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Aqueous garlic extract protects against sepsis-induced toxicity in pulmonary and ileal tissues

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Abstract

Objective: Based on the potent antioxidant effects of aqueous garlic extract (AGE), the present study was designed to characterize the potential of AGE to modify blood coagulation parameters as well as and pulmonary and ileal injury in septic rats. Sepsis was induced using the caecal ligation and perforation (CLP) method.

Material and Method: Twenty-four hours after sepsisinduction, rats were decapitated and trunk blood was collected for the measurement of platelet counts, fibrinogen, prothrombin time, activated partial thromboplastin time (APTT) and d-dimer levels. Then, pulmonary and ileal tissue samples were immediately obtained and stored at-70 °C for malondialdehyde (MDA), glutathione (GSH), myeloperoxidase (MPO) and superoxide dismutase (SOD) activity assays.

Results: Sepsis was associated with a decrease in platelet count and fibrinogen and an increase in APTT and International normalized ratio. It also caused a significant decrease in GSH levels and SOD activity in both pulmonary and ileal tissue samples. On the other hand, AGE treatment in rats with CLP caused significantly augmented the level of these antioxidants. As a result of CLP induction increased MPO activity and MDA levels and decreased thromboplastic activity were reversed with AGE treatment.

Conclusion: AGE treatment, through its antioxidant effects, protects against oxidative pulmonary and ileal injury and normalizes the impaired coagulation in sepsis.

Keywords: Oxidative stress, thromboplastic activity, septic, rat, pulmonary injury, ileal injury

Introduction

Septic shock is an infectious complication in which toxins initiate an inflammatory response involving all systems. Therefore, it is defined as an excessive and irregular systemic inflammatory response to an infectious state, involving various organ systems, that leads to hemodynamic changes, and ultimately results in shock, organ failure or even death. Excessive production of reactive oxygen species (ROS) by activated immune cells causes oxidative damage, which is thought to play a significant role in the pathogenesis of sepsis induced organ damage (1,2). These radicals lead to lipid peroxidation, impair cell membranes, and give rise to oxidative damage in deoxyribonucleic acid and proteins (3). Several experimental and clinical studies have shown beneficial effects of antioxidants in preventing organ failure and decreasing mortality in sepsis (4,5).

Garlic Allium sativum 'A. sativum' has been widely used as a foodstuff and also a traditional medicine for many centuries throughout the world (6). The antibacterial effects of garlic against a wide range of bacteria (7) and the intrinsic antioxidant activity of garlic, garlic extracts and some garlic constituents (8-10) have been widely documented in vivo (11,12) and in vitro (8,13). Furthermore, garlic acts as an enhancer of cellular antioxidant enzymes; superoxide dismutase (SOD), catalase, and glutathione (GSH) peroxidase, in addition to increasing cellular GSH levels (14-16). These properties of garlic increase the antioxidant capacity of the body and provide effective scavenging of free radicals, thereby improving immunity (17,18).

Based on the potent antibacterial and antioxidant effects of garlic, we investigated the putative protective role of aqueous

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garlic extract (AGE) against sepsis-induced oxidative damage in pulmonary and ileal tissues as well as its effects on certain coagulation parameters.

Material and Methods

Animals

Wistar albino rats of either sex, weighing 200 to 250 g, were kept in a room at a constant temperature (22±2 °C) with 12-h light and dark cycles and were fed a standard rat chow. Rats were fasted for 12 h before experiments, but were allowed free access to water. Experimental protocol was approved by the Marmara University Animal Care and Ethics Committee.

Preparation of garlic extract

Garlic, from which the study preparations were derived, was harvested in August from Kastamonu region of Turkey and was kept in dry storage conditions protected from light. Peeled garlic (30 g) was crushed with distilled water in a mortar. The crushed material was carefully decanted by pressing, and 60 mL of aqueous extract was extracted. One milliliter of aqueous extract contained material from 500 mg of garlic (19,20). The aqueous extract was stored at 4 °C.

