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Nigella sativa AS A POTENTIAL THERAPY FOR THE TREATMENT OF LUNG INJURY CAUSED BY CECAL LIGATION AND PUNCTURE-INDUCED SEPSIS MODEL IN RATS

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Abstract

We investigated the potential protective effects of *Nigella sativa* (NS) on mortality, serum levels of proinflammatory cytokines, oxidative stress and histopathological changes in lung tissues, in cecal ligation and puncture (CLP)-induced sepsis model in rats. Sepsis induction by CLP, determination of serum cytokine levels by ELISA, spectrophotometric determination of oxidative stress parameters, and histological examination of lung tissues. The rat groups were: 1) CLP group, 2) sham group, 3) NS500-sham group, 4) NS125, 5) NS250, 6)NS500 groups. NS treatment significantly decreased proinflammatory cytokine levels in serum; LPO level, MPO activity, and pathological changes in lung tissues, in CLP-induced sepsis, while significantly increasing GSH levels and SOD activity in the lung tissue. NS treatment after CLP potentially reduced mortality and may exert effects through the reduction in tissue oxidative stress and serum cytokines. The histopathological changes were minimized in lung tissue by NS, under sepsis conditions. We can suggest that NS reverses the systemic inflammatory reaction to polymicrobial sepsis and thereby reduces multiple organ failure. It may be suggested that role of the NS ethanolic extract in preventing formation of CLP induced sepsis, is due to the anti-inflammatory and antioxidant effects of the different compounds of the black seeds.

Key words: *Nigella sativa*, Polymicrobial sepsis, CLP, Cytokines, Oxidative stress, Rat.

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INTRODUCTION

Sepsis or septic shock, systemic inflammatory states resulting from the systemic response to severe bacterial infection, are complications that constitute major causes of morbidity and mortality for patients with severe trauma or blood loss (5, 15). In sepsis, many factors are involved in the pathogenesis characterized by progressive metabolic acidosis, multi-organ failure including the endocrine and systemic inflammatory response syndrome (SIRS), tissue damage, endothelial injury, genetic susceptibility, acute respiratory distress syndrome (ARDS) and/or acute lung injury (ALI) and even death (13, 33, 34). Sepsis is still one of the most prevalent causes of morbidity and mortality in the intensive care units and at hospital discharge all around the world (19). In sepsis, increased vascular permeability of the endothelium occurs with multiple organ dysfunction, leading to plasma extravasations, and the subsequent bacterial translocation may play an important role in the development of multiple tissue injury (43). In multiple tissue injury, the similarity of inflammatory response, despite the variety of underlying etiologies, increased evidence including the production of proinflammatory cytokines, particularly early tumor necrosis factor-α (TNF-α) and interleukins, vasoactive mediators and reactive oxygen species in the immuno-inflammatory process common to both SIRS and sepsis (32, 36). In sepsis, one of the important mechanisms, particularly in cecal ligation and puncture (CLP, a model of polymicrobial sepsis) induced sepsis multiple tissue damage increases with the resultant state of oxidative stress, due to the generation of free radicals and other reactive oxygen species. Oxidants are known to play a major role in inflammation and multiple tissue injury (28). Under normal physiological conditions, a homeostatic balance exists between the formation of oxygen or nitrogen species and their removal by endogenous antioxidant scavenging compounds (17). If homeostatic balance is impaired, overproduction of oxygen free radicals occurs. Thus, the natural scavenging mechanisms, which are the processes that are implicated in microvascular dysfunction, are unbalanced and followed by organ dysfunction including essential organs, such as the lung, kidney, liver, and heart (15, 28, 36). It has been reported that during septic shock as well as ARDS and ALI, which are characterized by the accumulation of a large number of neutrophils in the lungs, generation of reactive oxygen

species (ROS) and the production of cytokines increase (2, 45). Neutrophil accumulation is particularly remarkable in the lungs. Traditional treatment models have not successfully managed ARDS and ALI (26). There are lots of experimental studies such as antioxidant and plant origin drugs, which investigated sepsis management.

