

## Effects of gamma oryzanol on factors of oxidative stress and sepsis-induced lung injury in experimental animal model

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### ABSTRACT

**Objective (s):** There is corroborating evidence to substantiate redox imbalance and oxidative stress in sepsis that finally leads to organ damage or even death. Gamma oryzanol (GO) is one of the major bioactive components in rice bran has been considered to function as an antioxidant. The present study was carried out to evaluate the antioxidant activity of gamma oryzanol *in vitro* and its efficacy in sepsis.

**Materials and Methods:** To induce sepsis, cecal ligation and puncture (CLP) method was performed on the rats. A study group of forty male Wistar rats were divided into the following groups: sham group; CLP group; 50 mg/kg GO- treated CLP group and 100 mg/kg GO- treated CLP group. GO was administered with an oral gavage 2 hr prior to inducing sepsis. Tissue and blood samples were collected 12 hr after CLP to prepare tissue sections for histopathological study and assay the oxidative stress biomarkers including: SOD (Superoxide Dismutase), TAC (total antioxidant capacity), MDA (Malondialdehyde), MPO (Myeloperoxidase) and PAI-1 (Plasminogen Activator Inhibitor-1). Data are given as mean  $\pm$  SD. The ANOVA with Tukey *post hoc* test was used to determine the differences between groups and  $P < 0.05$  was considered as statistical significance.

**Results:** TAC level increased in GO- treated CLP groups ( $P < 0.05$ ). Inflammation score of lung tissue and MPO activity were significantly lower in GO treated CLP group ( $P < 0.05$ ).

**Conclusion:** It seems that GO has a protective effect on lung inflammation and improves the body redox capacity during sepsis.

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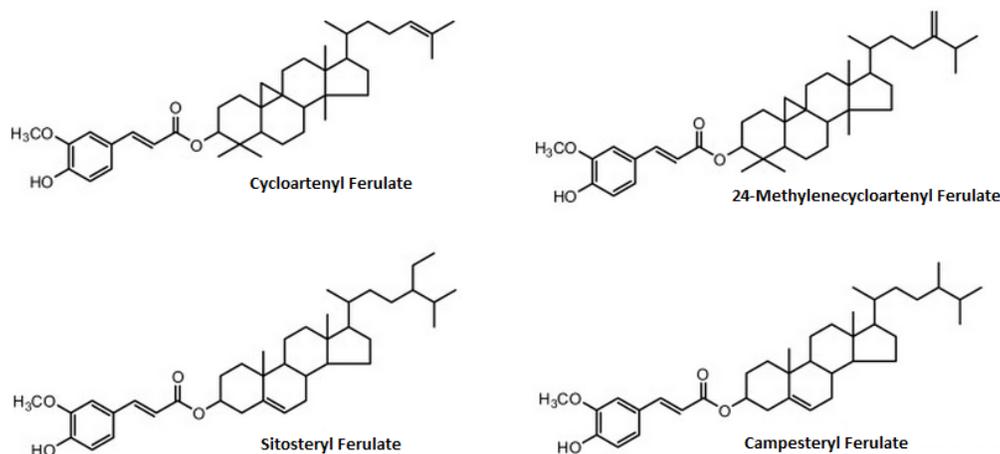
### Introduction

Sepsis is the condition of the presence of an infection with evidences of a systemic response that is called the systemic inflammatory response syndrome (SIRS) and is one of the most important reasons that results in admitting patients to intensive care units (ICUs) in the world (1). Following the invasion of microorganism, macrophages and neutrophils immigrate to the site of inflammation and release free radicals to destroy pathogens. Free radicals that are released following activation of immune system lead to peroxidation of lipids, proteins and DNA; moreover, reactive oxygen species (ROS) produced by macrophage cause lung tissue endothelium damage and acute respiratory distress syndrome (ARDS) (2). The imbalance between ROS production and its degradation by endogenous antioxidants during

sepsis causes free radicals overload and production of inflammatory mediators (3). There is increasing evidence that oxidative stress following free radical overload plays an essential role in the development of multi organ dysfunction (MOD) (4). Antioxidants can control the sepsis-induced inflammation and tissue damage by directly scavenging free radicals or enhancing endogenous antioxidant defence system (5, 6). Various studies established that the administration of a natural antioxidant in the septic animal showed a remarkable positive effect on improving the sepsis manifestations (7, 8).

