

## The Effect of Pomegranate Extract on Survival and Peritoneal Bacterial Load in Cecal Ligation and Perforation Model of Sepsis in Rats

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### ABSTRACT

**Background:** Sepsis is one of the major causes of death in intensive care units. Oxidative stress and hyper-inflammation has been shown to be major cause of mortality and morbidity in septic cases. Pomegranate is a fruit considered for its antioxidant and anti-inflammatory properties. The aim of this study is to evaluate the effect of a standard pomegranate fruit liquid extract (POMx), on mortality and peritoneal bacterial load in cecal ligation and perforation (CLP) sepsis model.

**Methods:** Male wistar rats were divided into four groups of 24 each: sham; CLP; prevention (consumed POMx [250 mg of polyphenols/kg/day] for 4 weeks before CLP); treatment (received a single drink of POMx [250 mg of polyphenols/kg] after CLP). Each group was divided into three subgroups, each containing eight animals, for bacterial load and survival (with and without antibiotics) studies. Sepsis was induced by CLP surgery. Ten day survival rate was recorded. Peritoneal bacterial load was also assessed. Data were analyzed using Log-rank and Kruskal-Wallis tests.

**Results:** There was no significant difference in survival rate of CLP, prevention and treatment groups, in subgroups without antibiotics. However, in subgroups with antibiotics, the prevention group had significantly lower survival rate than sham group ( $P < 0.05$ ). Conversely, the bacterial load of prevention and treatment groups were significantly higher than sham group ( $P < 0.01$ ).

**Conclusions:** Our study demonstrates for the first time that pomegranate extract could increase mortality rate via increasing peritoneal cavity bacterial load, in CLP sepsis model. More studies to assess mechanisms of this effect are warranted.

**Keywords:** Bacterial load, cecal ligation and perforation, mortality, pomegranate, sepsis, survival rate

### INTRODUCTION

Sepsis and septic shock are one of the leading causes of mortality in intensive care units (ICU).<sup>[1]</sup> Severe sepsis results in

more than 200,000 annual fatalities and the number of cases are projected to increase. Epidemiological studies of the incidence of sepsis indicate that between 11% and 27% of ICU admissions have severe sepsis, with mortality rates ranging from 20% to more than 50%.<sup>[2]</sup>

Sepsis is a severe systemic illness caused by invasion of pathogens to normally sterile parts of body. This local infection causes a systemic response which leads to septic shock and death. It is a complex disease, which causes oxidative stress and also unbalance between pro-inflammatory and anti-inflammatory processes.<sup>[3,4]</sup> Free radical production is implicated in this process, both as a mechanism for direct cellular injury and in activation of intracellular signaling cascades within inflammatory cells resulting in progression of the inflammatory responses.<sup>[5]</sup> The clear evidence for oxidative stress in sepsis and the link with inflammatory gene expression (including cyclooxygenase-2 [COX-2], inducible nitric oxide synthase [iNOS]) and intracellular signaling pathways (including nuclear factor  $\kappa$ B [NF- $\kappa$ B] and mitogen-activated protein kinase (MAPK) pathways), have provided a foundation for interventions to either reduce oxidative stress which could be through using antioxidant therapy or inhibit transcriptional activation.<sup>[6,7]</sup> Multiple organ dysfunction syndrome (MODS), one of the major causes of death from sepsis, is also resulted from oxidative stress and hyper-inflammatory state.<sup>[8]</sup> Some studies using antioxidants and anti-inflammatory reagents found clinical benefits and survival improvement.<sup>[5,9,10]</sup>

Pomegranate (*Punica granatum*, Punicaceae), a fruit with high content of polyphenols, has been focus of recent studies because of its antioxidant and anti-inflammatory properties.<sup>[11]</sup> The polyphenol content and antioxidant activity of pomegranate has been shown to be more and stronger than many potent antioxidants such as green tea.<sup>[12]</sup> Its anti-inflammatory effects happen via inhibition of cell signaling pathways including suppression of COX-2 and iNOS expression, inhibition of activation of NF- $\kappa$ B, and inhibition of phosphorylation of MAPKs proteins.<sup>[13-15]</sup>

The effect of some antioxidants and polyphenols on sepsis have been studied before, and most of the studies reported beneficial effects of polyphenol administration in sepsis models.<sup>[16-19]</sup> The reported

protective effects of polyphenols in sepsis includes: Improving mortality rate,<sup>[19]</sup> and controlling inflammatory and oxidative outburst.<sup>[10,20]</sup> However, there are some recent studies presenting no effect of polyphenols in similar condition.<sup>[21]</sup> To the best of our knowledge, the effect of pomegranate on sepsis has not been investigated yet. According to high polyphenol content and anti-oxidative and anti-inflammatory effects of pomegranate, we hypothesized that pomegranate may protect against sepsis mortality, thus, the aim of the current study is to evaluate the effect of POMx, a standard pomegranate extract, on mortality and peritoneal bacterial load in cecal ligation and perforation (CLP) model of sepsis in rats.