The seeds of Nigella sativa (NS) (family Ranunculaceae), commonly known as black seed or black cumin, have been used as a natural remedy for a number of diseases and conditions such as asthma, cough, bronchitis, headache, eczema, fever, dizziness, influenza, lung inflammation; have contraceptive and anti-inflammatory effects; and have been consumed as condiment (3, 10, 21, 23, 46). NS contains fixed and volatile oil. The volatile oil has been shown to contain thymoquinone and monoterpenes such as p-cymene and alpha-pinene (46). The seed contains alkaloids and glycoside flavonoids such as quercetin, kaempferol and quercetin-3. Recently, clinical and animal studies have shown that an extract of NS has many therapeutic effects, including anti-proliferative, anti-inflammatory, antitumor, anti-cancer, multiple myeloma, antibacterial, estrogenic and antioxidant activity (6, 9, 14, 25, 27, 30, 35, 38, 44). It has been also reported that the crude NS seeds did not produce any adverse side effects in the doses tested (47).

In this study, we induced sepsis/septic shock in rats with CLP, a model of polymicrobial sepsis, and hypothesized that ethanol extract of Nigella sativa could prevent CLP-induced tissue injury in the lungs, through the proinflammatory cytokine response and the ROS generation triggered by polymicrobial sepsis in rats.

MATERIALS AND METHODS

Animals

In the present study, a total of 120 male Wistar rats were used for the experiments. Each rat weighed 230–250 g, and all were obtained from Ataturk University's Experimental Animal Laboratory of Medicinal and Experimental Application and Research Center (ATADEM). Animal experiments and procedures were performed in accordance with the national guidelines for the use and care of laboratory animals, and were approved by Ataturk University's local animal care committee. (No:B30.2.ATA.023.85-60) The rats were housed in standard plastic cages on sawdust bedding in an air-conditioned room at 22±1°C under lighting controls (14 h light/10 h dark cycle). Standard rat food and tap water were given ad libitum.

Chemicals

All of the chemicals used in our laboratory experiments were purchased from Sigma Chemical Co. (Germany).

Preparations of NS Extract

Nigella sativa seeds were purchased from a local herbalist in Erzurum. The seeds were botanically authenticated by Dr.Y. Karagoz. A specimen has been preserved at 4°C. The seeds (100 g) were cleaned, dried, mechanically powdered and extracted with 96% ethanol (300 mL x 3). The extracts were filtered, combined, and the solvent was evaporated under reduced pressure with a rotary to render the extract alcohol free that yielded a blackish-brown concentrate liquid (yield % 25) and kept in a domestic refrigerator at 4°C. Extract was emulsified in water and administered with oral gavage.

Experimental Design

The rats were allocated into six groups, each composed of 20 individual rats: I. CLP group, II. sham group, III. 500 mg/kg NS-sham group (NS500-Sham), IV. 125 mg/kg NS-treated CLP group (NS125), V. 250 mg/kg NS-treated CLP group (NS250), VI. 500 mg/kg NS-treated CLP group (NS500). The groups were housed separately in different cages.

Sepsis Model

A CLP polymicrobial sepsis model was applied to the rats. Polymicrobial sepsis was induced through cecal ligation and two-hole puncture. Anesthesia was induced through intraperitoneal administration of thiopental 25 mg/kg. After the abdomen was shaved, the peritoneum was opened. Once the diaphragm exposed the abdominal organs, the cecum was isolated and ligated with a 3/0 silk ligature just distal to the ileocecal valve. Two punctures were made with a 12-gauge needle through the cecum distal to the point of ligation, and the cecum was returned to the peritoneal cavity. The abdominal incision was then closed with a 4/0 sterile synthetic, absorbable suture. The wound was bathed in 1% lidocaine solution to ensure analgesia. The sham-operated groups received laparotomies, and the groups' cecum were manipulated, but not ligated or perforated. All of the animals were given 2 ml/100 g body weight of normal saline subcutaneously at the time of surgery and 6 h after the operations, for fluid resuscitation. Immediately after the surgical procedure, the rats in the NS-sham (500 mg/kg) and the NS-treated CLP groups received 125, 250, and 500 mg/kg doses of NS suspended in saline, which were administered with an oral gavage (Table 1). An equal volume of saline was administered to the sham group and the CLP group. The rats were deprived of food postoperatively but had free access to water for the next 16 h until they were sacrificed. All six groups were sacrificed 16 h later with an overdose of a general anesthetic (thiopental sodium, 50 mg/kg). Cardiac blood samples were collected immediately and transferred to the laboratory to facilitate the estimation of the inflammatory cytokines, IL-1β, IL-6 and TNF-α levels in the serum. The lungs were rapidly removed from each sacrificed rat, left and right lobes were separated, all left lobes were washed in ice-cold saline and all right lobes were fixed in 10% formalin. The organs were labeled and stored at –80°C until the biochemical analysis was conducted.

