Original Communication

High Dose Pomegranate Extract Suppresses Neutrophil Myeloperoxidase and Induces Oxidative Stress in a Rat Model of Sepsis

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Abstract: Introduction: The effect of using high dose pomegranate extract on sepsis and its safety is not clarified. Considering the fact that proper immune and inflammatory responses are needed to cope with infection, the aim of current study was to assess the effect of high dose pomegranate extract consumption on oxidative and inflammatory responses after disease induction in rat model of sepsis. Methods: Sepsis was induced by Cecal Ligation and Perforation (CLP) surgery. Adult male Wistar rats were divided into three groups of eight animals: Sham; CLP and POMx [consumed POMx (250 mg of pomegranate fruit extract/kg/day) for four weeks before CLP]. Results: Peritoneal neutrophil myeloperoxidase activity was significantly lower in POMx compared with Sham and CLP groups ($p < 0.01$ and $p < 0.05$, respectively). Although antioxidant enzymes were higher in POMx group after sepsis induction, lower serum total antioxidant status (TAS) ($p < 0.01$ compared with both CLP and Sham groups) and higher liver thiobarbituric acid reactive species (TBARS) levels were observed in this group ($p < 0.01$ and $p < 0.05$, compared with Sham and CLP groups, respectively). Conclusion: High dose POMx consumption prior to sepsis induction, suppressed the vital function of neutrophils in early hours after sepsis initiation, resulting in higher oxidative stress. These findings indicate that caution should be made in using high dose pomegranate products. The main message of current study is that such useful compounds as antioxidants including pomegranate juice which have beneficial effects on general health status may have detrimental effects if misused or used in high doses.

Keywords: Sepsis, pomegranate, oxidative stress, myeloperoxidase, inflammation, rat

Introduction

Sepsis and systemic inflammatory response syndrome (SIRS) are major causes of patient admission and mortality at intensive care units (ICU). Sepsis is a systemic complex inflammatory and oxidative condition that occurs as a result of systemic manifestations of infection. Despite considerable number of studies performed to discover the therapeutic strategies for this disease, it is still a major clinical challenge and has high morbidity and mortality [1, 2].

Acute inflammation and oxidative stress are shown to be the central mechanisms of disease pathogenesis. For this reason limiting the hyperactivity of the host immune system and controlling the production of reactive oxygen species

(ROS) is pivotal to disease control [3, 4]. On the other hand, although the overproduction of ROS and inflammatory mediators leads to SIRS, septic shock and death, the proper levels of them are necessary to mount suitable responses against infection. Incomplete levels of inflammatory responses may prone the organism to outgrowth of infectious agents and consequently development of more severe disease [5].

Neutrophils are key effectors of the innate immune response and their migration to infectious focus is critical for the rapid eradication of bacterial pathogens. Although their hyper-activation may contribute to the development of multiple organ failure in sepsis, their insufficient activation at the beginning of disease establishment also may aggravate the disease and enhance its severity [6, 7]. Early enhanced local neutrophil recruitment accompanied by higher neutrophil myeloperoxidase (MPO) activity in the animal model of sepsis was shown to improve bacterial clearance and animal's survival [7]. Myeloperoxidase is a neutrophil enzyme which produces free radicals to combat with the bacterial pathogens and its deficiency was shown to impair survival in animal model of sepsis [8].

Pomegranate (*Punica granatum L.*), a fruit with high content of polyphenols, has been studied for its anti-inflammatory and anti-oxidative properties [9, 10]. Various mediators of inflammation and oxidative stress including MPO have been shown to be suppressed by pomegranate, which is the basis for pomegranate's beneficial effect in such chronic diseases [10, 11]. Pomegranate peel extract attenuated LPS-induced murine lung inflammation through neutrophil MPO suppression [12].

Although the safety of pomegranate fruit extract consumption in healthy rats has been addressed earlier [13], safety aspects of using these products in different pathological conditions including acute inflammation is not assessed thoroughly so far. Infection is an important complication in cancer patients, which can lead to acute organ dysfunction (severe sepsis) and eventually death. Cancer patients are known to be at higher risk for infection and subsequent complications, compared to the general population [14]. Considering the fact that proper immune responses of neutrophils and MPO activity are needed to cope with infection, an immediate question rises on how the extent of sepsis and its subsequent complications are affected by high doses of pomegranate extracts. This issue is more highlighted when high-dose pomegranate extract supplementation is suggested to be associated with a better outcome in cancer patients [15]. Our recent finding shows higher mortality rate in septic rats, which used high dose pomegranate extract for 4 weeks prior to sepsis induction [16]. Hence the aim of current study is to assess the effect of high dose pomegranate extract consumption on oxidative and inflammatory responses in a rat model of sepsis.

Materials and methods

Animals and sepsis model

Rat sepsis model was set up as we published recently [16]. Male Wistar rats, 3–4 months old; 300–350 g, were purchased from Pasteur Institute of Iran (Tehran, Iran) and housed in plastic cages (group of two) at standard condition: 22 ± 2 °C, standard humidity, 12 h cycle illumination and free access to food and drinking water. The animals were acclimatized for one week before the experiments. All the experiments were performed 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 and Avicenna Research Institute.

Sepsis was induced by cecal Ligation and Perforation (CLP) [17]. In brief, after abdominal wall incision; cecum was ligated at the middle below the ileocecal valve and perforated twice with an 18–G needle distal to ligation place to induce a moderate severity sepsis with approximately 50% mortality. A little fecal material was extruded from both holes. A sham surgery was also made in control group rats with the same procedure except the ligation and perforation step. All the experiences were performed in the same time of the day to avoid fluctuations due to circadian rhythm [18].