Experimental protocol and induction of sepsis

Rats were divided into four groups, 2 control and 2 sepsis groups, with 8 animals in each. Rats were supplemented with either saline or AGE (250 mg/kg/day orally) for 15 days prior to sham operation or caecal ligation and perforation (CLP), and also immediately postoperatively.

In the sham operated control groups, after laparotomy, the cecum was manipulated but left intact (without ligation or perforation). In the sepsis groups rats underwent CLP technique according to the method described by Fujimura et al. (21). Briefly, under ether anesthesia, a midline laparotomy was made using minimal dissection and the cecum was ligated just below the ileocaecal valve with 3-0 silk ligatures so that intestinal continuity was maintained. On the antimesentric surface of the cecum, using an 18-gauge needle, the cecum was perforated at two locations 1 cm apart and the cecum was gently compressed until the feces were extruded. The bowel was then returned to the abdomen and the incision was closed. At the end of the operation, all rats were resuscitated with saline (3 mL/100 g body weight) administered subcutaneously.

Twenty-four hours after the sepsis-induction, rats were decapitated and trunk blood was collected for the measurement of platelet counts, fibrinogen, International normalized ratio (INR) and activated partial thromboplastin time (APTT) levels. Furthermore lung and ileum tissue samples were immediately taken and stored at -70 °C to analyze SOD, myeloperoxidase (MPO), and thromboplastic activities, as well as malondialdehyde (MDA) and GSH levels.

Determination of coagulation parameters in blood

Trunk blood was collected into plastic syringes containing one-tenth in a volume of 3·8% (w/v) trisodium citrate or into plastic syringes containing sodium ethylenediaminetetraacetic acid (EDTA). Blood samples taken into 3·8 % (w/v) trisodium citrate were centrifuged at 2000 g for 10 min for the measurement of prothrombin time (prothrombin time, INR) (Cat. no. 52601003, Agappe, Switzerland), APTT (Cat. no. 52602001, Agappe, Switzerland), fibrinogen (Cat. no. 840155, Pacific Hemostasis, UK), and d-dimer (Cat. no. D2050-000, Teco, Germany). In d-dimer test, agglutination occurs within 180-200 seconds for samples containing more than 250 ng/mL. If agglutination is observed within 180-200 seconds a pathological condition probably exists. Platelet count was determined in the whole blood samples drawn into sodium EDTA by using an automated analyzer (KT 6200 VET, Genius, China).

Measurement of tissue superoxide dismutase activity

SOD activity in the lung and ileum tissue samples was measured in accordance with a previously described method (22). Briefly, measurements were performed in cuvettes containing 2.8 mL 50 mM potassium phosphate (pH=7.8) with 0.1 mM EDTA, 0.1 mM 0.39 mM riboflavin in 10 mM potassium phosphate (pH=7.5), 0.1 mL of 6 mM O-dianisidin.2 HCl in deionized water, and tissue extract (50, 100 mL). Cuvettes with all their components were illuminated with 20-W Sylvania Grow Lux fluorescent tubes that were placed 5 cm above and to one side of cuvettes maintaining a temperature of 37 °C. Absorbance were measured at 460 nm with a Shimadzu UV-02 model spectrophotometer. A standard curve was prepared routinely with bovine SOD (Sigma Chemical Co, ST-2515-3000 U) as reference. Absorbance readings were taken at 0 and 8 min of illumination and the net absorbance were calculated.

Measurement of tissue myeloperoxidase activity

MPO activity was measured in tissues in a procedure similar to that documented by Hillegass et al. (23). Tissue samples were homogenized in 50 mM potassium phosphate buffer (PB, pH=6.0), and centrifuged at 41,400 g (10 min); pellets were suspended in 50 mM PB containing 0.5% hexadecyltrimethylammonium bromide. After three freeze and thaw cycles, with sonication between cycles, the samples were centrifuged at 41,400 g for 10 min. Aliquots (0.3 mL) were added to 2.3 mL of reaction mixture containing 50 mM PB, O-dianisidine, and 20 mM H_2O_2 solution. One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance measured at 460 nm for 3 min. MPO activity was expressed as U/g tissue.

