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Prophylactic Effects of Parsley (Petroselinum crispum), on Sepsis Model Via Cecal Ligation and Puncture Procedure

Yasir Furkan ÇAĞIN[1]
İhsan KULAKSIZ[2]
Nigar VARDI[3]
Hakan PARLAKPINAR[4]
Onural ÖZHAN[4]
Azibe YILDIZ[3]
Alaaddin POLAT[5]
Kevser TANBEK[5]
Ahmet Kadir ARSLAN[6]
Yahya ATAYAN[7]

[1]İnönü University, Faculty of Medicine, Department of Gastroenterology, Malatya, Türkiye
[2]Istanbul University, Çapa, Faculty of Medicine, Department of Microbiology, İstanbul, Türkiye
[3]İnönü University, Faculty of Medicine, Department of Histology and Embryology, Malatya, Türkiye
[4]İnönü University, Faculty of Medicine, Department of Medical Pharmacology, Malatya, Türkiye
[5]İnönü University, Faculty of Medicine, Department of Physiology, Malatya, Türkiye
[6]İnönü University, Faculty of Medicine, Department of Biostatistics and Medical Informatics, Malatya, Türkiye
[7]Malatya Turgut Özal University, Faculty of Medicine, Department of Gastroenterology, Malatya, Türkiye

ABSTRACT

Objective: Sepsis causes the release of free oxygen radicals that disrupt membrane integrity, and damage to Mitochondria due to the production of free oxygen radicals and oxidation leads to exacerbation of sepsis, Cecal ligation and puncture (CLP) in rats mimics the characteristics and course of clinical sepsis (Hubbard et al., 2005).

Methods: We evaluated the antioxidant effects of Petroselinum crispum (Pc) in a cecal ligation and puncture (CLP)-induced rat sepsis model, in a rat model of sepsis caused by cecal ligation and puncture (CLP). *Wistar albino* rats were separated into four groups of eight groups: a sham group with incised and sutured abdomens; a Pc extract (PcE) group, which was given 2 g/kg parsley extract for 14 days by gastric gavage; a CLP group, which was subjected to sepsis caused by CLP; and a PcE + CLP group, which was given parsley extract for 14 days. PcE was given for 14 days, after which sepsis was induced via the CLP procedure. The groups were compared in terms of hemogram, biochemical and histological characteristics.

Results: The administration of PcE before CLP-induced sepsis increases neutrophil counts, PLTs and TASs, which decrease with sepsis, and decreases biochemical changes (BUN, AST, ALT, LDH, TOS, and OSI), which increase with sepsis, to protect against sepsis. Compared with that in the CLP group, the severity of intestinal infiltration was significantly lower in the PcE + CLP group; however, the degree of epithelial damage in the PcE + CLP group was similar to that in the CLP group. In the PcE + CLP group, the crypt and villus lengths were greater, and the decrease in Paneth cell degranulation intensity was greater than that in the CLP group.

Conclusion: Additionally, the morphology of the cells in the PcE + CLP group was similar to that in the sham group. PcE has potential as a prophylactic agent for sepsis.

Keywords: Oxidative stress; parsley; Petroselinum crispum; rat; sepsis

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Corresponding author:
Yahya Atayan
E-mail: yahyaatayan@hotmail.com

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Introduction

Sepsis is an irregular and excessive systemic inflammatory response to an infection. Despite improvements in treatments, it has a high mortality rate and requires early intervention (Neviere et al., 2017). The mortality rate for sepsis is approximately 30%; mortality increases as it progresses from severe sepsis to septic shock and multiple organ failure syndromes (Martin et al., 2003). One factor contributing to the pathogenesis of sepsis is excess production of free oxygen radicals. Mitochondria are most susceptible to damage by oxidation. The release of free oxygen radicals eliminates the membrane potential and disrupts membrane integrity (Bone, 1991; Cohen, 2002; Zhou et al., 2015).

Cecal ligation and puncture (CLP) in rats mimics the characteristics and course of clinical sepsis (Hubbard et al., 2005). The CLP model can be used to vary the severity of sepsis by controlling the size of the perforation (Walley et al., 1996).

Petroselinum crispum, also known as parsley, can be grown almost anywhere and has been used traditionally for the treatment of many maladies. Apigenin, cosmosiin oxypeucedanin hydrate, myristicin, apiol, cnidil and apiin have been detected in aqueous extracts of Petroselinum crispum leaves.