Gamma oryzanol (GO), with the structure shown in Figure 1 (9), is a part of unsaponifiable matter of crude rice bran oil, which is achieved in the milling process of rice (10). It has been demonstrated that GO is a mixture of ferulic acid esters of phytoosterol

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**Figure 1.** Chemical structures of four major components of gamma oryzanol (9)

and triterpene alcohols. Cycloartenyl ferulate, 24-methylene cycloartenyl ferulate, sitosteryl ferulate and campesteryl ferulate are the major components of GO (11). It is demonstrated that GO, a natural product, is a powerful scavenger of diphenyl picrylhydrazyl (DPPH), hydroxyl, and superoxide radicals (12). It is able to protect cells against lipid peroxidation in oxidative status. It has also been reported that GO poses anti-inflammatory effects. A report showed that GO treatment could suppress colitis and mucosal inflammation in mice by inhibiting the transcription of NF $\kappa$ B, interleukin 1 (IL-1) and IL-6 (13). Furthermore, it is shown that GO had the potential to attenuate the amounts of total cholesterol, very low-density lipoprotein (VLDL) and low-density lipoprotein (LDL), and to enhance plasma high-density lipoprotein (HDL) in hypercholesterolemic hamsters (14). In another study, in which the hypocholesterolemic activity of GO had been investigated, it is demonstrated that the cholesterol-lowering effects of GO is due to inhibition of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase activity and preventing from cholesterol uptake by intestine epithelial cells (15).

In order to create a condition similar to sepsis, we used cecal ligation and puncture (CLP) model on the test group of rats to evaluate the antioxidant effects of GO in animal model of sepsis. Furthermore, we theorized that GO, a known antioxidant, may prevent acute lung injury (ALI) during sepsis; therefore, investigation of the microscopic evidence on lung tissues of septic rats was performed.

## Materials and Methods

### Animals

The experiments performed on male Wistar rats weighing 250 to 300 g, and each group included ten

rats. The animals were housed in standard polypropylene cages, four per cage, under a 12:12 hr light/dark schedule at an ambient temperature of  $23\pm 2$  °C. Animals had free access to food and water. All experiments performed under ethical guidelines for the care and use of laboratory animals of Tabriz University of Medical Sciences, Tabriz, Iran.

The rats were divided into the four groups: (I) sham group, rats were subjected to laparotomy without any other manipulation; (II) CLP group, rats were subjected to CLP without any treatment; (III) 50 mg/kg GO- treated CLP group; and (IV) 100 mg/kg GO- treated CLP group.

GO powder was received as a gift from Tsuno Rice Fine Chemicals Co., Ltd. (Wakayama, Japan). GO is a white or yellowish white, odorless, crystalline powder with the purity  $\geq 99\%$  and is oil soluble. It was dissolved in olive oil and prepared freshly on the day of the experiment. As regards the maximum plasma level of GO is nearly 2 hr after oral administration (16), it was administered with an oral gavage 2 hr before CLP. The rats had free access to food and water after surgery, until they were sacrificed.

### Treatment protocol

Since, daily dosage of GO in humans is 500 mg/day (17), 50 and 100 mg/kg body weight were selected to be administered with an oral gavage in rats, based on the body surface area (18).

### Surgical procedure

A CLP model was used for induction of polymicrobial sepsis on the rats. Each rat was anesthetized by administering ketamine hydrochloride (80–100 mg/kg) and xylazine hydrochloride (5–15 mg/kg) IM. The abdominal area was shaved and disinfected; an incision of peritoneal cavity was made to

expose the cecum. The cecum was isolated and ligated with 4/0 silk suture, just distal to the ileocecal valve. One puncture wound was made piercing the cecum with an 18-gauge needle, the cecum was repositioned into the peritoneal cavity. Finally, closure of the abdominal incision with sterile 4/0 silk sutures was performed. Laparotomies performed on the sham-operated group with no perforation of the cecum. Pre-warmed normal saline (2 ml/100 g body weight) was administered subcutaneously for fluid resuscitation (19) post-operatively.

### **Specimen Collection**

All four groups were anaesthetized after 12 hr and 4 ml of blood drawn by cardiac puncture. Three ml of the blood was extracted and transferred to a plastic vial and centrifuged for 10 min at 7000 rpm/min. The plasma separated and stored at -70 °C until analyse. The 1 ml whole-blood sample was transferred to plastic vial and stored at -70 °C until analyse. The lungs were surgically removed and washed in an ice-cold saline solution. One specimen of lung tissue was stored at -70 °C and retained for biochemical analyses; the second specimen of lung tissue fixed in 10% formalin solution for histopathological analyses.

