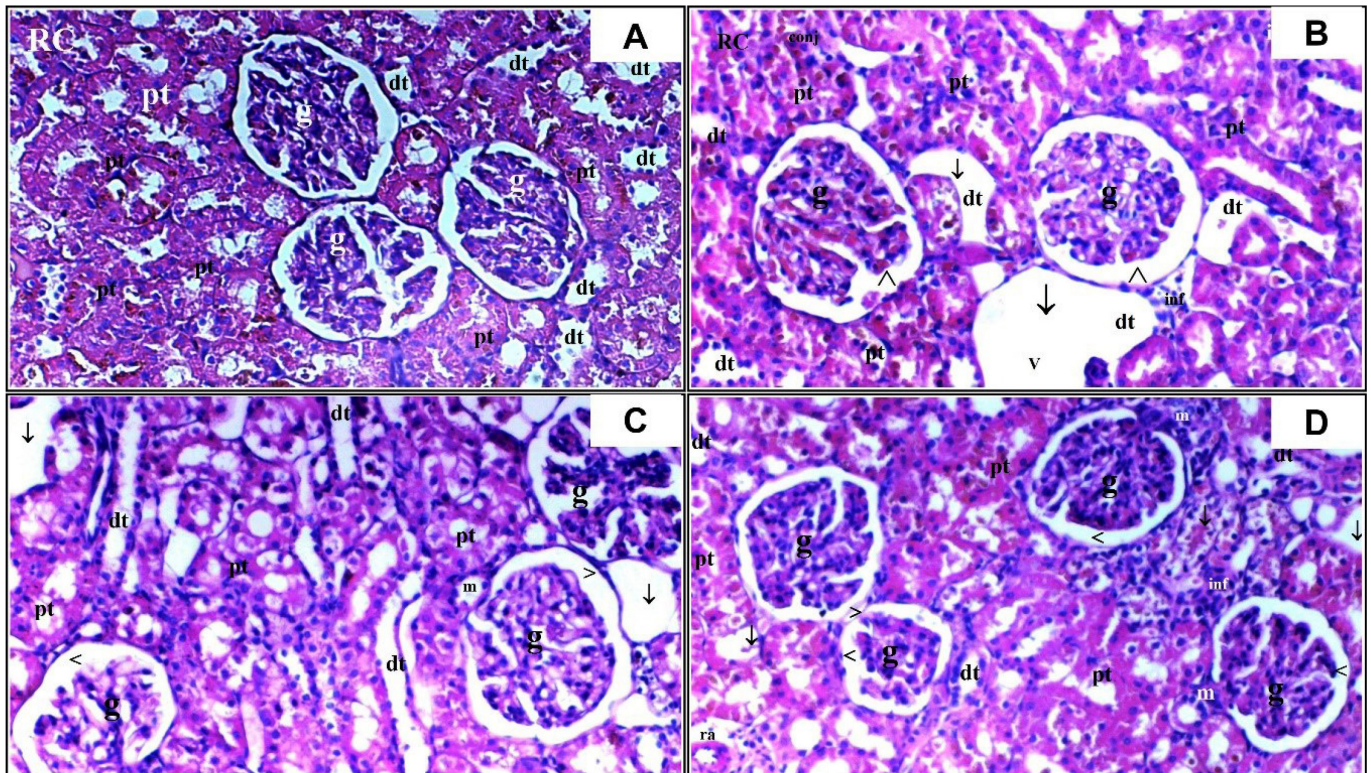
statistically significant ($p < 0.001$). TBARS levels of the CLP were higher than C group ($p = 0.001$). Besides that, PSO groups that were CLP + LD and CLP + HD had lower TBARS in comparison to CLP ($p = 0.017$, $p = 0.001$, in order of writing) (Table 3). SOD enzyme activity was significantly different between the groups ($p < 0.001$). In comparison to C, the SOD enzyme activity in the CLP group decreased ($p = 0.001$). In addition, SOD enzyme activity was detected at an

Table 2. Kidney histopathological analysis (Mean \pm SE).

	Group C (n = 6)	Group CLP (n = 8)	Group CLP + LD (n = 8)	Group CLP + HD (n = 8)	<i>p</i> **
Glomerular vacuolization (GV)	0.33 \pm 0.21	1.38 \pm 0.38*	0.63 \pm 0.26	0.50 \pm 0.19 [#]	0.041
Tubular dilation (TD)	0.33 \pm 0.21	1.38 \pm 0.38*	0.75 \pm 0.16	0.38 \pm 0.18 [#]	0.042
Vascular vacuolization and hypertrophy (VVH)	0.33 \pm 0.21	1.13 \pm 0.30	0.63 \pm 0.26	0.50 \pm 0.19	0.162
Tubular cell degeneration and necrosis (THDN)	0.17 \pm 0.17	1.25 \pm 0.25*	0.63 \pm 0.18	0.63 \pm 0.18	0.026
Bowman space dilation (BSD)	0.33 \pm 0.21	1.25 \pm 0.25*	0.50 \pm 0.19 [#]	0.25 \pm 0.16 [#]	0.017
Tubular hyaline cylinders (THC)	0.33 \pm 0.21	1.38 \pm 0.18*	0.63 \pm 0.18 [#]	0.38 \pm 0.18 [#]	0.006
Lymphocyte infiltration (LI)	0.33 \pm 0.21	1.13 \pm 0.23*	0.75 \pm 0.23	0.38 \pm 0.18 [#]	0.034
Tubular cell shedding (TCS)	0.33 \pm 0.21	1.13 \pm 0.23	0.63 \pm 0.18	0.50 \pm 0.19	0.062

* $p < 0.05$: compared with group C, [#] $p < 0.05$: compared with group CLP.