Additionally, coumarins, carotenoids and other compounds are present in various parts of parsley plants (Farzaei et al., 2013). Some compounds in parsley exhibit antioxidant properties (Wong and Kitts, 2006).

We investigated the protective effects of Petroselinum crispum extract on preventing the formation of free oxygen radicals and oxidative stress due to sepsis.

Methods

Animals

The Ethics Committee for Experimental Animal Research approved our study (Reference no. 2015/A-34). Our study was performed according to the principles of the Animal Research Guidelines of the International Health Board in the Laboratory of Experimental Animal Production (EAP) (https://www.ncbi.nlm.nih.gov/books/NBK54044/) with the permission of the Ethics Committee of Experimental Animals (We used 32 180–220 g female *Wistar albino* rats obtained from the EAP Center. The rats were housed at 21 ± 2 °C and 60 ± 5% humidity with a 12 h light:12 h dark cycle. Before the experiment, the rats were provided standard feed and water ad libitum. Experimental animals that did not develop sepsis with cecal ligation were excluded from the study.

Cecal ligation and puncture (CLP)

The sepsis model was created via CLP. A 1 cm midline incision was made in the abdominal wall under anesthesia.

The cecum was exposed and ligated with a 4–0 silk suture and then punctured twice with a 22-gauge needle. A small amount of fecal content was released by gently squeezing the cecum and then repositioning it with a 4–0 silk suture. After the procedure, 1 ml of saline solution (SS) was administered subcutaneously to the animals in each group. After sepsis symptoms, including lethargy, fever, piloerection and diarrhea, were observed after 24 h, the animals were euthanized by bleeding after a 5 ml blood sample was collected from the inferior vena cava, and the experiment was terminated (Toscano et al., 2011).

Experimental design

The rats were allocated to our groups of eight. 1) Sham-operated group. 2) Petroselinum crispum extract (PcE) group: 2 g/kg PcE was administered once daily for 14 days by orogastric gavage. 3) CLP group. 4) In the PcE + CLP group, after the application of 2 g/kg PcE by gavage once daily for 14 days, the rats were septic after CLP.

Tissue samples

On day 15, under anesthesia with 100 mg/kg ketamine

Table 1. Comparison of the Serum Hemogram and Biochemistry Parameters among the Study Groups

Parameters	Groups				
	Sham	PcE	CLP	PcE+CLP	*p* value
WBC (10^9/µL)	5.69 (2.31-9.92)	6.35 (3.71-14.04)	4.09 (3.26-9.76)	4.36 (2.36-10.03)	.4073
HGB (g/dL)	15.9 (13.9-18.4)	15.1^{b} (14.1-16)	18.7^{c} (14.7-20.9)	14.55 (13.3-18.2)	**.0301**
LY (%)	64$^{b.c}$ (35.4-71.9)	64.7$^{b.c}$ (31.8-85.2)	21.3 (14.7-40.3)	20.8 (10-41.7)	**.0002**
MO (%)	4.6^{b} (3.5-8.9)	3.4$^{b.c}$ (2.6-23.2)	9.4 (5.5-46.2)	18.05 (2.8-32)	**.015**
NE (%)	31.2$^{b.c}$ (24.6-55.3)	31.6$^{b.c}$ (11.8-50.1)	49.9 (31.4-75.5)	54.55 (42.4-77.6)	**.003**
EO (%)	0.2 (0-0.3)	0.1 (0-7.7)	0 (0-1.3)	0.1 (0-5.7)	.5803
BA (%)	0.2 (0-0.3)	0.2 (0-0.4)	0.3 (0-0.5)	0.2 (0-0.3)	.6258
PLT (10^9/µL)	683 (2,4-910)	764 (675-875)	623 (418-1395)	647 (51-856)	.3103
Glucose (mg/dL)	114 (49-170)	78 (62-132)	32 (12-151)	62.5 (32-114)	.1485
BUN (mg/dL)	18.63(12.06-4.17)	14.82$^{b.c}$(12.75-16.04)	67.32(12.89-22.09)	49.65(12.94-08.52)	**.0307**
Creatinine (mg/dL)	0.54^{a} (0.47-0.63)	0.45$^{b.c}$ (0.42-0.51)	0.69 (0.49-1.24)	0.54 (0.45-0.92)	**.0013**
AST (U/L)	23.8^{b} (18.9-65.3)	44.3 (26.9-71.8)	70.6 (22.0-177.3)	47.1 (16.7-72.3)	**.0388**
ALT (U/L)	40^{b} (27-82)	51^{b} (39-83)	197 (28-1060)	127 (24-287)	**.0339**
LDH (U/L)	186.8^{b} (171.0-258.8)	190.2^{b} (110.8-274.6)	332.5 (166.4-332.5)	247.9 (147.9-332.5)	**.0213**

Results are expressed as median (min–max). n= 8.
a: *p*<.05 versus PcE group. b: *p*<.05 versus CLP group . c: *p*<.05 versus PcE+CLP group.