Pomegranate treatments and experimental design

POMx liquid (POM Wonderful, CA, USA) was used for all experiments. POMx liquid is a commercial product containing 1000 mg/5 ml of pomegranate fruit extract, which is equal to 8 oz glass of pomegranate Juice. The extract is produced as follows: after expelling most of the juice from the fruit, the remaining whole pomegranate (containing peels and membrans) are treated enzymatically and processed to get pomegranate polyphenol extract. This extract contains total phenolics [130 000 μg/mL of gallic acid equivalents (GAE) (13%)], mainly consist of hydrolyzable tannins (ellagitannins), such as oligomers and punicalagin/punicalin; and also smaller amounts of ellagic acid, anthocyanins (delphinidin, cyaniding, pelargonidin) and their glycosides. Other components which were mentioned by Aviram et al. include sugars (52%); organic acid (2.0%) and ash (2.0%) [19].

Animals were divided into three groups of eight animals: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent CLP surgery without any other intervention) and POMx (animals consumed POMx,250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before CLP surgery).

The pomegranate extract dosage was selected according to the previous reports [20, 21] showing to suppress most of the inflammatory mediators which are similar in both chronic and acute inflammation pathways, e.g. COX-2, iNOS and inflammatory cytokines. Both studies used 250 mg/kg/day pomegranate extract; however the product used in those studies were different with the extract we used. Using dose translation formula based on body surface area [22], human equivalent dose of pomegranate extract was estimated to be about 40 mg/kg or 3000 mg pomegranate fruit extract/75 kg, which is 3 times as the recommended dose by the manufacturer. This dose was also considered as 'high dose' in the study by Paller et al. [15] who assessed the effect of POMx (the same extract as we used) on the outcome of cancer in patients with prostate carcinoma.

Sampling procedure

First blood sampling was performed just before CLP surgery and the subsequent samples were taken 24 hour after surgery. All the animals were sacrificed humanitarily to collect their blood, peritoneal neutrophils and tissue samples. Blood was collected by cardiac puncture and the sera were separated and kept in −80 °C before analysis.

Peritoneal cavity neutrophils were collected via abdominal lavage with sterile phosphate buffered saline (PBS). Liver, lung and kidney samples were collected and rinsed with PBS to remove any RBCs or clots. Tissue homogenates (10% w/v) were prepared using cold buffers [KCL (Merck, Germany) 1.15% (w/v) for lipid peroxidation determination assay, potassium phosphate buffer 50 mM (pH = 7) containing 1 mM Ethylenediaminetetraacetic acid (EDTA) (Merck, Germany) for CAT and GluPx assay, potassium phosphate buffer 50 mM (pH = 7) containing 1 mM EDTA, 210 mM mannitol (Merck, Germany) and 70 mM sucrose (Merck, Germany) for SOD assay, and RIPA buffer containing protease inhibitor cocktail (PIC), phenylmethylsulfonyl fluoride (PMSF) and sodium orthovanadate (Santa Cruz-USA) for western blot (WB) analyses]. The homogenates were kept in −80 °C before analysis. Additional tissue samples were fixed in 10% formalin for 72 hours at room temperature and embedded in paraffin for immunohistochemical and histological examinations.

Peritoneal neutrophil separation and MPO activity assay

Cell suspensions from peritoneal fluid were used to separate peritoneal neutrophils. The peritoneal neutrophils were separated by density gradient centrifugation method using Ficoll-Paque (Amersham, USA) and hypotonic lysis to eliminate contaminating erythrocytes [23]. The separated cells were resuspended in 10 ml cold PBS and final cell concentration and viability was determined by using a haemocytometer and trypan blue exclusion test. A volume of solution containing 10^7 cells was aliquoted to extract MPO and centrifuged at 250 × g for 6 minutes. The pellets were suspended in 0.5% hexadecyltrimethylammonium bromide (HETAB) (Sigma, USA) in 50 mM potassium phosphate buffer, pH 6 and sonicated (Bandelin, Germany) in an ice bath for 10 seconds. The samples were freeze-thawed 3 times, after which sonication was repeated. The final suspensions were centrifuged at 12000 × g for 15 minutes at 4 °C and supernatant were used to assay MPO [23].

Peritoneal neutrophil MPO activity was assayed according to the method described by Suzuki et al [24], with slight modifications. This Method employs hydrogen peroxide and tetramethylbenzidine (TMB) as substrate and chromogen, respectively to measure MPO activity. The assay solution consisted of TMB solution [20 mM TMB (Sigma, USA) in Dimethylformamide (Merck, Germany)], neutrophil supernatant, 80 mM sodium phosphate buffer (pH 5.4), 0.03% H_2O_2 (Merck-Germany) and PBS. Since the reaction is so rapid and may reach plateau in assay time, different substrate concentrations were used and all concentrations were assayed in triplicate. Primarily, 4 µl TMB solution, neutrophil supernatant (0.5, 1, 2, 3, 4 µl), 20 µl sodium phosphate buffer and DDW (up to 28.4 µl) were mixed in a 96 well plate (Nunc, Denmark) and incubated for 5 min in room temperature. The primary absorbance was measured by a microplate reader (Bio Tek, USA) at 630 nm. The mixture of H_2O_2 and PBS (21.6 µl, 1.6:20 v/v) was rapidly added and the plate was incubated for 3 min at 37 °C and then immediately placed on ice. The reaction was terminated by the addition of 175 µl of 200 mM sodium acetate buffer (pH 3.0) and the final absorbance was measured at 630 nm. The difference of 3 minutes and baseline absorbances (ΔA_{630}) was calculated and MPO activity was expressed as ΔA_{630}/min/ml.