### **Measurement of tissue myeloperoxidase (MPO) activity**

Myeloperoxidase activity was assayed according to the Bradley *et al* method (20). The lung tissue specimen was homogenized in 0.5% hexadecyl trimethyl ammonium bromide (HTAB) solution (prepared with potassium phosphate buffer, 50 mM; pH=6) for 3 min, at 8500 rpm. The homogenates were sonicated for 10 sec, frozen and thawed 3 times, then centrifuged at 45000 rpm, in 4 °C for 45 min. 100 µl of supernatant was added to 2.9 ml of o-Dianisidine dihydrochlorid and hydrogen peroxide solution. After five min, the reaction was stopped by adding 0.1 ml of hydrochloric acid (1.2 M). Lastly, the absorbance was measured spectrophotometrically at 400 nm and concentrations expressed as MPO Unit/mg tissue.

### **Measurement of plasma and tissue malondialdehyde (MDA)**

MDA levels in the lung tissue and plasma were measured according to the Olgen *et al* method (21). Tissues were mixed in 1.15% KCl to achieve a 10% (W/V) homogenate. One ml of each supernatant and plasma was added to a mixture of O-phosphorous acid (1%) and thiobarbituric acid (0.67%) in an aqueous solution. The reaction mixture was heated for 60 min up to 90°C and then was cooled to room temperature. Three ml of *n*-BuOH (*butanol*) was

added and centrifuged. The absorbance of the *n*-BuOH (*butanol*) phase was measured spectrophotometrically at 532 nm, and MDA levels reported as nmol MDA/mg tissue and nmol MDA/ml plasma.

### **Measurement of tissue superoxide dismutase (SOD), whole blood total antioxidant capacity (TAC) and plasma plasminogen activator inhibitor-1 (PAI-1)**

SOD activity in lung tissue, TAC level of whole blood and PAI-1 level of plasma were measured according to the manufacturer's instructions and guidelines using assay kits (Randox Laboratories Ltd., Crumlin, UK for SOD and TAC, Glory science Co., Ltd, USA for PAI-1). SOD activity was reported as Unit/mg protein, TAC level was reported as mmol/l and PAI-1 level was reported as ng/ml.

### **Histopathological examination of lung tissue**

Lung tissues samples were fixed in 10% formalin, and after fixation they were embedded in paraffin. Five to six µm sections were taken from tissue samples and stained with hematoxylin-eosin (HE). After staining, sections were examined under light microscopy and then scored based on the degree of inflammation and neutrophil infiltration by an experienced observer blinded to treatment. The severity of tissue injury was scored as follows: 1: normal; 2: mild; 3: moderate; and 4: severe.

### **Data analysis**

Data are expressed as the mean ± SD. Kolmogorov-Smirnov test demonstrated that all data in each group distributed normally. The one-way analysis of variance (ANOVA) with Tukey *post hoc* test was used to determine the differences in the oxidative stress plasma and tissue extract levels.  $P < 0.05$  was accepted as the statistically significant difference.

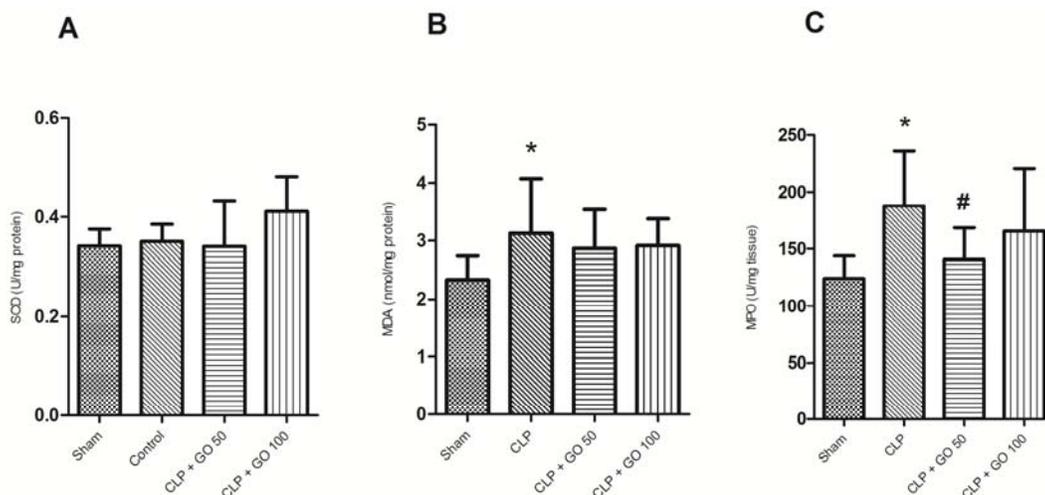
## **Results**

### **The levels of oxidative stress factors in lung tissue**

There was no significant difference between groups in tissue SOD enzyme activity ( $P > 0.05$ ) (Figure 2).