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Measurement of tissue thromboplastic activity

Thromboplastic activity of lung and ileum tissues was evaluated according to Quick's onestage method using normal plasma (24). This was performed by mixing 0.1 mL tissue homogenate with 0.1 mL 0.02 M $CaCl_2$; the clotting reaction was started upon the addition of 0.1 mL plasma. All reagents were brought to the reaction temperature (37 °C) before mixing. Thromboplastic activity was expressed as seconds. The lengthening of the clotting time is an indication of decreased tissue factor (TF) activity.

Measurement of tissue malondialdehyde and glutathione levels

Lung and ileum tissue samples were homogenized with ice-cold 150 mM chloride for the determination of MDA and GSH levels. MDA levels were assayed for products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously (25). Lipid peroxidation was expressed in terms of MDA equivalents using an extinction coefficient of $1.56x10^5$ M^{-1} cm^{-1} and results are expressed as nmol MDA/g tissue. GSH measurements were performed using a modification of the Ellman procedure (26). Briefly, after centrifugation at 3000 rev/min for 10 min, 0.5 mL of supernatant was added to 2 mL of 0.3 mol/l $Na_2HPO_4.2H_2O$ solution. A 0.2 mL solution of dithiobisnitrobenzoate (0.4 mg/mL 1% sodium citrate) was added and the absorbance at 412 nm was measured immediately after mixing. GSH levels were calculated using an extinction coefficient of 1.36x104 M^{-1} cm^{-1}. Results are expressed in µmol GSH/g tissue.

Statistical analysis

Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA) and all data were expressed as means ± standard error of mean. Groups of data were compared with an analysis of variance (ANOVA) followed by Tukey's multiple comparison tests. A p value of less than 0.05 was considered significant.

Results

As shown in Table 1 sepsis was associated with reduced platelet and fibrinogen, and increased APTT and INR levels. D-dimer levels also increased after sepsis induction (Table 2). On the other hand AGE treatment did not have a significant effect on these parameters except for d-dimer, which was significantly reduced by AGE treatment (Table 2).

Sepsis induced significant decrease in GSH and SOD in both lung ($p<0.001$, Figure 1) and ileum ($p<0.01$, Figure 2) tissue samples, while AGE treatment in rats with CLP gave rise to significant increases in both of these antioxidants ($p<0.5$).

As a result of CLP induction, MPO activity and MDA levels were found to increase in both lung and ileum tissues ($p<0.001$, Figure 3 and Figure 4, respectively). On the other hand, AGE treatment in the CLP group caused a decline in these values ($p<0.005$-0.001) restoring baseline levels.

Since the clotting time is inversely proportional to the TF activity, prolonged clotting time is indicative of decreased TF activity. Accordingly, CLP caused decrease in TF activity of lung and ileum tissues ($p<0.001$, Figure 3c, 4c). On the other hand AGE treatment in CLP rats caused an increase in TF activity in both lung ($p<0.05$) and ileum ($p<0.001$) tissues.