Serum total antioxidant status (TAS) and tissue lipid peroxidation level

To confirm the changes in antioxidant capacity associated with the pomegranate consumption, serum TAS was assayed before and after sepsis induction using a commercially available kit (Rel Assay Diagnostics, Turkey).

The lipid peroxidation level of lung and liver tissues were determined using the method of Ohkawa et al. [25]. The method was based on measuring thiobarbituric acid reactive species (TBARS) level in animal tissue. The reaction mixture [contained 0.1 ml of ten percent (w/v) tissue homogenates, 0.2 ml of 8.1% sodium dodecylsulfate (SDS)

(Merck, Germany), 1.5 ml of 20% acetic acid (pH = 3.5) (Merck, Germany), 1.5 ml of 0.8% aqueous solution of thiobarbituric acid (TBA) (Sigma, USA) and 0.7 ml distilled water] was heated at 95 °C for 60 min. After cooling with tap water, 1.0 ml distilled water and 5.0 ml n-butanol (Merck, Germany) were added and the mixture was shaken vigorously. After centrifugation at 3200 × g for 10 min at 4 °C, the organic layer (upper layer) was used for measurement. The measurement were performed fluorometrically with a fluorimeter (Multilable counter, Perkin Elmer, USA) equipped with a band-pass filter (excitation: 515 nm, emission: 550 nm) and the results were expressed as mM/lit.

Tissue SOD, CAT and GluPx activity measurements

Lung and liver homogenates (10% w/v) were used to assay tissue SOD, CAT and GluPx activity. Tissue SOD and GluPx were determined using commercially available kits (Cayman chemical company, Ann Arbor, MI, USA). Tissue CAT was assayed using the method of Aebi [26]. The method was based on the catalytic activity of CAT and the absorbance of H_2O_2 at 240 nm.

Serum nitrite and nitrate assay

Serum nitrite and nitrate (NO_x) levels were assayed based on modified method of Griess assay described by Miranda et al [27]. The principle of this assay is reduction of nitrate to nitrite by vanadium combined with detection of nitrite by the acidic Griess reaction. Briefly, serum samples were deproteinized by pre-chilled ethanol (Merck, Germany) (−20° C) [28]. One hundred µl of deproteinized serum samples were incubated with equal volumes of VCl_3 (Sigma, USA) and Griess reagents containing Sulfanilamide (Sigma, USA) and N-(1-naphthyl)-ethylenediamine hydrochloride (Sigma, USA) overnight. The absorbance was measured by a microplate reader (Bio Tek, USA) at 540 nm. NO_x concentration was calculated using a $NaNO_2$ standard curve and expressed as µmol/L [27, 28].

Serum cytokine assay

Levels of tumor necrosis factor (TNF)-α, IL-6 and IL-10 were measured in animal sera by sandwich ELISA according to manufacturers' recommendations (BD Bioscience, USA). All measurements were done in duplicate. Minimal detection limits for TNF-α, IL-6 and IL-10 were 31.3, 78 and 15.6 pg/mL, respectively.

WB analysis of iNOS and COX-2 expression

Liver and lung tissue homogenates were used for WB analyses of iNOS and COX-2 expression. Protein concentration of samples was measured using BCA protein assay kit (Pierce-USA). Western blotting of iNOS and COX-2 were performed according to the protocol published elsewhere with some modifications [29]. A total of 50 µg and 20 µg protein per lane for iNOS and COX-2, respectively, were added to sample buffer [containing 5% 2-Mercapto Ethanol (Sigma, USA)] and subjected to SDS-PAGE electrophoresis at 100 V for 90 min. The proteins were subsequently transferred onto nitrocellulose membrane (Millipore, USA). After blocking with 5% non-fat dry milk in PBS containing 0.1% Tween (PBST) overnight at 4 °C, blots were incubated in primary antibody [1:10000 of polyclonal anti-iNOS antibody (Sigma, U.S.A.), overnight at 4 °C or 1:100 of polyclonal anti-COX-2 antibody (Thermo Scientific, USA) for 2 hours at room temperature). Blots were washed and then incubated 1 h in HRP-conjugated anti-rabbit IgG (Avicenna Research Institute, Iran, 1:8000). iNOS and COX-2 bands were detected using the enhanced chemiluminescence detection system (Amersham-UK) according to the manufacturer's protocol. LPS-treated mouse macrophage cell line (RAW 264.7 cell line, Avicenna Research Institute) was used as positive control. Beta-actin western blotting was employed as loading control. Analysis of WB bands was performed using AlphaEase FC software (Alpha Innotech, CA, USA) according to the protocol we published elsewhere [29, 30]. Relative expression of iNOS and COX-2 was presented as the percent of iNOS or COX-2 band density relative to corresponding β-actin density.

Immunohistochemical analysis of iNOS and COX-2 expression

Immunohistochemistry (IHC) was performed on 5 micron paraffin sections of liver and lung tissues as we published elsewhere with some modifications [31]. LPS-treated RAW 264.7 cells were used as positive control. Briefly, after deparaffinization and rehydration, tissue sections were subjected to heat-activated antigen retrieval. Following washing steps, endogenous peroxidase activity was quenched by 1% H_2O_2 followed by additional washing steps and blocking of non-specific binding sites with 5% normal sheep serum diluted in protein block (Dako, CA, USA). Tissue sections subjected to iNOS analysis were also incubated with Avidin-Biotin blocking solutions (Dako, Glostrup, Denmark) to block endogenous biotin activity. Anti-iNOS (Millipore, USA) (1:5000) or anti-COX-2 (Thermo Scientific, USA) (1:200) antibodies were added to the slides (overnight at 4 °C for iNOS and 90 min at room temperature for COX-2). iNOS slides were sequentially