The lung tissue level of MDA was significantly higher in the CLP group as compared to sham group ( $P < 0.05$ ). MDA level has a relative decrease in GO-treated CLP groups as compared to CLP group, but it was not statistically significant ( $P > 0.05$ ) (Figure 2).

The activity of MPO in lung tissue was significantly higher in the CLP group. Treatment with GO 50 mg/kg significantly depressed the elevation in MPO activity ( $P < 0.05$ ) (Figure 2).



**Figure 2.** The parameters of lung tissue oxidative stress measured 12 hr after cecal ligation and puncture (n= 10). (A) SOD activity. (B) MDA level. (C) MPO activity. CLP, cecal ligation and puncture; GO 50, Gamma oryzanol 50 mg/kg peroral. 2 hr before CLP; GO 100, Gamma oryzanol 100 mg/kg p.o. 2 hr before CLP. \* -indicates  $P < 0.05$  compared to sham group. # -indicates  $P < 0.05$  compared to control group. Values are expressed as mean $\pm$ SD

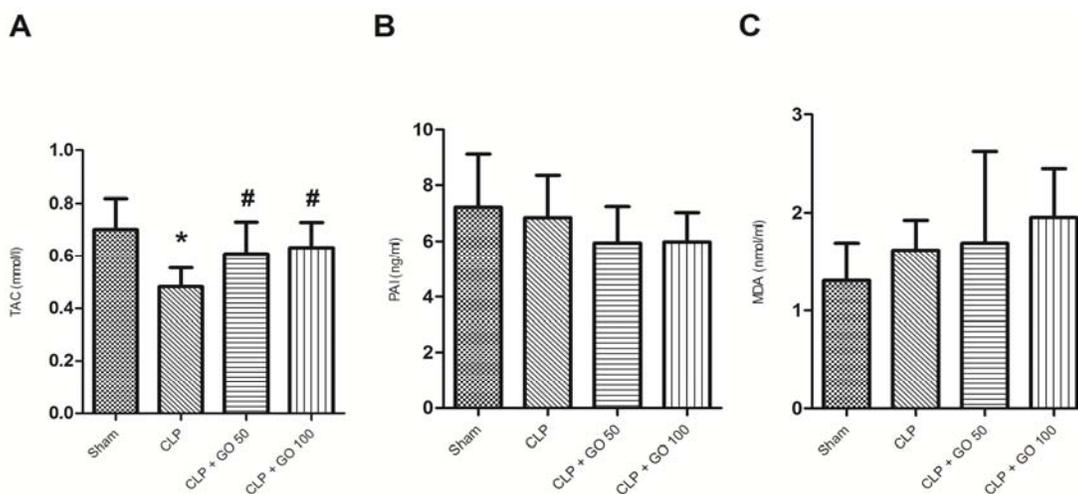
### The levels of oxidative stress factors in plasma and whole blood

The level of TAC in whole blood samples was significantly lower in CLP group as compared to sham group. However, a significant increase occurred in the GO- treated CLP groups as compared to CLP group ( $P < 0.05$ ) (Figure 3).

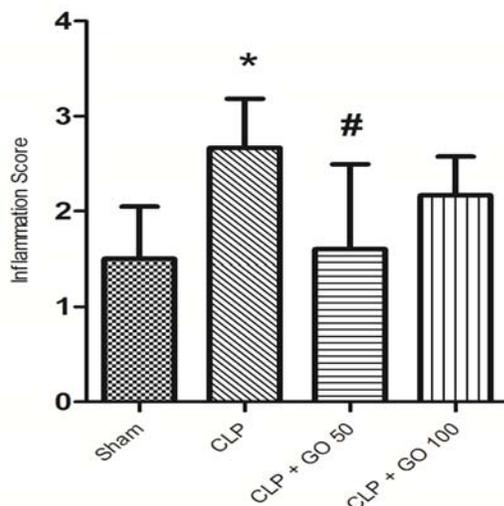
There was no significant difference between the groups in plasma levels of PAI and MDA ( $P > 0.05$ ) (Figure 3).