Table 2. Comparison of the tissue TAS, TOS and OSI among the study groups in terms of intestinal tissue

Parameters	Groups				
	Sham	PcE	CLP	PcE+CLP	*p* value
TAS (Trolox Eq/L)	0.94 (0.79-1.08)	0.95 (0.78-1.12)	0.91 (0.83-1.14)	1.05 (0.95-1.17)	.0654
TOS (µmolH2O2 Eqv/L)	5.43 (4.3-5.85)	8.46 (5.35-12.61)	8.46 (5.35-10.5)	8.31 (5-11.98)	.0575
OSI (Arbitrary unit)	5.52 [a]-(4.36-6.45)	8.19 (4.96-10.98)	8.89[b](6.06-5.01)	7.38 (4.29-12.55)	.0405

The results are expressed as the median (min–max), n= 8.
a: *p*<.05 versus the CLP group, b: *p*<.05 versus the PcE+CLP group

and 10 mg/kg xylazine, the skin was shaved and sterilized with iodine, and laparotomy was performed. The rats were sacrificed by extracting 5 ml of blood from the inferior vena cava 24 h after CLP. The characteristics measured included a hemogram, LDH, AST, ALT, BUN, creatinine, glucose, bicarbonate, CRP and ASO. A 10 cm portion of intestinal tissue from the ascending colon, including the cecum, was placed in a 10% formaldehyde or histopathological examination; the remaining portion was frozen in liquid nitrogen and stored in a -35 °C freezer until biochemical measurements were performed. Two hundred milligrams of frozen tissue samples cut into pieces on dry ice were homogenized in PBS buffer (1:9, w/v) via a manual glass homogenizer for approximately 5 min and flushed with centrifugation at 3500 × g for approximately 45 min to remove large debris. The supernatant was used for TAS and TOS analysis.

Total antioxidant status (TAS)

TAS was measured via the automated colorimetric measurement method Biotek Synergy HT plate reader immunostimulant gene 5 software and a Rel Assay (Rel Assay Diagnostics Kit, Mega Tip, Gaziantep, Türkiye) developed by Erel (Erel, 2004b). The hydroxyl radical is produced by the Fenton reaction and reacts with the colorless substrate, O-dianisidine, to produce the dianisyl radical, which is bright yellowish brown. Upon the addition of tissue samples, the oxidative reactions initiated by the hydroxyl radicals in the reaction mixture are suppressed by the antioxidant components of the sample, which prevents the color change from providing a measure of the total antioxidant capacity of the sample. The antioxidants in the sample increase the color in proportion to their concentration. The reaction can be monitored spectrophotometrically, and the opening ratio in color is proportional to TAS for the sample. The assay is calibrated with a stable antioxidant standard solution, which is traditionally referred to as Trolox equivalent, which is a vitamin E analog. The results are expressed as mmol Trolox equiv/L.

Total oxidant status (TOS)

TOS measurements were performed via a TOS Biotek Synergy HT plate reader with immunostimulant gene 5 software and a Rel Assay (Rel Assay Diagnostics Kit, Mega Tip, Gaziantep, Türkiye) developed by Erel. Oxidants in the tissue samples oxidize the ferrous ion–O-dianisidine complex to ferric ions. The oxidation reaction is enhanced by glycerol molecules in the reaction medium. Ferric iron produces a colored compound with a chromogen in an acidic medium. This chromogen is orange and a reagent. The assay kit is calibrated in a similar manner. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The assay was calibrated using H_2O_2, and the results are expressed as µmol H_2O_2 equiv/l.