incubated with biotin-conjugated goat anti-rabbit antibody (Sigma, USA) (1:800 for 45 min) and streptavidin-horseradish peroxidase (Biosource, Camarillo, CA) (1:750, 30 minutes). Signals were visualized after incubation with 3,3'-diaminobenzide tetrahydrochloride (DAB) (Roche, USA) as the substrate. COX-2 signals were visualized by indirect immunostaining system [peroxidase-conjugated sheep anti-mouse Ig (Avicenna Research Institute, Tehran, Iran) and DAB]. In negative reagent control slides, primary antibodies were substituted by the same concentrations of pre-immune rabbit serum and rabbit IgG for iNOS and COX-2, respectively. Slides were counterstained with Harris hematoxylin, dehydrated and mounted. Digital images were captured by BX51 microscope and DP70 CCD camera (Olympus, Japan). The cells positive for COX-2 were enumerated and the level of iNOS signals was scored visually.

Organ failure examination

The severity of organ failure was studied by histological and biochemical examination. Liver, lung and kidney tissue 5 micron paraffin sections were stained with hematoxylin and eosin and examined blindly under light microscopy by an experienced pathologist. Edema, congestion, inflammatory cell infiltration, necrosis and degeneration were evaluated to establish the presence or absence of tissue injury. Serum blood urea nitrogen (BUN), creatinine (Cr), aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin (Bil) and lactate dehydrogenase (LDH) were measured using an automatic analyzer (Eurolyser, Austria).

Statistical analysis

Data were analyzed statistically using the SPSS 22.0 software. The normality of data distribution was tested by one-sample Kolmogorov Smirnov test. Since the data were not normally distributed, the difference between groups was studied by Mann-Whitney U non parametric test. The graphs were plotted using Graph-Pad Prism version 5.00 for Windows (GraphPad Software, CA, USA). The p value < 0.05 was set as statistical significance.

Results

Effect of POMx treatment on peritoneal neutrophil MPO activity

MPO activity was assessed in order to test whether peritoneal neutrophils maintain such activity necessary to kill bacteria after POMx treatment [7]. The MPO activity of POMx group was significantly lower than sham and CLP groups ($p < 0.01$ and $p < 0.05$, respectively) (Fig. 1); however no significant difference was observed between CLP and Sham groups.

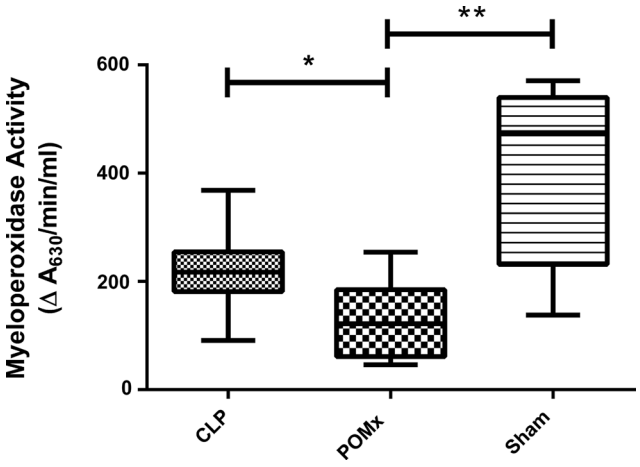

Figure 1. The effect of pomegranate polyphenol liquid extract on peritoneal neutrophil Myeloperoxidase (MPO) activity after cecal ligation and perforation surgery. Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention), and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each. Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (*$p <$ 0.05, **$p < 0.01$).

Effect of POMx treatment on serum TAS

Since TAS reflects the effect of dietary antioxidants on circulatory antioxidant status and also shows oxidative stress severity [32], serum TAS was evaluated before and after sepsis induction. The result of animals consuming POMx for four weeks showed significantly higher TAS values compared with normal animals before CLP surgery ($p < 0.001$) (Fig. 2a). After sepsis induction, the TAS levels of CLP and POMx groups were significantly lower than Sham group ($p < 0.01$ for both analyses) and the POMx group had lower TAS levels compared with CLP group ($p < 0.01$) (Fig. 2b).

Effect of POMx treatment on tissue TBARS level

In order to examine lipid peroxidation, as an indicator of oxidative stress-induced tissue damage, the level of TBARS in liver and lung homogenates were determined [25]. The liver samples of POMx group had higher amounts of TBARS