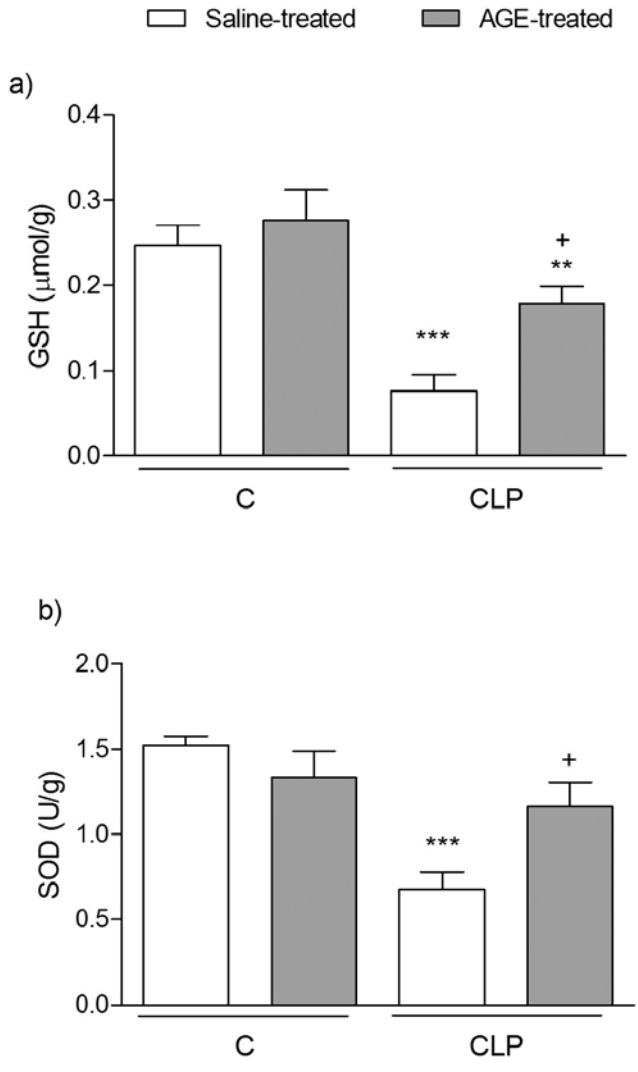

Figure 1. Glutathione levels and b) Superoxide dismutase activities in the lung tissue samples of saline-and aqueous garlic extract-treated control and sepsis groups, **p<0.01, ***p<0.001 versus saline-treated control group, $^{+}$p<0.05 versus saline treated-sepsis group, for each group n=8, GSH: Glutathione, SOD: Superoxide dismutase, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract

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Discussion

Being one of the most popular herbal remedies, garlic has been widely used for the treatment of diseases since ancient times, despite the scarcity of data in the current literature on the effects of AGE on pulmonary and ileal tissues in sepsis. In the present study, pulmonary and ileal pathologic changes induced by oxidative damage due to experimentally-induced sepsis and the potential protective effects of AGE against this damage were investigated. Our results showed an alleviation of sepsis-induced oxidative damage in the lung and intestinal tissues by AGE, as suggested by significantly reduced MDA and MPO levels and increased GSH and SOD. Furthermore, thromboplastic activity, which decreased due to sepsis, was augmented by AGE