Oxidative stress index (OSI)

The percent ratio of TOS to TAS is the oxidative stress index (OSI), an indicator of the degree of oxidative stress (Erel, 2004a, 2005). To perform the calculation, the resulting unit of TAS, mmol Trolox equivalent/L, was converted to µmol equivalent/L, and the OSI value was calculated via the following formula:

OSI (arbitrary unit) = [(TOS, µmol/l)/(TAS, mmol Trolox equivalent/l) × 100].

Histopathology

The tissue samples were fixed with 10% formaldehyde. After the tissue was subjected to follow-up procedures, the samples were dehydrated through 80%, 95% and absolute ethyl alcohol, cleared in xylene, and embedded in paraffin, and the tissue sections were cut into 4–5 µm thick paraffin

Table 3. Histopathological score findings

Groups (n= 8)	İnfiltration	Epitelial damage
Sham	0.0 (0.0-1.0)	0.0 (0.0-2.0)
PcE	0.0 (0.0-1.0)	0.0 (0.0-2.0)
CLP	0.0 (0.0-3.0)[a]	0.0 (0.0-3.0)[a]
PcE+CLP	0.0 (0.0-2.0)[b]	0.0 (0.0-3.0)

The results are expressed as the median (min–max). n= 8.
[a]*p*<.05 versus the Sham group. [b]*p*<.05 versus the CLP group.

Table 4. Average crypt debth, villus length (AM ± SD), severity of caspase-3 immunoreactivity and Paneth cell degranulation severity in each group.

Groups (n= 8)	Crypt debth (µm)	Villi length (µm)	Caspase-3 immunoreactivity	Paneth cell degranulation severity
Sham	166.25±36.02	260.52±50.05	6.0 (2.0-12.0)	1.0 (0.0-3.0)
PcE	165.90±37.49	281.55±42.46	6.0 (2.0-12.0)	0.0 (0.0-3.0)
CLP	147.05±36.41[a]	237.13±49.70[a]	3.0 (1.0-12.0)[a]	2.0 (0.0-3.0)[a]
PcE+CLP	175.02±34.33[b]	264.61±49.84[b]	6.0 (2.0-12.0)[b]	1.0 (0.0-3.0)[b]

AM ± SD: Arithmetic mean ± Standard deviation
The results are expressed as the median (min–max). n= 8.
[a]$p<.05$ versus the Sham group. [b]$p<.05$ versus the CLP group.

blocks. The sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) (Bancroft & Gamble, 2008) for examination of morphology. Sections of the intestinal mucosa were examined for epithelial spillage and mononuclear cell infiltration. Alterations in structure were evaluated via a semiquantitative method according to the following scale: 0 (absent), 1 (mild), 2 (moderate), and 3 (severe) (Taslidere et al., 2018). Twenty villus and crypt lengths were measured in each section. Additionally, 100 Paneth cells/section were evaluated for degree of degranulation (Adolph et al., 2013). Analyses were performed via the Leica Q Win Image Analysis System (Leica Micros Imaging Solutions Ltd., Cambridge, UK) with a Leica DFC-280 research microscope.

Immunohistochemistry

After deparaffinization and rehydration, the sections were placed in citrate buffer (pH 6.0) and antigen retrieval solution and boiled in a pressure cooker for 20 min. Then, the sections were washed with phosphate-buffered saline (PBS). After washing, 3% hydrogen peroxide solution was applied to block endogenous peroxide for 12 min at room temperature, after which the samples were washed with PBS. A protein blocker was applied to the sections to prevent nonspecific background staining. The sections were incubated with a caspase-3 primary antibody (rabbit polyclonal; Thermo Fisher Scientific, Anatomical Pathology, Fremont, USA) for 60 min, rinsed with PBS, and then incubated with biotinylated goat anti-polyvalent for 10 min and streptavidin-peroxidase for 10 min at room temperature. Staining was completed with chromogen (AEC substrate system; AEC chromogen and AEC substrate; 20 µl of AEC chromogen was added to 1 ml of AEC substrate) (Thermo Fisher Scientific, Anatomical Pathology, Fremont, USA) + substrate for 10 min, followed by counterstaining with Mayer's hematoxylin for 1 min, rinsing in tap water and dehydration. Caspase-3-positive cells were stained brown.