IL-1β, IL-6 and TNF-α Cytokine Serum Measurements

Sera from the six rat groups were separated and stored at −80°C until they were thawed for the assay. IL-1β, IL-6 and TNF-α from each sample were measured with highly sensitive ELISA kits; Invitrogen-KRC0011 (Grand Island, USA), RayBiotech-ELR-IL6.001 (Norcross, USA), and Invitrogen-KRC3011 (Grand Island, USA), respectively. Kits were specifically designed for rat cytokines, and all measurements were performed according to the manufacturer's instructions. Cytokine assays for each animal and its correlated control were run in the same lot.

Biochemical Investigation of Lung Tissues

After macroscopic analyses, activities of superoxide dismutase (SOD) and myeloperoxidase (MPO); and amounts

of lipid peroxides (LPO) and glutathione (GSH) enzyme levels in the rat lung tissues were determined. To prepare the tissue homogenates, the tissues were ground with liquid nitrogen in a mortar. The ground tissues (0.5 g each) were then treated with 4.5 mL of the appropriate buffer. The mixtures were homogenized on ice using an Ultra-Turrax Homogenizer for 5 min. The homogenates were filtered and centrifuged using a refrigerated centrifuge at 4°C. These supernatants were then used to determine the enzymatic activity. The enzymatic activities have been recorded on a UV–vis spectrophotometer. All assays were performed at room temperature in triplicate. In the biochemical analyses, all tissue homogenates subjected to the same procedures that reduced standard variation rates to minimum.

Superoxide Dismutase (SOD) Activity

Measurements were made according to Sun et al. (21). SOD estimation was based on the generation of superoxide radicals produced by xanthine and xanthine oxidase, which react with nitro blue tetrazolium (NTB) to form formazan dye. SOD activity was then measured at 560 nm by the degree of inhibition of this reaction, and was expressed as millimole per minute per milligram of tissue (mmol / mg tissue per minute).

Myeloperoxidase (MPO) Activity

Myeloperoxidase activity was measured according to Bradley et al. (1982) with minor modification (22). The homogenized samples were frozen and thawed three times, and then centrifuged at 1500 g for 10 min at 4°C. MPO activity was determined by adding 100 µl of the supernatant to 1.9 ml of 10 mmol/L phosphate buffer (pH 6.0) and 1 ml of 1.5 mmol/L o-dianisidine hydrochloride containing 0.0005% (wt/vol) hydrogen peroxide. The changes in each sample's absorbance at 450 nm were recorded on a UV–vis spectrophotometer. MPO activity in all tissues was expressed as micromole per minute per milligram of tissue (µmol / mg tissue per minute).

Determination of Lipid Peroxidation (LPO)

LPO in tissue was determined by estimating the level of malondialdehyde (MDA) using the thiobarbituric acid test (23). The rats' tissues were promptly excised and rinsed with cold saline. To minimize the possibility of the interference of hemoglobin with the free radicals, any blood adhering to the mucosa was carefully removed. The corpus mucosa was scraped, weighed, and homogenized in 10 mL of 100 g/L KCl. The homogenate (0.5 mL) was added to a solution containing 0.2 mL of 80 g/L sodium laurylsulfate, 1.5 mL of 200 g/L acetic acid, 1.5 mL of 8 g/L 2-thiobarbiturate, and 0.3 mL of distilled water. The mixture was incubated at 98°C for 1 h. After the mixture had cooled, 5 mL of n-butanol:pyridine (15: l) was added. The mixture was centrifuged for 30 min at 4000 rpm. The supernatant was measured at 532 nm, and a standard curve was obtained using 1,1,3,3-tetramethoxypropane. The recovery was more than 90%. The results were expressed as nanomole of MDA per gram of tissue (nmol MDA / g tissue).