### Histopathological results for lung tissues

There was a significant difference between sham group and CLP group in terms of inflammation scores ( $P < 0.05$ ) (Figure 4, 5). The mean inflammation score in sham group was 1.5 and 2.6 in the CLP group. On the contrary, the results of histopathological study showed significant decrease in lung tissue damage in GO 50 mg/kg- treated CLP group compared to CLP group ( $P < 0.05$ ). Inflammation scores had a slight decrease in the GO 100 mg/kg- treated CLP group compared to CLP group, but it was not statistically significant ( $P > 0.05$ ).



**Figure 3.** The whole blood and parameters of plasma oxidative stress measured 12 hr after cecal ligation and puncture (n= 10). (A) TAC level. (B) PAI level. (C) MDA level. CLP, cecal ligation and puncture; GO 50, gamma Oryzanol 50 mg/kg p.o. 2 hr before CLP; GO 100, gamma oryzanol 100 mg/kg p.o. 2 hr before CLP. \* -indicates  $P < 0.05$  compared to sham group. # -indicates  $P < 0.05$  compared to control group. Values are expressed as mean $\pm$ SD



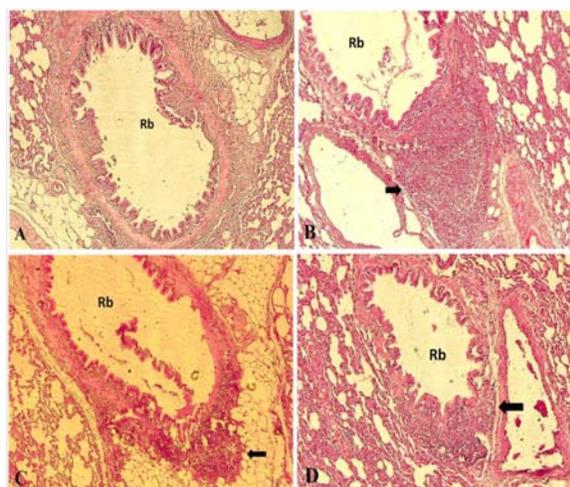
**Figure 4.** The lung tissue inflammation scores measured 12 hr after cecal ligation and puncture (n=6). CLP, cecal ligation and puncture; GO 50, gamma oryzanol 50 mg/kg p.o. 2 hr before CLP; GO 100, gamma oryzanol 100 mg/kg p.o. 2 hr before CLP. \* -indicates  $P < 0.05$  compared to sham group, # -indicates  $P < 0.05$  compared to control group. Values are expressed as mean  $\pm$  SD

## Discussion

Sepsis causes progressive damage in multiple organs and if progress to septic shock with a significant drop in blood pressure can lead to death. Treatment with antibiotics alone is not an adequate treatment plan to increase the possible survival rate of the septic patient. Considering this fact, it is thought that the use of antioxidants may have an important role in the treatment of septic patients (22).

GO, as a natural product, has a potent antioxidant properties (23). The result of an animal study showed that GO has protective effects on liver injury induced by chronic ethanol administration (24). Furthermore, there is scientific evidence reporting its immunomodulatory properties and the effects of GO in stimulating immune responses in experimental animal models of immunity (17).

Oxidative stress influences the molecular mechanisms that control inflammation and directly cause tissue damage. This type of tissue damage is believed to be one of the most important mechanisms those results in multiple organ failure (MOF) in septic patients (4). The lung is the primary organ that is affected initially and most severely in intra-abdominal sepsis (25). Under normal physiological conditions, there is a balance between the levels of antioxidants and oxidants in the lungs, and a disruption in this balance is considered to be one of the primary events that can cause an inflammatory response in the lungs during a septic infection (26). The accumulation and activation of neutrophils in the lungs is an important early event in the development of an ALI in the experimental