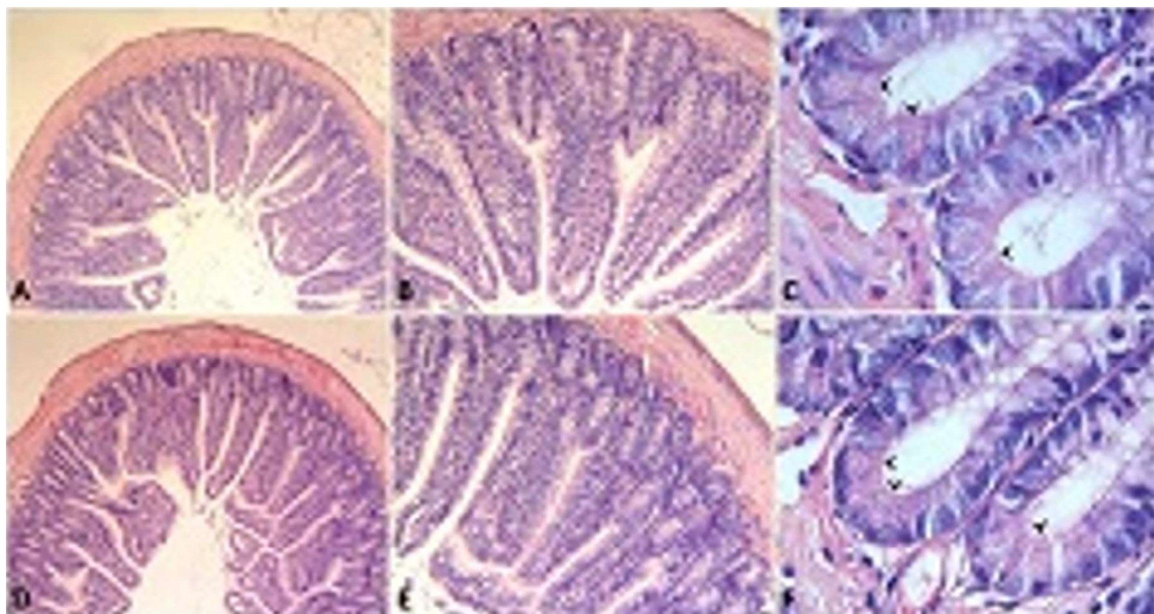

Figure 1. Paneth cells were characterized by eosinophilic granules located at the base of the intestinal tissue and crypts with a normal histological appearance in the Sham (A; 10×, B; 20×, C; 100×) and PcE (D; 10×, E; 20×, F; 100×) groups. Arrowheads indicate Paneth cells.

The sections were graded according to the degree of staining as follows: 1 = 0–25% staining; 2 = 26–50% staining; 3 = staining 51–75%; and 4 = staining 76–100%. The sections were graded according to their staining intensity as follows: 0 = no staining; 1 = weak but detectable staining; 2 = distinct; and 3 = intense staining. The total staining score was obtained as (prevalence)X(intensity) (Parlakpinar et al., 2019). All the sections were evaluated via a Leica DFC280 light microscope and a Leica Q Win image analysis system (Leica Micros Imaging Solutions Ltd., Cambridge, UK).

Statistical analyses.

IBM SPSS statistics version 17.0 for Windows was used for the statistical analyses. Normally distributed data were analyzed by ANOVA (Tamhane). For nonnormally distributed data, comparisons between groups were performed with the Kruskal–Wallis H test. After the Kruskal–Wallis H test, multiple comparisons were made via the Conover test. The results are expressed as the median (minimum–maximum) and mean ± standard deviation. Values for $p \leq .05$ were considered statistically significant.

Results

Biochemistry

Significant increases in the Hb, lymphocyte, neutrophil, BUN, Crea, ALT, AST and LDH levels were detected between the CLP, PcE+CLP and sham groups ($p<.05$), but no significant differences were detected between the CLP and PcE+CLP groups (Table 1).

Compared with those in the sham group, a significant increase in only the OSI was detected in the TOS, TAS and OSI in the CLP group ($p<.05$), but no significant difference in any parameter was observed between the CLP and PcE+CLP groups (Table 2).

Histopathology

The Sham and PcE groups presented a normal histological appearance of the intestinal mucosa, except for slight epithelial spillage and infiltration, which were observed in a few sections (Figure 1A, B and D, E). The crypta length was 166.25 ± 36.02 µm in the sham group and 165.90 ± 37.49 µm in the PcE group; the villus length was 260.52 ± 50.05 µm in the sham group and 281.55 ± 42.46 µm in the PcE group. Paneth cells were noted for the presence of densely packed eosinophilic granules in the Sham and PcE groups (Figure 1C, F).