Total Glutathione (GSH) Determination

The amount of GSH in the tissues was measured according to Sedlak and Lindsay's method (24). The mucosal surface of the tissue was collected by scraping, and was then weighed and homogenized in 2 mL of 50 mM Tris–HCl buffer containing 20 mM EDTA and 0.2 M sucrose, pH 7.5. The homogenate was immediately precipitated with 0.1 ml of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4200 rpm for 40 min at 4°C. The supernatant was used to determine GSH using 5,5'-dithiobis (2-nitrobenzoic acid). Absorbance was measured at 412 nm using a spectrophotometer. The results of the GSH levels in the tissues were expressed as nanomoles per milligram of tissue (nmol / mg tissue).

Histological procedures

The lungs were fixed in a 10% buffered formalin solution for 48 hours. After paraffinization, deparaffinization, and rehydration, the sections were stained with hematoxylin and eosin (HE). All lungs were evaluated the semi-quantitative scoring system in a blind manner by histopathologists. Lung injury including interstitial edema, infiltration of polymorph nuclear leukocytes and monocytes, hemorrhage, vascular congestion, and cellular hyperplasia were semi-quantitatively scored. Each feature was graded as absent, mild, moderate, or severe, with a score of 0–3. The total scores were calculated as the sum of the scores given for each criterion.

Inflammation scoring

Sections were prepared from paraffin blocks of systematic and randomly sampled (16) lung pieces and stained with H&E. Because of the pattern of perivascular cell accumulation in acute inflammation, we designed a system to evaluate that. In the acute inflammation, the grading system was as follows: 0 = no or occasional cells; 1 = few loosely arranged cells; 2 = many cells in the peripheral parts of the perivascular space; and 3 = numerous cells in the perivascular space.

Statistical Analysis

Data for the serum cytokine levels and SOD, MPO, GSH, LPO levels and histological inflammation scoring in **s**ections were subjected to one-way analysis of variance (ANOVA) using SPSS 13.0 software. Differences among the groups were obtained using the Scheffe option and were considered significant at $p<0.01$. Significance between mortality rates were determined with the chi-square test and were considered significant at $p<0.01$. All the results were expressed as mean ± SD (Standard Deviation) for rats in each group.

RESULTS

Survival

Five (5/20 – 25%) rats survived between 12 and 16 h after CLP-induced sepsis in the CLP group, while 10 (10/20 – 50%) survived in the NS125 group and 11 (11/20 – 55%) in the NS250 group, finally 11 (11/20-55%) survived in NS500 group. The protective effects of all doses of NS were significant ($p<0.01$). No mortality was observed in the sham group or the NS500-sham group ($p<0.01$) (Table 1, Figure 1). We did not utilize samples from dead animals for serum examination and biochemistry. We selected all animals from CLP and NS-treated CLP groups to use for future serum examination and biochemistry experiments, and 10 from each of sham and NS sham groups.

Table 1. Experimental protocol groups of Sepsis and Survival Rates

	GROUPS	0th hour	1st hour	16th hour After creation of sepsis
1	**CLP**	Oral water	creation of sepsis	I.P. Thiopental Sodyum-50 mg/kg
2	**Sham**	Oral Water		
3	**NS500-Sham**	Oral NS 500 mg/kg		
4	**NS125**	Oral NS 125 mg/kg	creation of sepsis	
5	**NS250**	Oral NS 250 mg/kg	creation of sepsis	
6	**NS500**	Oral NS 500 mg/kg+	creation of sepsis	

NS: *Nigella sativa*, CLP: Cecal ligation and puncture

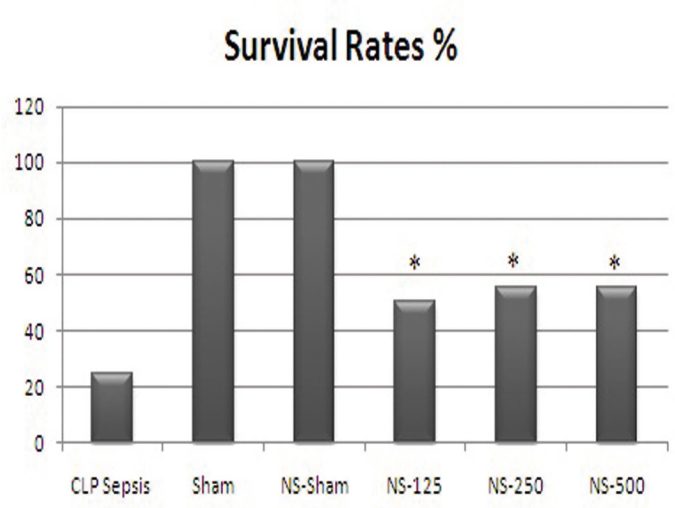

Figure 1. Survival rates (%) of the experimental groups. CLP sepsis group was compared to NS treated. * Significant at $p < 0.01$. Results are means. **NS**: *Nigella sativa*, **CLP**: Cecal ligation and puncture applied, **Sham:** sham-operated.