## METHODS

### Animals

Male Wistar rats, 3-4 months old (300-350 g), were purchased from Pasteur Institute of Iran (Tehran, Iran). All the animals were housed in plastic cages (2 in a cage) at standard condition including: 22°C  $\pm$  2°C, 12 h light/dark cycle, standard humidity and free access to both food (standard rat chow) and water. The animals were allowed to acclimatize for 1 week before the experiment. All the procedures were carried out in accordance with the National Institutes of Health guidelines for the humane use of laboratory animals. The study was approved by Ethics Committee for Animal Experimentation of Tehran University of Medical Sciences.

### Sepsis model

The CLP method was used to induce sepsis in rats.<sup>[22,23]</sup> Briefly, rats were anesthetized with intra peritoneal injection of Ketamine (80-100 mg/Kg) and Xylazine (5-10 mg/Kg). After deep anesthesia, rats were placed in a supine position and the abdominal area was shaved. A three cm incision was made in abdominal wall and cecum was exposed. The cecum was ligated at the middle, to induce a moderate severity sepsis,<sup>[22]</sup> and was perforated twice distal to ligation place, with a 18-G needle. Small droplets of feces were extruded from both of holes. After relocating the cecum to the abdominal cavity, the abdominal wall was repaired in two layers, using a 4-0 sterile silk suture. A sham surgery was also made in control group with same procedure except the ligation and perforation step.

All the animals were resuscitated by injecting pre-warmed normal saline (37°C; 5 ml per 100 g body weight) subcutaneously and were kept under close observation in a warm room until recovery. All the animals had free access to food and water after recovery.

### **Pomegranate treatments and experimental design**

Pomegranate fruit liquid extract (POMx) was purchased from POM Wonderful (Los Angeles, CA). The POMx is a liquid concentrated extract that is produced by extraction of the remaining fruit residue obtained after the first pressing for pomegranate juice and contains natural pomegranate polyphenol extract. Each 5 ml of extract contains 1000 mg of pomegranate polyphenols.

The rats were randomly divided to 4 groups of 24 each: sham, the animals underwent the sham surgery without any other intervention; CLP, the animals underwent CLP surgery without any other intervention; prevention, the rats consumed POMx (250 mg of polyphenols/kg/day) in their drinking water for 4 weeks before undergoing CLP surgery; and treatment, the rats received a single drink of POMx (250 mg of polyphenols/kg) after CLP surgery. The POMx dose were selected according to previous studies,<sup>[24]</sup> which was hypothesized to modulate the oxidative stress and inflammatory outburst in sepsis. Due to light sensitive nature of POMx, fresh POMx was added to dark bottles containing drinking water on a daily basis.

### **Survival study**

As mentioned earlier, 24 rats were allocated in each group of study. Immediately after CLP induction, 16 rats were selected randomly for survival study and divided into two subgroups: with and without antibiotics (AB<sup>+</sup> and AB<sup>-</sup>). The group with antibiotics received Imipenem/cilastatin 25 mg/kg every 12 h subcutaneously for 3 days. Survival was monitored for 10 days. The remaining 8 rats were tested for bacterial load study.

### **Bacterial load**

Eight rats selected for bacterial load study were sacrificed humanitarily 24 h after surgery and the bacterial load of peritoneal cavity was assessed as follows: Briefly, the peritoneal cavity lavage

was performed with 10 ml phosphate buffered saline (PBS). Serial dilutions of peritoneal lavage in PBS were plated onto nutrient agar plates. Colony-forming units (CFU) were counted after incubation at 37°C for 24 h and calculated as CFU per peritoneal cavity.

### **Statistical analysis**

Survival rate was analyzed with log-rank test. The difference between bacterial load of groups were analyzed with Kruskal-Wallis test. The level of significance was set at 5%. All analyses were performed with the SPSS statistical package 15.0 (SPSS Inc., Chicago, IL, USA). The graphs were created using GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., San Diego California USA).