Figure 2. Kidney histopathology. **A.** Control group (RC: renal cortex, pt: proximal tubule, dt: distal tubule, g: glomerule), (H&EX40); **B.** Cecal ligation perforation group (RC: renal cortex, pt: proximal tubule, dt: distal tubule, g: glomerule, ↓: dilated tubule, a: artery, conj: congestion, ^: bowman gap, inf: inflammation) (H&EX40); **C.** Cecal ligation perforation + low dose pomegranate seed oil group (pt: proximal tubule, dt: distal tubule, g: glomerule, ↓: dilated tubule, m: macula densa) (H&EX40); **D.** Cecal ligation perforation + high dose pomegranate seed oil group (pt: proximal tubule, dt: distal tubule, g: glomerule, m: macula densa, ↓: dilated tubule, inf: inflammation, ra: renal artery) (H&EX40).



elevated level in PSO groups versus CLP ($p = 0.002$, $p < 0.001$, in order of writing) (Table 3).

All groups had significantly different TBARS levels in the kidney ($p < 0.001$). The TBARS level in the CLP was defined to be higher than in the C group ($p < 0.001$). In addition, PSO groups' TBARS levels were diminished against CLP ($p < 0.001$, $p < 0.001$, in order of writing) (Table 4). All groups had different SOD enzyme activity, which was statistically significant ($p = 0.018$). The SOD enzyme activity in the CLP group was found to be lower in comparison to C ($p = 0.015$). Another point is that in PSO groups, SOD enzyme activity was detected at a decreased level opposite to CLP in significant ($p = 0.035$, $p = 0.010$, in order of writing) (Table 4).

Discussion

Sepsis is a heterogeneous syndrome defined as an irregular systemic response. Physiological disorders and failure in two or more organs in acute sepsis are called MODS [1]. The most damaged organs in MODS are the lungs and kidneys. MODS lung injury is acute respiratory distress syndrome. In this syndrome, as a result of atelectasis and intravascular thrombosis, ventilation impairment occurs. Inflammatory cells and factors induce oxidative stress and apoptosis [13]. MODS renal injury is a renal or prerenal failure. Systemic vasodilatation and renal vasoconstriction occur due to inflammatory cell cytokines. Microthrombus occurs as a result of increased coagulation cascade activation. This causes tubular necrosis and excretory dysfunction [13,14]. Therefore, inflammation and oxidative stress should be reduced to prevent sepsis-related injury. Based on this, we aimed to reduce the damage caused by sepsis on kidney and lung tissues by applying PSO doses in our study.

The sepsis model can be created by surgical (CLP) and the injection of gram-negative bacterial wall component lipopolysaccharide (LPS) [15]. LPS

infections depend on toll-like receptor 4 (TLR4) signaling, which provides a specific immune response. However, there are multiple complex interactions among signaling pathways during the progression of sepsis. As a result, only TLR can reveal the complex interactions of sepsis. Nevertheless, higher cytokine levels are seen after the LPS injection than in human sepsis [16]. This method is called endotoxic shock rather than sepsis in rodents. Thus, these parameters can reduce the truth of the study [17]. To prevent this, we preferred the CLP sepsis model in our study. However, the amount of cecum bound in the CLP model the rate of cecum stool during punction, cecum sizes, and bacterial habitation vary between animals [16,18]. However, the number of punctures and needle sizes affect disease severity differently [19]. These will affect the truth and reproducibility of animal CLP studies. The same investigator followed standard protocols to avoid this situation to reduce potential inconsistencies.

To begin, we chose neutrophils for their first role in inflammation and their information about the progression of inflammation. During sepsis, anti-apoptotic protein Bcl-xl expression increases [20]. On the other hand, myeloid cell leukemia (Mcl-1), extracellularly regulated protein kinases (ERK) 1/2, phosphoinositide3 kinases (PI3K) in neutrophils, Akt activation, and Bad phosphorylation suppress apoptosis [21]. Furthermore, the activation of caspase-8 by Src homology domain 2 tyrosine phosphatase1 (SHP1) decreases. This anti-apoptotic process increases the physiological half-life of neutrophils, which is 7-12 hours [22]. Also, neutrophil circulation is increased [23]. Besides, granulocyte colony-stimulating factor (G-CSF) and bacterial products promote leukocytosis [24]. In light of these mechanisms, we can consider that the increase in neutrophil infiltration/aggregation seen in the CLP may result from a high neutrophil half-life and leukocytosis [20-24]. Makled *et al.* showed that pomegranate extract attenuated inflammatory cell

Table 3. Lung oxidant parameters (Mean ± SE).