The Effects of Nigella sativa on Serum IL-1β, IL-6 and TNF-α Levels in Cecal Ligated and Puncture-induced Septic Rats

In the present study, the serum levels of inflammatory cytokines IL-1β, IL-6 and TNF-α were studied in the CLP model based on the sera from the rats (Table 2). The levels of cytokines were found to be higher in the CLP group when compared to the sham animals (shamgroup) ($p<0.01$). In contrast to the CLP group, the serum levels of IL-1β, IL-6 and TNF-α were found to be lower in treatment groups (NS125, NS250 and NS500) in the septic rats ($p<0.01$). 250 and 500 mg/kg doses of NS were more effective than 125 mg/kg in terms of decreasing serum levels of inflammatory cytokines. It is clear from the same figure that the administration of NS alone in the sham-operated rats did not affect the serum levels of cytokines when compared to the sham group.

Biochemical Results for Oxidant and Antioxidant Levels of Lung Tissue in Rats

The antioxidant levels (SOD and GSH) were evaluated in all lung tissues. In addition, the levels of oxidant parameters, such as lipid peroxidation levels and the enzymatic activity of MPO, were also evaluated in all lung tissues. The results, presented in Table 3, show that SOD activity and GSH levels were lower and the MPO and LPO levels were higher for the CLP-induced sepsis group than those of the sham rat group ($p<0.01$). All NS treated CLP groups had higher SOD activity and GSH levels in the lung tissues when compared to CLP group. In addition, all doses of NS were found to decrease the oxidant parameters (MPO and MDA) significantly when compared to the CLP group ($p<0.01$). As shown in Table 3, the administration of NS alone in the sham rats did not affect the oxidant and antioxidant levels of lung tissue when compared to the sham group ($p>0.01$).

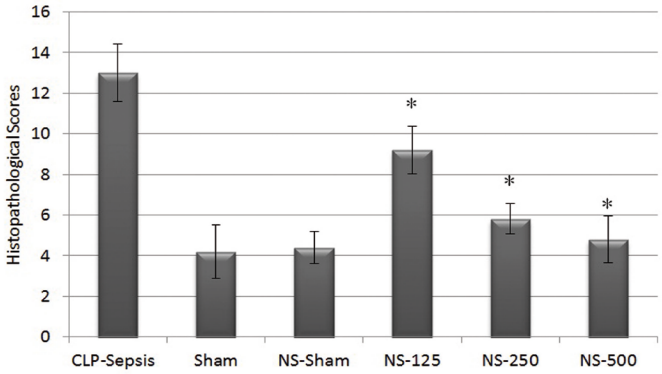

Figure 2. Histopathological scoring of lung injury on the total histology score. The sums of histology scores of the five parameters were calculated. CLP sepsis group was compared to NS treated. * Significant at $p < 0.01$. Results are means. **NS**: *Nigella sativa*, **CLP**: Cecal ligation and puncture applied, **Sham:** sham-operated.

Histopathological Changes of Lung Tissues

The general architectures of the lung in the sham and NS sham groups were of normal histological structure (Figure 3b, and 3c). There were also no statistically significant difference between both the sham group and NS500-sham group ($P<0.01$). However, lung tissue in the CLP-control and NS125 groups showed histopathological changes in the alveolar walls (Figure 3a and 1d). Also, interstitial edema, infiltration of polymorphnuclear leukocytes and monocytes, hemorrhage, vascular congestion, and cellular hyperplasia were observed. Inflammatory cell types were generally neutrophils and macrophages. But the lungs of the NS250 group and NS500 groups had normal histological structure, when compared with the CLP group (Figure 3a, 3e and 3f; Figure 2). According to total histology score, NS250 and NS500 groups had significantly reduced scores of lung injury induced by sepsis (Figure 3e, and 3f

Table 2. Effects of *Nigella sativa* (NS) treatments on changes in serum levels of interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in serum of rats.