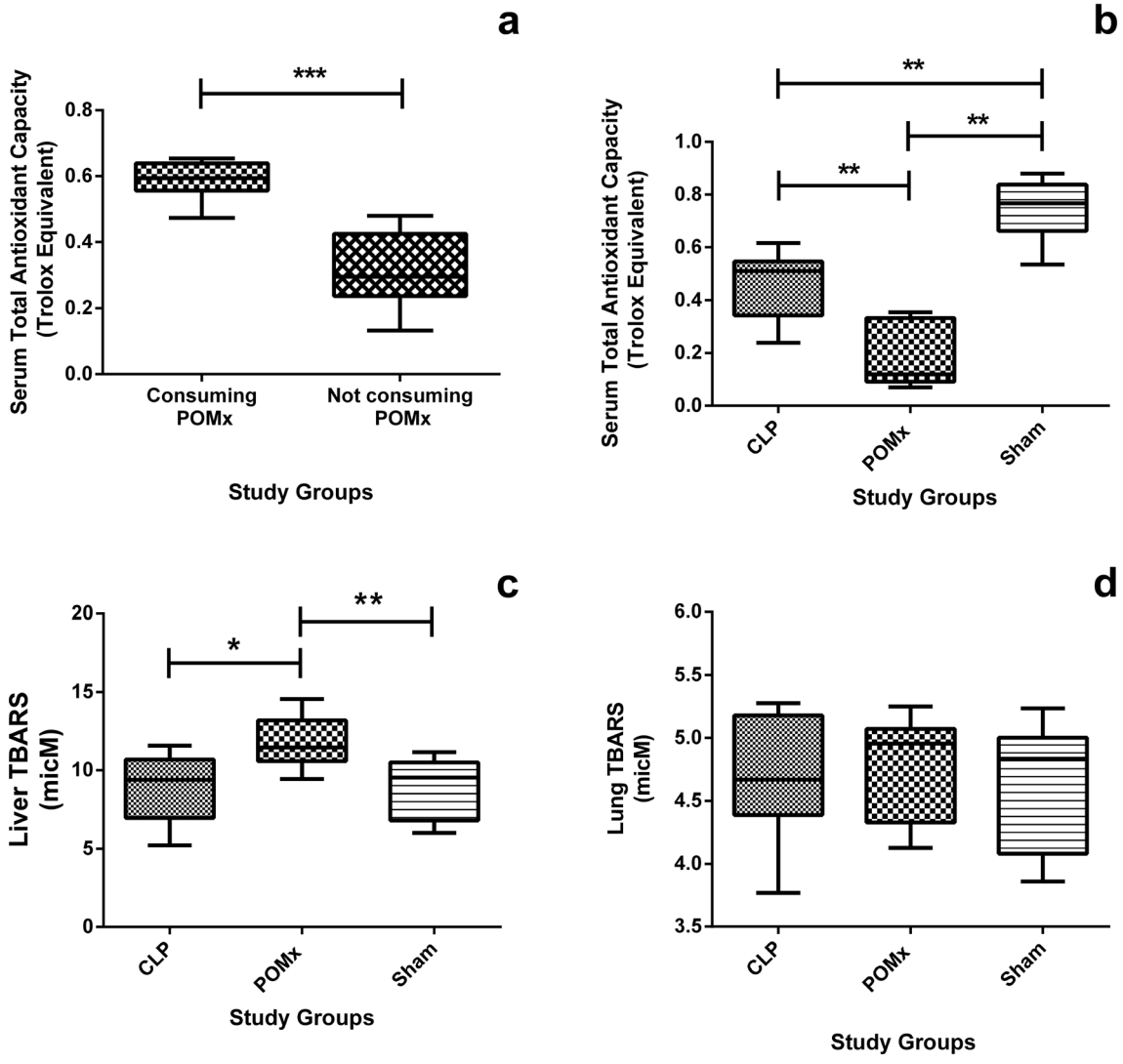

Figure 2. The effect of pomegranate polyphenol liquid extract on serum total antioxidant status before (a) and after (b) cecal ligation and perforation surgery and thiobarbituric acid reactive species(TBARS) level in liver (c) and lung (d) tissue after cecal ligation and perforation surgery. The consuming POMx group consumed pomegranate polyphenol liquid extract (250 mg of pomegranate fruit extract/kg/day) in their drinking water for four weeks (n = 8) and not consuming POMx group were normal rats before randomization in to groups and having cecal ligation and perforation surgery (n = 16) (a). Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each (b, c, d). Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

compared to CLP and Sham groups ($p < 0.05$ and $p < 0.01$, respectively) (Fig. 2c). The level of TBARS in lung samples did not differ significantly between all groups (Fig. 2d).

Effect of POMx treatment on tissue SOD, CAT and GluPx activities

SOD, CAT and GluPx activities were assayed as these enzymes have a crucial role in internal antioxidant defense [33]. The levels of SOD activity were assayed in liver and lung of experimental groups. There was no significant difference between SOD activity of groups in both tissues (Fig. 3a, b).

The result of CAT activity assay showed that the liver tissue of POMx group had higher enzyme activity compared to CLP groups ($p < 0.01$). Assessment of Lung samples, showed no difference between groups (Fig. 3c, d).

The POMx group had higher amounts of liver GluPx activity, compared to CLP group ($p < 0.05$). However, there were no significant differences in GluPx activity of experimental groups' lung tissue (Fig. 3e, f).