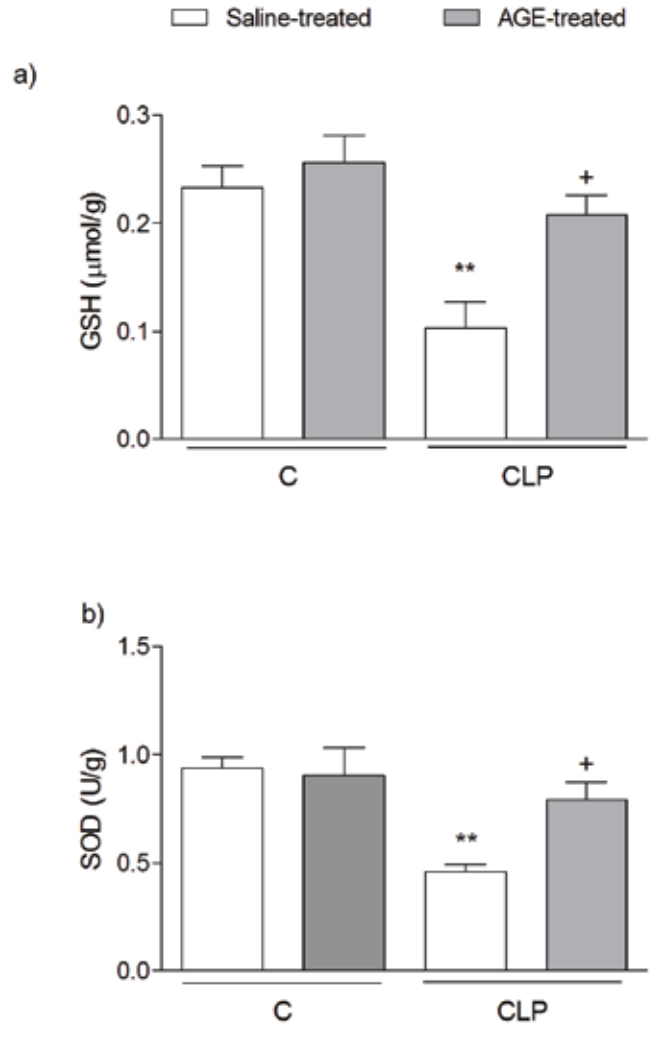

Figure 2. Glutathione levels and b) Superoxide dismutase activities in the ileal tissue samples of saline-and aqueous garlic extract-treated control and sepsis groups, **p<0.01 versus saline-treated control group, $^{+}$p<0.05 versus saline treated-sepsis group, for each group n=8, GSH: Glutathione, SOD: Superoxide dismutase, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract