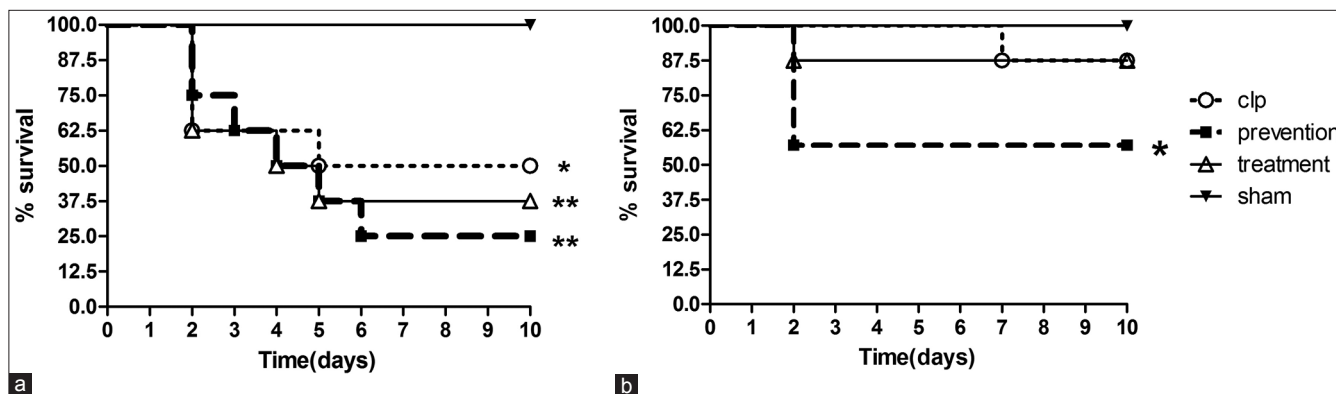
## **RESULTS**

### **Survival study**

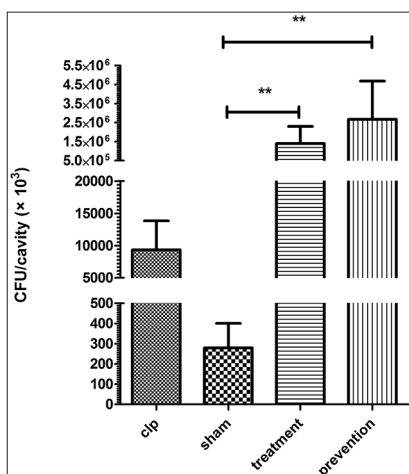
Survival rate of animals with or without antibiotic was determined. In AB<sup>-</sup> subgroups, CLP, treatment and prevention groups showed significantly lower survival rate compared to sham group ( $P < 0.05$ ,  $P < 0.01$  and  $P < 0.01$ , respectively). Although prevention and treatment groups had less survival rate than CLP group (25% and 37.5% vs. 50%), the difference between CLP and these groups were not significant [Figure 1a]. In AB<sup>+</sup> subgroups, antibiotic therapy improved the survival rate of CLP and treatment groups (87.5%,  $P = 0.317$  compared to sham group, for both groups). Although the survival rate of prevention group improved as well after antibiotic therapy, these animals exhibited significantly lower survival than sham group (57.1%,  $P = 0.046$ ) [Figure 1b]. The difference between CLP and POMx intervention groups were not significant.

### **Determination of bacterial load of peritoneal cavity**

To further examine the effect of POMx on sepsis, bacterial load of peritoneal cavity was evaluated. There was a significant difference between groups ( $P = 0.002$ ). The prevention and treatment group had significantly higher bacterial load comparing to sham group ( $P < 0.01$ ) [Figure 2], but their difference with CLP group was not significant.



**Figure 1:** Effect of pomegranate fruit liquid extract (POMx) on 10 day survival rate of rats with cecal ligation and perforation (CLP)-induced sepsis. Four subgroups of rats (sham, CLP, prevention and treatment) were subdivided to two subgroups, receiving Imipenem/cilastatin 25 mg/kg every 12 h for 3 days (b), or no antibiotic therapy (a). Ten day survival rate was assessed after CLP surgery. The prevention group consumed POMx (250 mg of polyphenols/kg/day) in their drinking water for 4 weeks before CLP surgery; and treatment group received a single drink of POMx (250 mg of polyphenols/kg) after CLP surgery. \* $P < 0.05$  and \*\* $P < 0.01$ , comparing to sham (log-rank test)



**Figure 2:** Peritoneal bacterial load of rats with cecal ligation and perforation (CLP)-induced sepsis. The prevention group consumed pomegranate fruit liquid extract (POMx) (250 mg of polyphenols/kg/day) in their drinking water for 4 weeks before CLP surgery; and treatment group received a single drink of POMx (250 mg of polyphenols/kg) after CLP surgery. \*\* $P < 0.01$ , comparing to sham (Kruskal-Wallis test)