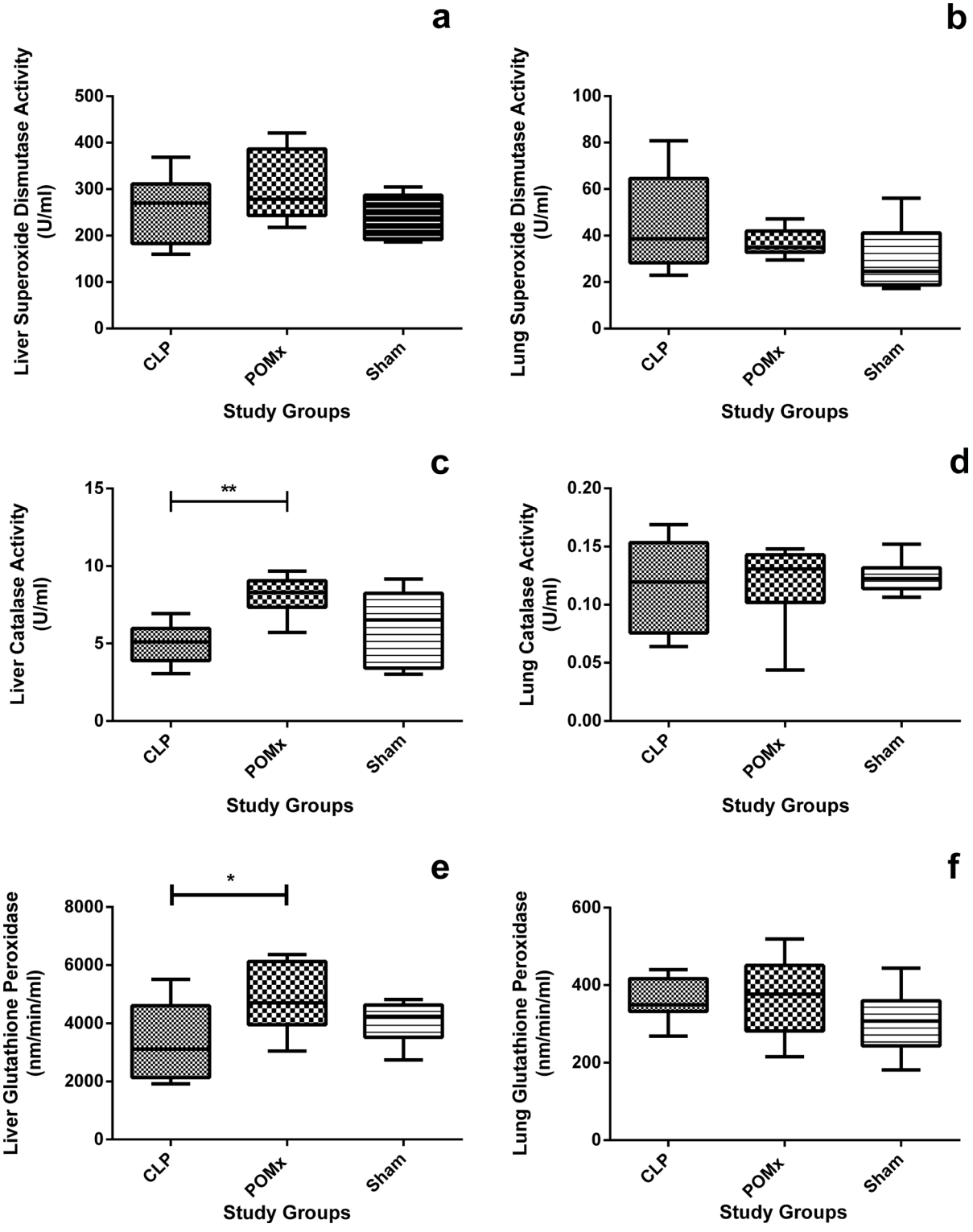

Figure 3. The effect of pomegranate polyphenol liquid extract on activity of antioxidant enzymes; superoxide dismutase (SOD) in liver (a) and lung (b), catalase (CAT) in liver (c) and lung (d) and glutathione peroxidase (GluPx) in liver (e) and lung (f). Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract,250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery) n = 8 each. Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (*$p < 0.05$, **$p < 0.01$).

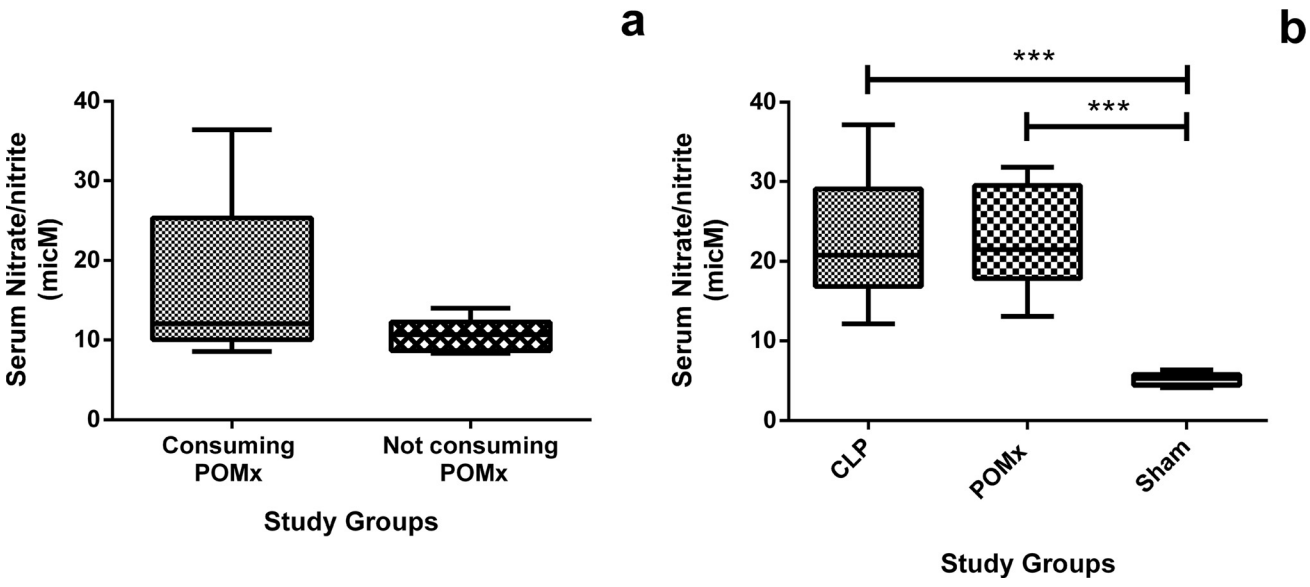

Figure 4. The effect of pomegranate polyphenol liquid extract on serum Nitrate/nitrite level before (a) and after (b) CLP surgery. The Consuming POMx group consumed pomegranate polyphenol liquid extract (250 mg of polyphenols/kg/day) in their drinking water for four weeks (n = 8) and Not consuming POMx group were normal rats before randomization in to groups and having cecal ligation and perforation surgery (n = 16) (a). Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each (b). Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (***$p < 0.001$).