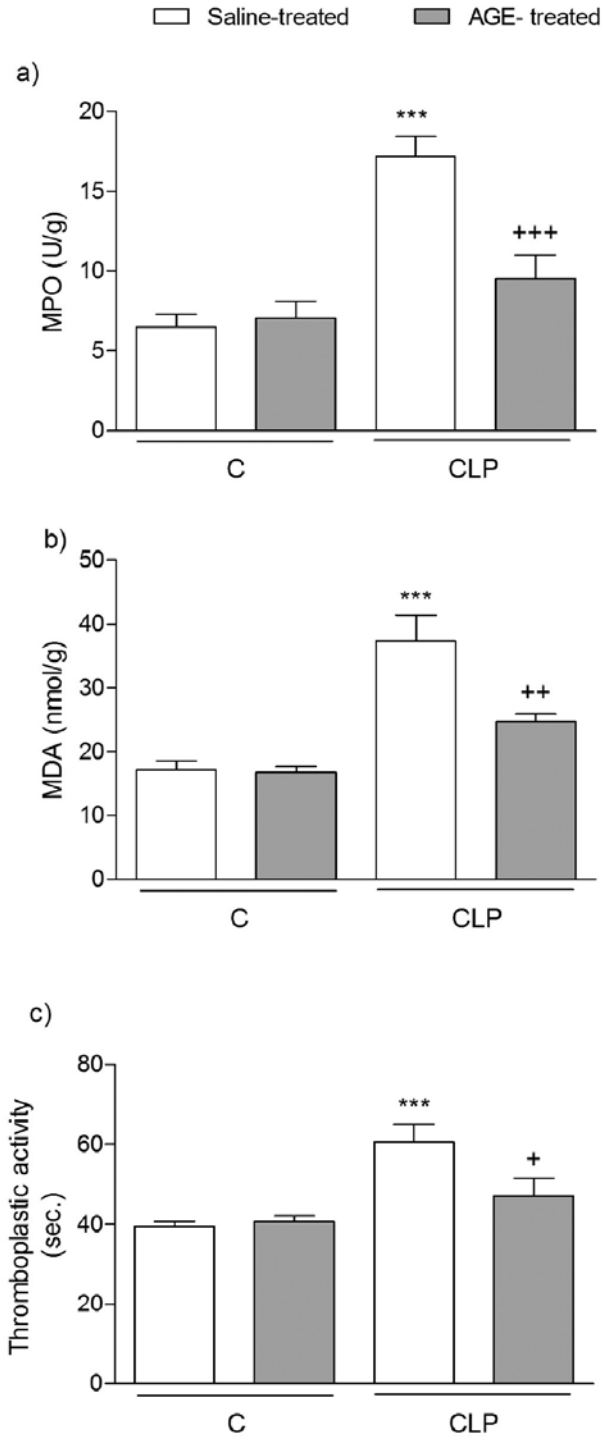

Figure 3. Myeloperoxidase activities, b) Malondialdehyde levels and c) Thromboplastic activities in the lung tissues of saline- and aqueous garlic extract-treated control and sepsis groups, ***p<0.001 versus saline-treated control group, $^{+}$p<0.05, $^{++}$p<0.01, $^{+++}$p<0.001 versus saline treated-sepsis group, for each group n=8, MPO: Myeloperoxidase, MDA: Malondialdehyde, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract

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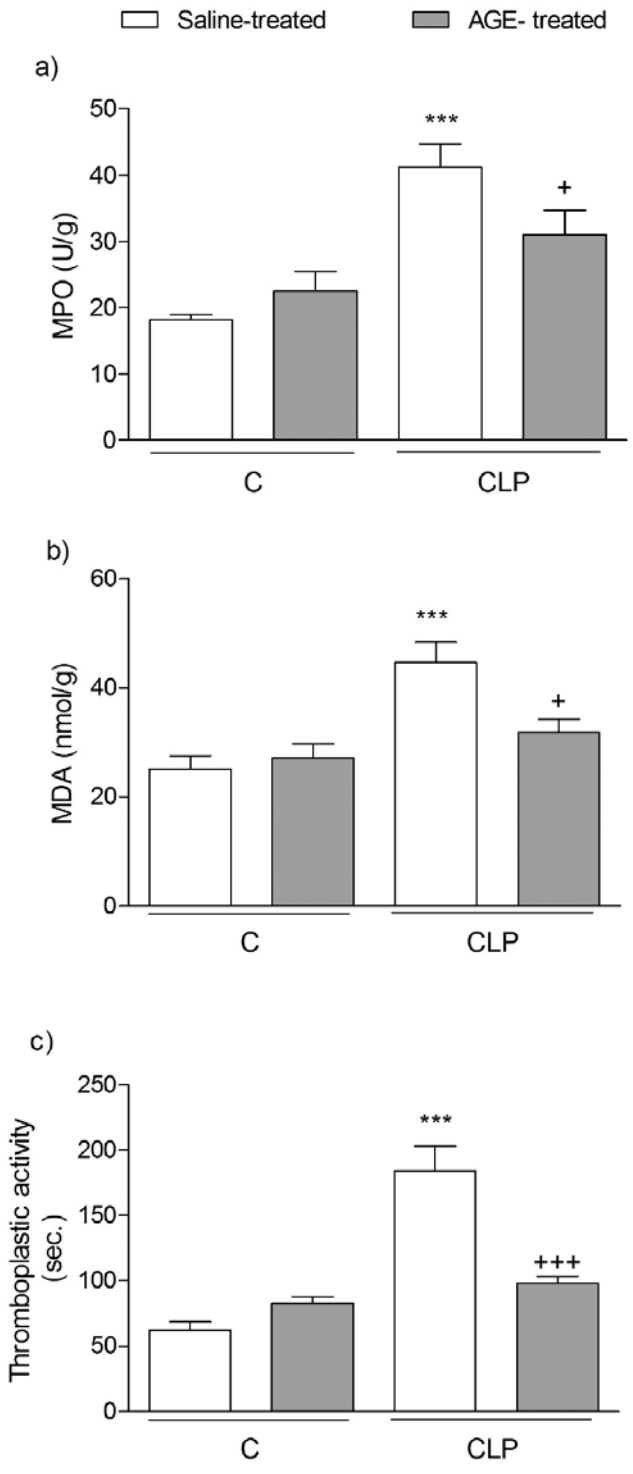