GROUPS	IL-1 β (pg/ml)	IL-6 (pg/ml)	TNF-α (pg/ml)
CLP Sepsis	151.65 ± 17.14	860.52 ± 129.95	150.33 ± 26.23
Sham	42.78 ± 5.35*	331.40 ± 44.33*	45.46 ± 11.24*
NS500-Sham	48.80 ± 8.00*	335.20 ± 70.04*	48.29 ± 6.25*
NS125	80.35 ± 9.09*	530.32 ± 98.45*	93.95 ± 11.30*
NS250	76.83 ± 10.27*	502.30 ± 94.50*	81.47 ± 11.20*
NS500	78.22 ± 11.47*	488.29 ± 80.48*	77.09 ± 9.40*

CLP sepsis group was compared to NS treated. * Significant at $p < 0.01$. Results are means ± SD. **NS**: *Nigella sativa*, **CLP**: Cecal ligation and puncture applied, **Sham:** sham-operated.

Table 3. Effects of *Nigella sativa* (NS) treatments on changes in activities of myeloperoxidase (MPO), superoxide dismutase (SOD) and with levels of lipid peroxidation (LPO) and total glutathion (GSH) in lung tissues of rats.

GROUPS	SOD	MPO	GSH	MDA
CLP Sepsis	102.60 ± 6.63	92.14 ± 3.75	2.23 ± 0.11	37.65 ± 1.14
Sham	181.93 ± 7.85*	16.73 ± 1.23*	3.86 ± 0.17*	18.55 ± 1.36*
NS500-Sham	164.11 ± 3.41*	16.09 ± 1.34*	3.78 ± 0.16*	19.65 ± 1.25*
NS125	149.39 ± 2.62*	57.53 ± 5.82*	3.11 ± 0.04*	27.45 ± 0.57*
NS250	151.05 ± 7.56*	40.20 ± 3.73*	3.35 ± 0.10*	25.15 ± 0.96*
NS500	161.98 ± 7.21*	37.01 ± 3.68*	3.50 ± 0.11*	24.00 ± 0.83*

CLP sepsis group was compared to NS treated. * Significant at $p < 0.01$. Results are means ± SD. **NS**: *Nigella sativa*, **CLP**: Cecal ligation and puncture applied, **Sham:** sham-operated.