Effect of POMx treatment on serum nitrite and nitrate

Serum NO_x level (the stable byproducts of nitric oxide observed in sepsis) was determined, as it has critical role in septic shock progression [34]. There was no difference between experimental groups regarding the pre-sepsis NO_x levels (Fig. 4a). The result of post-sepsis serum NO_x levels demonstrated a significant difference between sham and other groups ($p < 0.001$ for both analyses), whereas there was no difference between CLP and POMx groups (Fig. 4b).

Effect of POMx treatment on serum cytokines

The serum levels of TNF-α and IL-6 as inflammatory cytokines and IL-10 as anti-inflammatory cytokine were measured. The levels of TNF-α and IL-6 in CLP and POMx groups were significantly higher than sham group (respectively $p < 0.05$ and $p < 0.05$ for TNF-α and $p < 0.05$ and $p < 0.01$ for IL-6) (Fig. 6a, b). Similarly, the IL-10 level of CLP and POMx groups were significantly higher than sham groups ($p < 0.001$, for each analysis) (Fig. 5c). There was no statistical difference between CLP and POMx groups in all analyses.

Effect of POMx treatment on iNOS and COX-2 protein expression

Protein expression of iNOS and COX-2 was assessed by WB analyses and IHC. Neither iNOS nor COX-2 was detectable in lung tissue by both WB and IHC. Furthermore, liver iNOS protein expression was not detectable by WB. Eventually, iNOS expression was examined by IHC and COX-2 expression was examined by both WB and IHC in liver tissue of animals.

Regarding iNOS asseement, CLP group showed higher expression of iNOS compared with Sham group ($p < 0.05$) (Fig 6a–f, m). There was no difference between POMx and other experimental groups. The results of IHC analyses showed that the expression of COX-2 was higher in CLP compared with Sham group ($p < 0.05$) (Fig. 6g–l, n). However, there was no different between groups regarding W.B. (Fig 7).

Effect of POMx treatment on organ failure

Organ failure variables were assessed as markers of disease prognosis [35]. No difference was detected between experimental groups concerning biochemical (Tab. 1) and histological examination (Fig. 8).

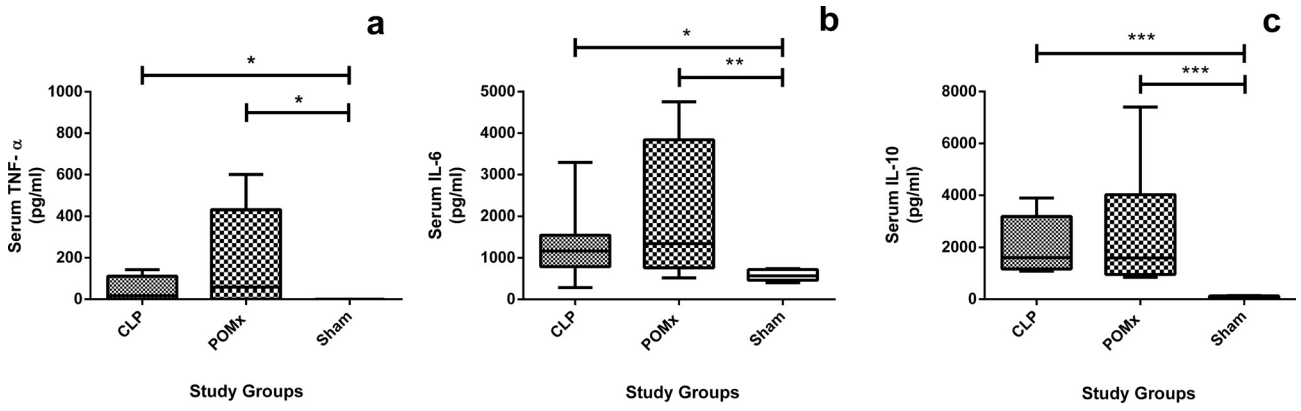

Figure 5. The effect of pomegranate polyphenol liquid extract on serum cytokines after cecal ligation and perforation surgery, tumor necrosis factor (TNF)-α (a), interleukin (IL)-6 (b), IL-10 (c). Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each. Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

Discussion

Since pomegranate extract has been studied for its anti-inflammatory and anti-cancer effects, a thorough assessment of its safety aspects, including its high dose effect on sepsis, a prevalent cancer complication, is mandatory.

Comparing CLP and POMx groups, peritoneal neutrophil MPO activity was significantly lower in POMx compared with CLP group. Our intervention, which was POMx consumption prior to sepsis induction, suppressed this vital function of neutrophils in early hours of sepsis induction, thus led to higher mortality and bacterial load in this group, as we showed previously [16]. The suppressive effect on neutrophil MPO was previously reported by Makled et al [36] that showed pomegranate fruit extract consumption prior to CLP induction decreased hepatic MPO activity.

SOD, CAT and GluPx are among antioxidant enzymes which are generally evaluated to monitor the effect of antioxidant supplementation. In line with literature [37, 38], our results showed that the POMx group had higher liver CAT and GluPx activity compared to CLP group. However, there was no difference in SOD activity between groups. There is a great controversy on potential effect of pomegranate on SOD activity; while some reports showed inductive effect [38], the results of the other studies failed to show such association [39]. Based on interaction between aforesaid antioxidant enzymes [3], one may expect that the net effect in POMx group would be in favor of decreasing hydrogen peroxide and hydroxyl radicals; a condition which potentially leads to lower liver oxidative stress and better outcome. Previous studies showed that elevation of SOD activity with no concomitant increase in CAT activity, a condition that occurs in severe sepsis, results in increased levels of hydrogen peroxide and could increase the mortality [40]. The overproduction of the hydrogen peroxide is accompanied with generation of higher levels of hydroxyl radicals that are thought to be the most hazardous reactive oxygen species [40].