Figure 4. Myeloperoxidase activities, b) Malondialdehyde levels and c) Thromboplastic activities in the ileal tissues of saline- and aqueous garlic extract-treated control and sepsis groups, ***p<0.001 versus saline-treated control group, $^{+}$p<0.05, $^{+++}$p<0.001 versus saline treated-sepsis group, for each group n=8, MPO: Myeloperoxidase, MDA: Malondialdehyde, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract

treatment and the sepsis-induced reduction in platelet count was partially reversed. On the other hand, INR and APTT, which were elevated in septic rats, were significantly decreased by AGE treatment. AGE treatment did not affect fibrinogen and d-dimer levels, which were decreased and elevated, respectively, during sepsis. Garlic containing preparations have recently been shown to exert beneficial effects against tumor promotion (27), in cardiovascular disorders, in hepatic damage (28) and in the process of aging (29) and these effects were mostly attributed to its anti-oxidant properties. Preventive or therapeutic strategies that incorporate the use of AGE might arguably be developed in this condition, considering the fact that sepsis is associated with oxidative damage in various organs (30) and that garlic is known to have antioxidant properties.

Sepsis is a generalized inflammatory response, involving various organ systems and causing disturbance of homeostasis through a currently uncontrollable cascade of excessive inflammation and coagulation with impaired fibrinolysis that contributes to an inflammatory response, microvascular hypoperfusion, organ dysfunction, and increased mortality. The magnitude of disruption in homeostasis is influenced by the virulence of the causative pathogens and the host's response to the infection (31-33). In this regard, the results of the present study are consistent with the above-mentioned hemostatic disturbance in sepsis. On the other hand, although garlic has been shown to have antithrombotic and antiplatelet properties (34,35), in the present study AGE decreased the platelet count only in the control group without an antithrombotic effect. However reversal of the sepsis-induced changes in platelet count, INR, APTT and d-dimer level by AGE treatment suggests that these effects of AGE in sepsis may not be directly linked with its antithrombotic and antiplatelet effects, and rather may stem from its antioxidant and antibacterial effects.

The CLP are widely used for the induction of sepsis in models of sepsis based on its clinical resemblance to sepsis in humans. Recent studies have shown that sepsis is associated with the enhanced generation of reactive oxygen metabolites (ROMs), leading to multiple organ dysfunction (36,37), most marked in lungs, liver, kidneys, heart, and intestines. These pathological changes are known to result from bacterial invasion, direct effects of bacterial toxins and enzymes, effects of mediators, impaired perfusion, and disseminated intravascular coagulation (38). Pulmonary involvement occurs early in sepsis and pulmonary complications are major factor contributing to poor prognosis. After pulmonary involvement, other common pathological conditions include the acute ischemic colitis in the intestines and zonal necrosis of the liver (39,40).

Lipids are a major target of free oxygen radicals, which initiate lipid peroxidation by receiving a hydrogen atom from

polyunsaturated fatty acids, giving rise to the formation of hydrogen peroxide. The result of this process is the disrupted cell membrane fluidity followed by cell death (41). In the present study, the levels of MDA, an end-product of lipid peroxidation, were significantly increased in pulmonary and ileal tissues, in line with the previous studies, in which elevated levels of lipid peroxidation products were increased from 40% to 80% above basal values as a result of oxidative stress (30,42,43). On the other hand, AGE treatment inhibited MDA elevations and restored the control levels suggesting that AGE might be protective against organ damage by maintaining cellular integrity.

There are enzymatic and non-enzymatic antioxidant mechanisms involved the removal of free radicals and for damage repair. Among enzymatic antioxidants, SOD is particularly important for intracellular killing of phagocytized bacteria and for granulocyte function (44). It catalyzes the conversion of superoxide to hydrogen peroxide and is primarily protective against oxyradicals. GSH, on the other hand, a non-enzymatic antioxidant, is an important constituent of intracellular protective mechanisms against various noxious stimuli including oxidative stress (45). In a previous study by our team, CLP was shown to cause significant decrease in GSH levels, which was reversed by the powerful antioxidant melatonin (46). Furthermore we also demonstrated that following a variety of various oxidative insults resulting in depletion of GSH, repletion could be accomplished by AGE (19,47). Similarly, Kilikdar et al. (28) demonstrated that AGE treatment provided an elevation in SOD activity in lead -induced hepatic injury in rats. In our study, pulmonary and ileal tissue GSH levels and SOD activities were lower in the sepsis group as compared to the control group, while AGE was effective in replenishing these antioxidants.