**Figure 5.** The histopathological view of lung section. (A) Sham group; (B) CLP (cecal ligation and puncture) group; (C) gamma oryzanol 50 mg/kg- treated CLP group; (D) gamma oryzanol 100 mg/kg- treated CLP group. Inflammatory cell infiltration (arrow indicated); Rb: respiratory bronchiole. All magnifications: 100 $\times$

animal models of sepsis (27). The measurement of MPO activity as a marker of active neutrophil accumulation in lung tissue showed that MPO activity enhanced in the lungs of septic animals (26). Decreasing level of MPO activity by treatment with GO 50 mg/kg may indicate that GO suppresses the severity of sepsis, and it may have lung protective effects in CLP model of sepsis. Furthermore, the results of histopathological findings demonstrated that GO prevented damage to the lung tissue. Severe sepsis has been associated with microvascular thrombosis, which is mediated by plasminogen activators (PAs). In disseminated intravascular coagulation (DIC) syndrome, intravascular activation of coagulation occurs that may delay sufficient blood supply to the organs. PAI-1 that is secreted by the endothelial cells increases in inflammatory conditions and causes fibrosis and thrombosis in otherwise healthy tissues (28). Sepsis-induced DIC might result in a poor prognosis; therefore, early diagnosis and treatment may improve the outcome of patients with sepsis (29). Findings of a study showed that PA and PAI levels were increased during sepsis and caused fibrin remaining in microvasculature, which led to inadequate organ perfusion (30). However, PAI-1 level as a marker for coagulopathy did not rise in the CLP group in this study as compared to the sham group.

Superoxide anion ( $O_2^-$ ) is a free radical that is used by SOD and is converted to hydrogen peroxide ( $H_2O_2$ ). Then  $H_2O_2$  is detoxified by neutrophil-accumulation peroxidases such as glutathione peroxidase. When SOD is activated sufficiently and

there is no compensatory elevation in the peroxidases, the excess amount of H<sub>2</sub>O<sub>2</sub> is converted to hydroxyl radicals (OH·), which is suggested to be the most dangerous radical (31). It is demonstrated that SOD activity is decreased in lung tissue during sepsis (32). In this regard, it was illustrated that SOD activity decreased 12 hr after inducing sepsis as a result of neutrophils activation and production of superoxide in response to the inflammation (8). On the other hand, the results of a study (33) demonstrated that CLP- induced sepsis caused an elevation in SOD activity of CLP group in response to overproduction of mitochondrial superoxide and expression of inflammatory mediators. However, in the present study SOD did not rise in CLP group as compared to the sham group.

MDA is the product of oxidative stress and is formed during the destruction of cellular membrane phospholipids. When phospholipids such as arachidonic acid (AA) are attacked by hydroxyl radical (OH·), lipid endoperoxide is formed and undergoes spontaneous breakage, which causes MDA production (34). Oxidized lipids and proteins play an important role in damaging cell membranes and are associated with septic mortality (32). Previous studies have shown elevated MDA levels following lipid peroxidation in rats with CLP- induced sepsis (7, 35). In the present study, measurement of MDA in the tissue extract showed significant increase in the CLP group as compared to the sham group, but it was not significant in the plasma samples. Therefore it seems that measurement of MDA in the tissue comparing it with plasma will predict sepsis more precisely.

In addition, the results of our study demonstrated that treatment with GO decreased MDA tissue levels, but depression was not significant.

The elevation of oxidants and free radicals and suppression of antioxidants in plasma samples of patients with sepsis has been established previously (36). The study of Gadek *et al* in patients with sepsis-induced ALI (37) demonstrated that administration of antioxidants prevented lung injury by reducing pro-inflammatory mediators; moreover, it is shown that patients who developed SIRS had a lower antioxidant status (38). On the other hand, some investigators noticed that TAC level was elevated in severe septic patients (39, 40). In the present study, TAC level was lower in the CLP group as compared to the sham group, and treatment with GO was able to increase the TAC level significantly in septic rats.

Based on the results of the present study and evidence-based approaches, the difference between the effects of 50 mg/kg GO and 100 mg/kg GO is not interpretable. However, trends of results shows that 50 mg/kg GO may be more effective than 100 mg/kg GO. Regarding that antioxidant level of plasma samples is increased in patients with septic shock (39), the balance between oxidants and antioxidants

and taking care in antioxidant-related therapy during sepsis is important and also very challenging topic.

### Limitations of study

As regards the present study was a pilot study to assess the effects of GO in sepsis and because of low sample size and lack of survival study due to ethical limitations, dose-response effects of GO may not be interpretable.

### Conclusion

In conclusion, it appears that GO has a protective effect on the lungs during an acute phase of sepsis. It attenuated lung inflammation and neutrophils infiltration in septic rats. GO improved the body redox capacity during the acute sepsis induced by CLP to overcome oxidative stress.

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