The severity of epithelial spillage and infiltration was significantly greater in the CLP group than in the sham group ($p<.05$) (Figure 2A, B). In this group, the mean length of the crypta (147.05 ± 36.41 µm) and the length of the villus (237.13 ± 49.70 µm) were also significantly shorter

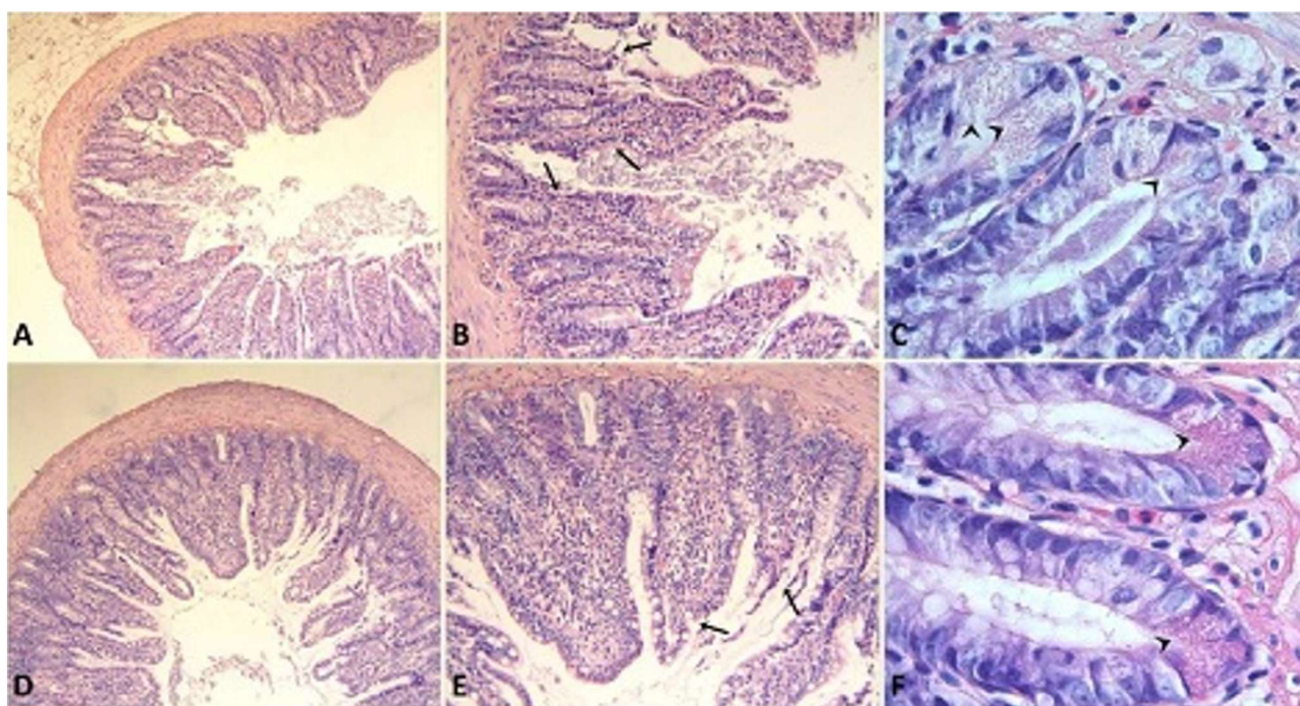

Figure 2. Appearance of villi, crypts and Paneth cells in the CLP (A; 10×, B; 20×, C; 100×) and PcE + CLP (D; 10×, E; 20×, F; 100×) groups. The arrows indicate epithelial damage, and the arrowheads indicate Paneth cells.

than those in the Sham group (*p*<.05). Notably, in the CLP group, Paneth cells had a hypertrophic appearance, and the granule content in their cytoplasm was significantly lower than that in the Sham group (*p*<.05) (Figure 2C, Table 4).

The severity of infiltration was significantly lower in the PcE+ CLP group than in the CLP group (*p*<.05); however, epithelial spillage in the PcE+ CLP group was similar to that in the CLP group (Figure 2D, E). In this group, the length of the crypta (175.02 ± 34.33 µm) and the length of the villus (264.61 ± 49.84 µm) were significantly greater than those in the CLP group (*p*<.05). The severity of Paneth cell degranulation was significantly lower in the PcE+CLP group than in the CLP group, and the appearance of Paneth cells was similar to that in the control group (*p*<.05) (Figure 2F, Table 4).