Studies have demonstrated that neutrophils are one of the major sources of ROMs (48). The heme enzyme MPO, found in neutrophils, uses a superoxide anion to produce hypochlorous acid, which is the major oxidant for its immune function. However, these MPO-derived oxidants also cause tissue and cellular damage (49). Thus, the tissue-associated MPO activity is considered to indicate the severity of inflammatory damage. Kettle and Winterbourn (50) suggested that inhibition of the enzyme activity could modulate the oxidant production and ultimately, tissue damage. In our study, increased MPO activity in both tissues suggests that neutrophil accumulation contributes to the sepsis-induced oxidative injury. Previously, in ischemia/reperfusion-or naphthalene-induced oxidative stress models (51), increased MPO activities have been reported to decrease with AGE treatment. Similarly in our study, pulmonary and ileal tissue MPO activities were significantly higher in sepsis group as compared to the control group, while a significant decrease occurred in the sepsis group treated with AGE, suggesting an anti-inflammatory effect for AGE.

Thromboplastin (TF, factor III), the primary cellular initiator of blood coagulation, is a transmembrane receptor that is expressed in a tissue-specific manner (52). Moreover, various tissues and body fluids are known to harbor thromboplastic activity (53,54). In the present study, although a significant decrease in the thromboplastic activity occurred in in the lung and pulmonary tissues in association with sepsis, AGE treatment resulted in a significant increase in thromboplastic activity in both tissues. Normalization of thromboplastic activity with AGE may also help eliminate the increased risk of bleeding due to the decreased thromboplastic activity in pulmonary and ileal tissues in sepsis.

Table 1: Platelet count, fibrinogen, International normalized ratio and activated partial thromboplastin time levels in plasma

	C		CLP	
	Saline-treated	**AGE-treated**	**Saline-treated**	**AGE-treated**
Platelet count (x10^3/mm^3)	493±17.1	420±23.3*	205±10.4***	280±21.7***, $^{+}$
Fibrinogen (mg/dL)	428±5.4	389±31.9	296±22.8**	355±30.1
INR	1.06±0.04	1.11±0.07	1.84±0.13***	1.40±0.06^{+}
APTT (sec.)	29.5±2.4	35.7±3.0	70.2±2.5***	54.4±1.3***, $^{+++}$

n=8 per group, *p<0.05, **p<0.01, ***p<0.001 vs. control group, $^{+}$p<0.05, $^{+++}$p<0.001 vs. saline-treated sepsis group, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract, INR: International normalized ratio, APTT: Activated partial thromboplastin time

Table 2: Changes in d-dimer for all groups

	C		CLP	
	Saline-treated	**AGE-treated**	**Saline-treated**	**AGE-treated**
D-dimer (ng/mL)	<250	<250	>16000***	500-1000*

<250 ng/mL: There is no agglutination in undiluted and serial dilutions until 1:64 dilutions 500-1000 ng/mL: There is agglutination in 1:2 dilutions and there is no agglutination in serial dilutions until 1:64 dilutions n=8 per group, *p<0.05, ***p<0.001 vs. control group, CLP: Caecal ligation and perforation, AGE: Aqueous garlic extract

Conclusion

In conclusion, the results of our study showed antioxidant and anti-inflammatory effects of AGE against tissue damage caused by free oxygen radicals and lipid peroxidation resulting from sepsis in an experimental rat model. However, further studies are warranted to better define the mechanisms of pulmonary and intestinal injury due to sepsis as well as the mechanisms of the benefit observed in AGE treatment.

Conflict of Interest: The authors declare that there is no conflict of interest arising out of this manuscript.

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