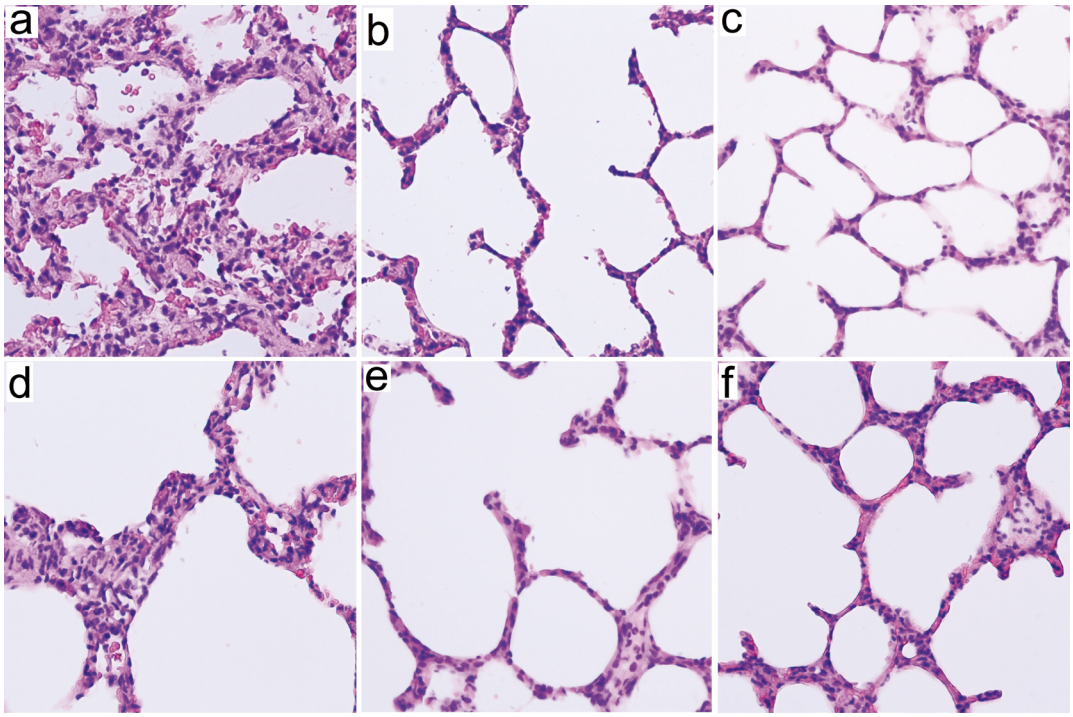

Figure 3. Morphologic features of the lungs tissue a) CLP Sepsis group, b) Sham group, c) NS500-Sham group, d) NS125 group, e) NS250 group, f) NS500 group. **NS**: *Nigella sativa*, **CLP**: Cecal ligation and puncture applied, **Sham:** sham-operated. Original magnification: 200X.

and Figure 2). The administration of NS125 had minimally protective effects on lung injury (Figure 3d) induced by sepsis. In NS250 and NS500 groups rats, the septic effects statically decreased when compared to CLP-sepsis group ($P<0.01$). So, there was no statistically significant difference in lung histological structures between sham group and especially NS250 and NS500 treated animals (Figure 3e, and 3f, Figure 2) ($P<0.01$).

DISCUSSION

Sepsis or septic shock is described as the presence of

an infection accompanied by evidence of a systemic response called the SIRS. Sepsis with its complications is still a major problem in medicine, affecting approximately 700,000 patients annually. Depending upon the standards of medical treatment, the world wide mortality rates in septic humans range from 30% to 70% (with an aggregate mortality rate of ≈ 50%) (5, 40). The current study investigated to find whether NS can provide protection against CLP (polymicrobial)-induced sepsis in rats. In this context, we investigated protective effects of NS extract in acute lung injury via determining the tissue levels of SOD, GSH, LPO, MPO, serum proinflammatory cytokines such as IL-1ß, IL-6, TNF- α and histopathological changes in lung sections.

There are previous studies on effects of thymoquinone obtained from *Nigella sativa* on sepsis (3, 4, 20). One of them reports protective effect of NS on oxidative injury of liver tissue and blood biochemical parameters in a model of LPS induced sepsis (20). The other one reports biochemical and immunological analyzes in a model of LPS *E. coli* induced sepsis (4). Our study differentiates from the mentioned studies at four major points. First, we used CLP model of sepsis, which is the most reliable and widely used among sepsis models (8, 40, 45). Second, our study tries to explain protective effect of NS based on changes of biochemical parameters of oxidative stress and histopathological changes in lung sections, and immunological parameters, including IL-1β, IL-6, TNF-α. Third, our study shows the dose dependent manner of protective effect of NS. Finally, the mentioned studies focused on thymoquinone, which is a volatile compound of NS seeds. We used ethanol extract of NS, major constituents of which include glycoside flavonoids such as kaempferol, quercetin, and quercetin-3 (1, 11, 18). Several studies report antioxidant activity of these compounds (18, 46). In short, our study reveals the effects of different compounds of NS and contributes to the literature about it.

Mortality is high in CLP induced-sepsis model (29, 40). Polymicrobial sepsis leads to a polymicrobial infection of the peritoneum in CLP model, which eventually results in bacteremia, ARDS, SIRS, sepsis, septic shock, severe blood loss and usually death. In the current study, the mortality rates at 16th hour were 75% in the CLP group, 45% in the NS250 and NS500 mg/kg groups, and 50% in the NS125 group. There was no mortality in the sham groups that received NS or saline.

Besides analysis of survival, the complex pattern of cytokine expression has been investigated extensively. Monocytes orchestrate the innate immune response to bacteria that follows tissue infiltration with immune cells, and the release of a cascade of pathophysiologically uncontrolled proinflammatory mediators occurs (32). Increased serum early release of proinflammatory cytokines is important in the pathogenesis of septic shock (7, 40). The main proinflammatory cytokines, IL-1β, IL-6 and TNF-α have been shown to increase following CLP. Further, it has been reported that high levels of IL-6 strongly correlate with survival after CLP, a phenomenon that also occurs in human sepsis (7, 22, 40, 41) Therefore, the IL-1β, IL-6 and TNF-α levels of serum in rats was measured. In the current study IL-1β, IL-6, and TNF-α in the serum of the CLP group significantly increased. These results were also in harmony with data from several other studies (4, 7, 12). NS treatment resulted in significant decreases in proinflammatory (IL-1β, IL-6 and TNF-α) cytokine levels following CLP-induced sepsis. These data suggest that the ability of NS given to rats to produce less inflammatory cytokines in response to CLP-induced sepsis may, in part, account for a significant increase in the survival of septic rats and a decrease in cytokine-related organ injury. It seems likely that the anti-inflammatory effect of NS in CLP-induced sepsis involves the suppression of a variety of proinflammatory mediators produced by the leukocytes and macrophages (46).