	Group C (n = 6)	Group CLP (n = 8)	Group CLP + LD (n = 8)	Group CLP + HD (n = 8)	<i>p</i> **
TBARS (nmol/mg protein)	1.00 ± 0.21	2.60 ± 0.40*	1.51 ± 0.11 [#]	1.03 ± 0.14 [#]	< 0.001
SOD (U/mg protein)	3.31 ± 0.12	1.98 ± 0.17*	2.90 ± 0.20 [#]	3.13 ± 0.17 [#]	< 0.001

* $p < 0.05$: compared with group C, [#] $p < 0.05$: compared with group CLP.

Table 4. 17720 Kidney oxidant parameters (Mean ± SE).

	Group C (n = 6)	Group CLP (n = 8)	Group CLP+LD (n = 8)	Group CLP+HD (n = 8)	<i>p</i> **
TBARS (nmol/mg protein)	0.88 ± 0.05	2.73 ± 0.36*	1.23 ± 0.10 [#]	1.03 ± 0.12 [#]	< 0.001
SOD (U/mg protein)	2.86 ± 0.32	1.46 ± 0.18*	2.60 ± 0.20 [#]	2.82 ± 0.41 [#]	0.018

* $p < 0.05$: compared with group C, [#] $p < 0.05$: compared with group CLP.

infiltration and TLR4 expression in CLP liver injury [25]. Biddle *et al.* reported that proinflammatory cytokines were decreased with PSO in necrotizing enterocolitis [26]. We demonstrate that a high dose of PSO (0.64 mg/kg) reduced neutrophil infiltration/aggregation. Significant reductions in the scores of alveolar wall thickness and total lung injury also demonstrate the protective efficacy of high-dose PSO. Hemmati *et al.* reported that pomegranate extract attenuated bleomycin-induced pulmonary fibrosis with antioxidant effects [27]. Şen *et al.* studied pomegranate extract and carvacrol together and found that pomegranate extract ameliorated methotrexate-induced lung injury [28]. Nevertheless, the effects of carvacrol and its extract on lung damage are not specific. As can be seen, these studies were conducted on extracts, but we studied the effectiveness of pomegranate seed oil in our study [25,27,28].

The protective effect of PSO may be associated with its antioxidant capacity in the lungs [27]. It has a higher antioxidant capacity than green tea extracts because it contains more flavonoids [29]. We demonstrated PSO oxidant capacity with TBARS and antioxidant capacity with SOD. TBARS is the most frequently used test to assess lipid peroxidation. MDA-TBA₂ produced as a result of the effect of MDA on TBA is called TBARS [30]. Our research found that TBARS oxidant capacity decreased and SOD antioxidant capacity increased after PSO. Thus, we reported that PSO has scavenging activity. Pomegranates do have not only scavenger activity but also metal chelates [26]. Studies about the chelate binding feature of PSO are absent from the literature. To talk about the total antioxidant activity of PSO, this feature should also be studied.

In this, we show that PSO reduces glomerular and tubular injuries. Similar to our findings, Sancaktutar *et al.* reported that pomegranate extract showed a protective effect by reducing tubular damage in ischemia/reperfusion [31]. Boroushaki *et al.* used the same doses as us and reported that PSO attenuated gentamicin-induced nephrotoxicity [32]. Last, of all, we concluded that PSO has a protective outcome in renal injury with its anti-oxidant capacity, taking as reference the studies with similar information.

The missing part of our study is that we should have shown an influential component in PSO. We knew the PSO content analysis that was used in our study. Nevertheless, in our study, we did not separate PSO into acids and did not show effective content in sepsis. If we had shown an antioxidant and an anti-inflammatory acid, we could have facilitated the approach to sepsis.

This missing part can be eliminated with the studies to be done by researchers in the future.

Conclusions

In our study, we explain that high-dose PSO has protective roles in lung and kidney tissues in sepsis. However, it isn't known which chemicals have these properties. Many conjugated fatty acids are present in the PSO chemical structure (4% palmitic, 2-3% stearic, and 6-7% oleic, 6-7% linoleic, as well as 0-1% arachidic, 0-3% 11-eicosenoic, and 79-84% punicic) [29]. Among these components, punicic acid is both an anti-oxidant and an anti-inflammatory [30]. Nevertheless, more research is needed to state clearly that conjugated fatty acids have anti-oxidant and anti-inflammatory properties.

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Authors' contributions

Ümmü Gülşen Bozok and Ayşegül Küçük did the design of the study. Mustafa Arslan created the experimental model. Aydan İremnur Ergörün and Aydın Yavuz performed pomegranate injections. Mustafa Kavutçu did biochemical studies, and Şaban Cem Sezen made histopathological analyzes. Mustafa Arslan and Ayşegül Küçük carried out statistical studies. Ümmü Gülşen Bozok wrote the study. All researchers have checked the written work, and they all agree.

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