Immunohistochemistry

Caspase-3 immunoreactivity was observed in surface and gland epithelial cells. The intensity of caspase-3 immunoreactivity was similar in the sham and PcE groups (Figure 3A, B). In contrast, we detected decreased caspase-3 immunoreactivity in the CLP group compared with the sham group (P = 0.001) (Figure 3C). The intensity of caspase-3 immunoreactivity in epithelial cells was significantly greater in the PcE+CLP group than in the CLP group (P = 0.001) (Figure 3D). Caspase 3 expression levels are summarized in Table 4.

Discussion

Sepsis is a complex, destructive condition accompanied by high mortality and limited treatment options. Despite improved surgical techniques and intensive care treatments, sepsis-related mortality rates remain high and continue to be a significant cause of hospital death (Iwashyna et al., 2012; Jones et al., 2008; Rodríguez et al., 2011). For these reasons, new and effective treatment methods for sepsis are highly important.

Multiple organ failure is the usual cause of death from sepsis. Lysosomal enzymes and free oxygen radicals, especially those released by neutrophils, participate in the pathogenesis of organ failure (Reinhart et al., 2005). Various antioxidants have been used to prevent the accumulation of free oxygen radicals, which contribute to mortality due to sepsis.

İn vitro and *in vivo*, extracts of Petroselinum crispum have been reported to possess antioxidant properties (Popović et al., 2007; Zhang et al., 2006); however, we have found no reports of its protective effects against sepsis. We

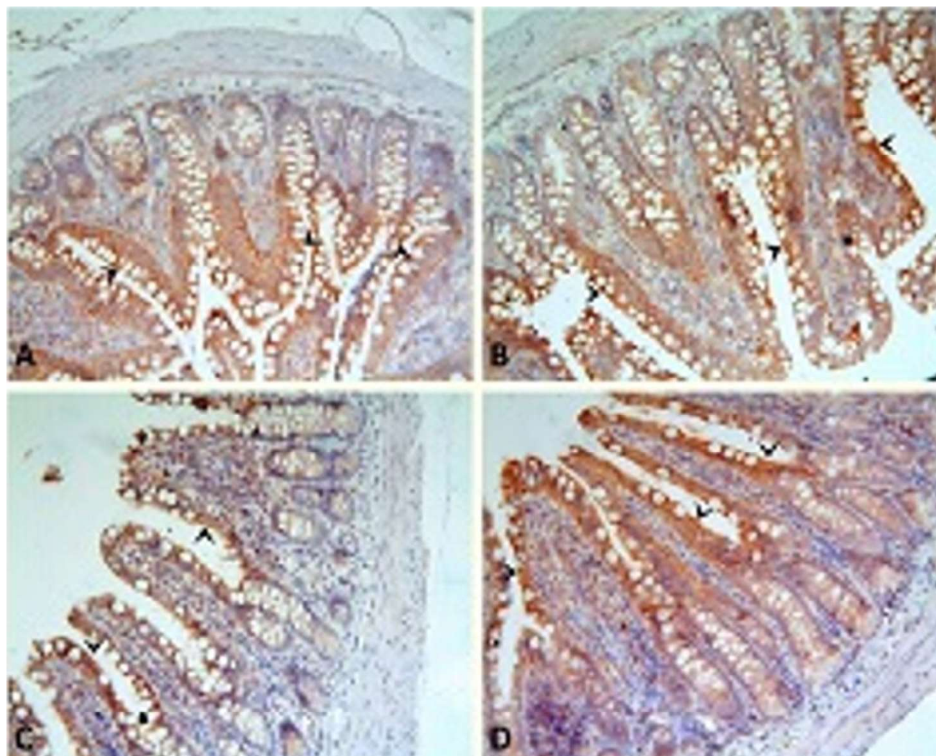

Figure 3. Similar caspase-3 immunoreactivity was observed in the sham (A) and PcE (B) groups. In the CLP group (C), a decrease in caspase-3 immunoreactivity was observed. On the other hand, a significant increase in caspase-3 immunoreactivity was noted with PcE treatment (D). Caspase-3 immunostaining; magnification ×20.

used CLP-induced sepsis as our model. Sepsis was verified by hemogram (neutrophils, lymphocytes), biochemical (AST, ALT, LDH, BUN, TAS, TOS and OSI levels) and histopathological changes, which are used to confirm sepsis. In the comparison between the sham and CLP groups, an increase in the OSI level indicates that CLP increases oxidative stress, whereas a decrease in the OSI between the CLP and PcE+CLP groups indicates that PcE reduces sepsis-induced oxidative stress.