## DISCUSSION

The current study examines the effect of POMx, a standard pomegranate extract, on mortality and peritoneal bacterial load in rat CLP model of sepsis. Opposite to our primary hypothesis and reported beneficial effects of some polyphenols such as curcumin,<sup>[16]</sup> we found aggravating effect of POMx on sepsis survival and bacterial load. In AB<sup>-</sup> subgroups, there was no significant statistical difference between CLP, treatment and prevention group, with all groups had significant lower survival

than sham group. With this assumption that POMx mainly control inflammatory outburst<sup>[25,26]</sup> which usually occur during sepsis, but could not *per se* reduce bacterial load, a subgroup of experimental groups were treated with a mixture of antibiotics to control both inflammation and bacterial load. Interestingly, antibiotic treatment normalized survival rate in both CLP and treatment groups, but in spite of improved survival rate in prevention group, the survival rate of this group still remained statistically lower than sham group. Based on aforesaid results, we hypothesized that the aggravating effect of POMx, may be attributed to the increased load of bacteria due to hypo-inflammatory state, which could potentially predispose the animals to more severe sepsis. The significant higher bacterial load of prevention and treatment groups supported these hypotheses. We think that a relatively high mortality rate and bacterial load in AB<sup>-</sup> subgroups may mask the aggravating effects of POMx in prevention group.

Interpretation of these findings needs understanding of sepsis pathogenesis and mechanisms through which other polyphenols affect sepsis outcome. After bacterial recognition, inflammatory response is stimulated through various signaling pathways. Although hyper-inflammation has been shown to cause MODS and mortality, proper inflammatory response is needed to control the infection and efficient function of immune system. An extreme hypo-inflammatory response, which leads to lack of inflammatory cytokines

and activation of adaptive immunity, will cause insufficient immune system response which subsequently leads to bacterial infection spreading all over the body.<sup>[21]</sup> Indeed, the mechanism of septic shock must be considered too. The vasodilation and myocardial dysfunction occurring at late sepsis is responsible for shock progression, with nitric oxide produced by iNOS has a major role in this context.<sup>[27]</sup>

Other point to consider is how polyphenols prevent or treat sepsis. It seems that anti-inflammatory properties of polyphenols are the major mechanism of their beneficial effects. In studies reported so far, polyphenols have been shown to inhibit MODS through modulation of primary inflammatory response in sepsis, which is accomplished by attenuation of local and systemic production of NF- $\kappa$ B-dependent inflammatory mediators, including tumor necrosis factor-alpha (TNF- $\alpha$ ), COX-2, and Intercellular Adhesion Molecule 1 (ICAM-1).<sup>[16]</sup> Suppression of iNOS expression by polyphenols is another mechanism by which they improve sepsis outcomes.<sup>[16]</sup> However, the safety and probable toxicity of polyphenols should not be forgotten. The harmful effects of polyphenols include potential for causing dose independent or dependent adverse effects, such as toxicity or endocrine system-disrupting effects of certain polyphenols.<sup>[28]</sup>

Considering above information, the results of our study put forward two distinct hypotheses to understand the cause of this aggravating effect. First, the detrimental effect may be due to anti-inflammatory effect of pomegranate. Since the bacterial load in prevention and treatment group is significantly higher compared to sham group, it seems that POMx may cause an extreme hypo-inflammatory response which leads to higher bacterial load and thereby increased mortality rate. Although, the beneficial and anti-inflammatory effect of similar dose of pomegranate extract was shown in other studies of chronic inflammatory states,<sup>[24]</sup> this dose of POMx seems to be unsuitable for sepsis, an acute inflammatory condition. It is yet to be determined whether other doses of POMx will improve the condition or cause adverse effect in sepsis, independent of dose. The second hypothesis is effect of pomegranate on iNOS and endothelial nitric oxide synthase (eNOS). Although, pomegranate has been shown to suppress iNOS expression, long-term consumption of pomegranate extract was demonstrated to increase arterial eNOS expression<sup>[29]</sup> and plasma nitrate and nitrite (NOx)

levels.<sup>[11]</sup> We hypothesized that this background high NOx may make the animals prone to septic shock. Besides, pro-inflammatory role for eNOS was shown in endotoxic shock models.<sup>[30]</sup> Proposed higher eNOS expression in prevention group may also explain the detrimental effect of POMx. Further studies are needed to test all these hypotheses.

To the best of our knowledge, this is the first study investigating the effect of pomegranate extract on CLP model of sepsis. However, we are aware that our work has limitations. First, we investigated a simple dose of POMx and the effect of other doses is yet to be investigated. Second, this study is a descriptive one which needs further steps to find out the mechanisms of pomegranate effect on sepsis.

## CONCLUSIONS

Our study demonstrates that pomegranate extract could increase mortality rate via increasing peritoneal cavity bacterial load, in CLP model of sepsis.

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