Under normal physiological conditions, a homeostatic balance exists between the formation of reactive oxygen and nitrogen species (ROS, RNS) and their removal by endogenous antioxidant scavenging compounds (17, 28). But during sepsis, oxygen free radicals are overproduced, the natural scavenging mechanisms are unbalanced, and the processes that are implicated in microvascular dysfunction are followed by organ dysfunction, which are characterized by the accumulation of a large number of neutrophils in the lungs, the increased generation of ROS and RNS (2, 37). Starkopf et al. showed an increase in the LPO levels and a decrease in serum antioxidant capacity by sepsis, in a clinical study (42). Similarly, others reported that LPO levels increased in septic shock induced by CLP in rats (24). In addition, MPO is a marker of accumulation of neutrophils during sepsis, as part of the acute inflammatory response (16). When stimulated, neutrophils produce excessive amounts of ROS from membrane bound NADPH oxidase which produces the oxygen free radical superoxide and hydroxyl radical (36). In our study, LPO levels and MPO activity increased in the lung tissue after CLP-induced sepsis, but NS attenuated this elevation in all tissues dose dependently. In addition to our biochemical data, a dose dependent decrease in septal neutrophils infiltration and septal-alveolar damage were observed in lung tissues of NS treatment rats when compared to septic rats in the histopathological evaluation. Therefore, we think NS may have prevented neutrophil accumulation and lipid peroxidation in the tissues by decreasing oxidative stress and protecting membrane permeability. Similar results related to the effects of NS on LPO and MPO have been reported in the literature (20, 46)

In parallel to increases in the MPO and MDA levels, the GSH levels were decreased in the lung tissue in CLP-induced sepsis group. GSH plays an important role in the maintenance of protein and lipid integrity, and provides major protection in oxidative injury against oxidative damage which Mandel et al. demonstrated that decreasing the level of GSH engaged with tissue injury induced by various stimuli (31). In the current study, the levels of GSH were significantly decreased in the lung tissue in CLP-induced sepsis group while NS dose dependently attenuated this elevation in all lung tissues. We suggest that the increase in the GSH level in the lung tissue may be a response of the lung tissue as an augmentation of antioxidant defense mechanisms against increased oxidative stress. Administration of NS immediately after the CLP procedure increased the lung GSH levels. These results suggest that NS attenuated the oxidative organ injury after CLP-induced sepsis by preventing oxidative stress. Lung SOD, LPO, MPO, and GSH concentrations correlated with serum proinflammatory cytokines (IL-1β, IL-6 and TNF-α) concentrations.

It is well known that SOD and GSH are important in-

dicators of the antioxidant capacity of the body that are resulted in protection against the damage caused with oxidative stress. Ritter et al. reported that SOD levels are markers of early mortality in septic rats (39). This may express why the SOD activity decreased in the septic rats in our current study. SOD activity increased in the lung tissues due to the administration of NS immediately after the CLP procedure, while CLP-induced sepsis group did not increase the activity of the SOD enzyme in lung tissues.

In conclusion; we demonstrated the ability of NS to reduce inflammatory cytokines such as IL-1β, IL-6 and TNF-α and ameliorate the negative alteration in the tissue levels of SOD, GSH, MPO, LPO and histopathological changes in lung sections under these conditions.

In light of these observations, we found that administration of NS prevented oxidative stress changes and decreased mortality induced by sepsis. It can be speculated that the role of the ethanolic NS extract in preventing formation of CLP induced sepsis, as seen in the present study, is in part due to the anti-inflammatory and antioxidant effects of the different compounds (qurecetin, kaempferol, etc.) of the black seeds. These compounds may interfere with antioxidant and anti-inflammatory mechanisms which may be crucial in reduction of CLP-induced lung dysfunction. Further prospective and randomized clinical controlled trials are required to better understand this novel candidate for use in treating sepsis and reducing sepsis-related mortality.

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