There was no significant decrease in TAS in intestinal tissue extracts in the CLP group compared to the Sham group. The increase in the PcE+CLP group compared to the CLP group was not statistically significant. CT was significant in that it showed that CLP triggered sepsis and PcE reduced it., the increase in the TAS in the PcE+CLP group compared with that in the CLP group was not statistically significant, indicating that CLP induced sepsis, whereas PcE reduced it. Previous studies have shown that the levels of antioxidant markers are decreased in sepsis via CLP. In a study by Ritter et al., the activity of antioxidant enzymes such as catalase and superoxide dismutase was reduced in essential organs involved in the septic response (Ritter et al., 2004). reported an increase in the glutathione level in parsley extract. These findings suggest that parsley extract has a protective effect due to its antioxidant properties. These previous studies revealed that sepsis increases oxidative stress and that the activity of the antioxidant system decreases. In the present study, this situation was shown by the changes in TOS, OSI and TAS levels. We did not identify these genes individually but rather identified them as oxidative stress markers (TOS, OSI) and antioxidant markers (TAS).

Oxygen-free radicals, including malondialdehyde (MDA), hydrogen peroxide and hydroxyl radicals, cause peroxidative stress. Reduced antioxidative defenses, such as superoxide dismutase, catalase, and glutathione (GSH), also contribute to oxidative stress. Then, organ failure occurs (Koksal et al., 2004; Ozsoy-Sacan et al., 2006). In this study, parsley extract had a protective effect against CLP, with biochemical and histopathological analyses showing that it reduces multiorgan damage. This protective effect is primarily due to the inhibition of oxidative stress, which is one of the most important mechanisms of organ injury in sepsis. In our current study, the administration of parsley extract before CLP-induced sepsis increased neutrophil counts, PLTs and TASs, which decrease with sepsis, and decreased biochemical changes (BUN, AST, ALT, LDH, TOS, and OSI), which increase with sepsis, to protect against sepsis.

Histopathological changes revealed that epithelial damage and infiltration severity increased, whereas crypt–villus length, Paneth cell hypertrophy, and cytoplasmic granules decreased in the CLP group, suggesting sepsis syndrome. These changes have been confirmed by previous studies (Leng et al., 2014). The intestinal histological changes, including significantly reduced infiltration severity, were reversed by the use of PcE. This finding revealed the protective effect of PcE on the intestinal mucosa in sepsis. In the present study, we evaluated apoptosis via caspase-3 activity. Compared with that in the sham group, caspase-3 activity was significantly lower in the CLP group and significantly greater in the PcE + CLP group than in the CLP group, possibly because parsley extract has apoptosis-inducing activities and protective effects. The histopathological and biochemical results obtained in this study were similar to those of previous studies (Sęczyk et al., 2016; Shukla & Gupta, 2010).

As shown in previous studies, in this study, the decrease in the TOS and OSI levels, as well as their histopathological changes, especially a reduction in intestinal infiltration, demonstrated the antioxidant properties of PcE (Al-Juhaimi & Ghafoor, 2011; Fejes et al., 2000). In further research, we believe that the content of parsley extract will be elucidated in more detail via chromatographic methods and that the active substances or plant-prepared bioactive fractions isolated from the plant will offer alternative treatment options.

As a result, especially in hospitalized patients or other patients at risk for sepsis, we believe that our research may constitute the first step for the development of drugs that can reduce the risk of sepsis and facilitate treatment.

Ethics Committee Approval: The Ethics Committee for Experimental Animal Research approved our study (Reference no. 2015/A-34).
Peer-review: Externally peer-reviewed.
Author Contributions: Concept - H.P., Y.A.; Design- Y.F.C, A.P., H.P.; Supervision- H.P., Y.A.; Resources- Y.A., H.P.; Data Collection and/or Processing- O.O., I.K., N.V., K.T., A.K.A.; Analysis and/or Interpretation- O.O., I.K., N.V., K.T., A.K.A.; Literature Search- Y.A.; Writing Manuscript- Y.A.; Critical Review- H.P., Y.A.
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