To investigate the effect of favorable pattern of antioxidant enzymes beside lower MPO activity in POMx group, the levels of TAS and TBARS were measured. TAS and TBARS are among the biomarkers that are generally used to evaluate the severity of oxidative stress and damage. TAS reflects the endogenous as well as food-derived circulating antioxidants. The more the TAS levels, the less oxidative stress exists [32]. The level of TBARS reflects sepsis severity and it is shown that TBARS is higher in lethal compared with non-lethal sepsis [41]. In line with the results of antioxidant enzymes, POMx group exhibited higher levels of TAS prior to sepsis induction, indicating reduced serum oxidative status. These findings are in consistent with previous reports on the effect of POMx on chronic diseases [42, 43]. However, post sepsis results of TAS and TBARS were in line for the lower MPO of POMx group, as this group had significantly lower TAS and higher TBARS values compared with CLP and Sham groups. In consistent with these findings we recently showed that consuming POMx before CLP induction increased mortality in a rat sepsis model [16]. Collectively, these results suggest that elevated levels of antioxidant enzymes following POMx consumption were not effective to combat the oxidative stress-induced lipid peroxidation and to reduce disease mortality and bacterial load, as we showed recently [16]. Such conclusion has also been reported earlier by Batra et al. in neonatal sepsis [44].

The effect of pomegranate extract consumption on NO regulation was assessed by measuring serum NOx and tissue iNOS expression. Although pomegranate was shown to decrease the expression of iNOS and subsequent

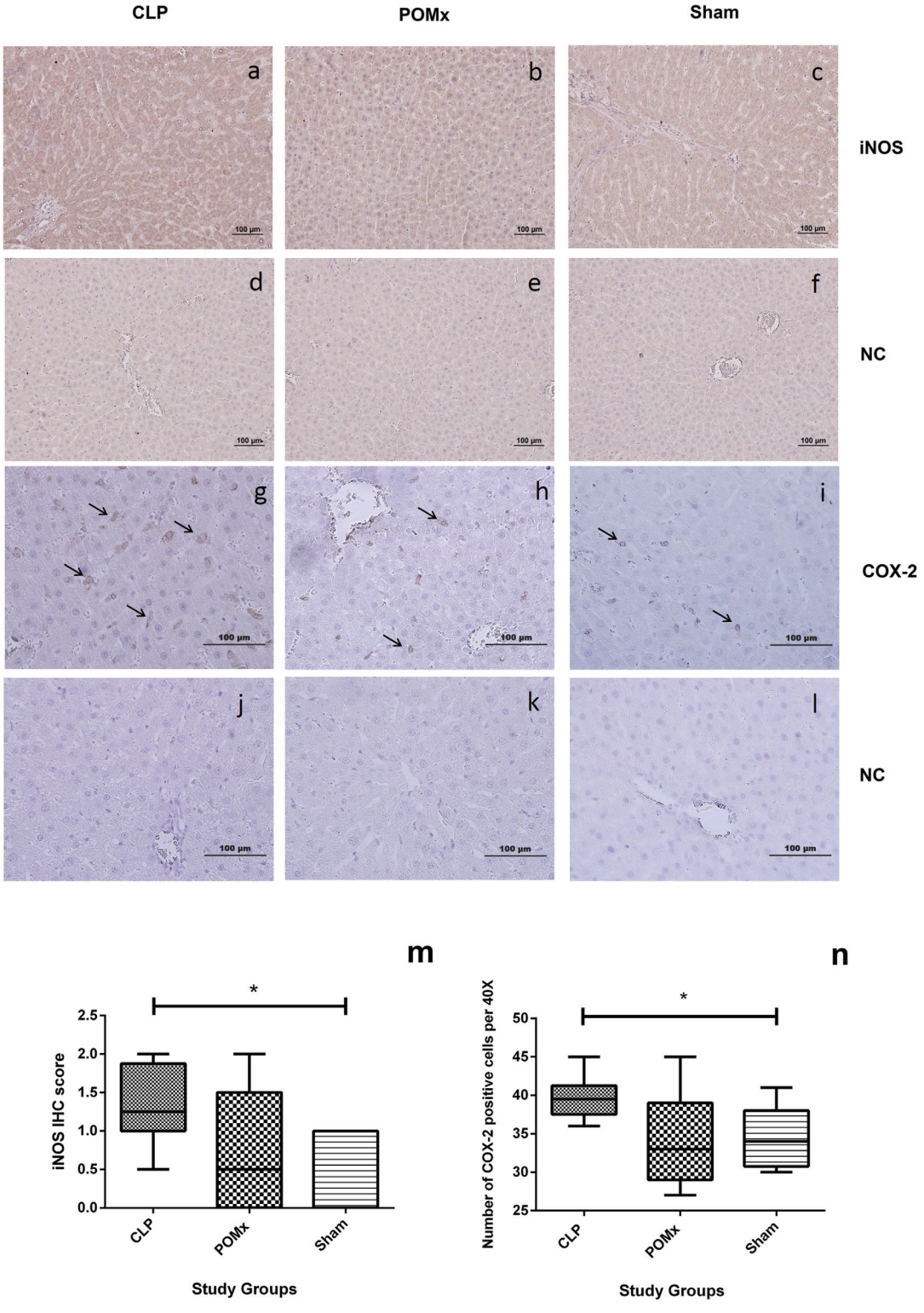

Figure 6. The effect of pomegranate polyphenol liquid extract on immunohistochemical analysis of inducible nitric oxide synthase (iNOS) (a–f, m) and cyclooxygenase 2 (COX-2) (g-l, n) in liver tissue of experimental groups. Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each. Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values. NC: Negative control (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

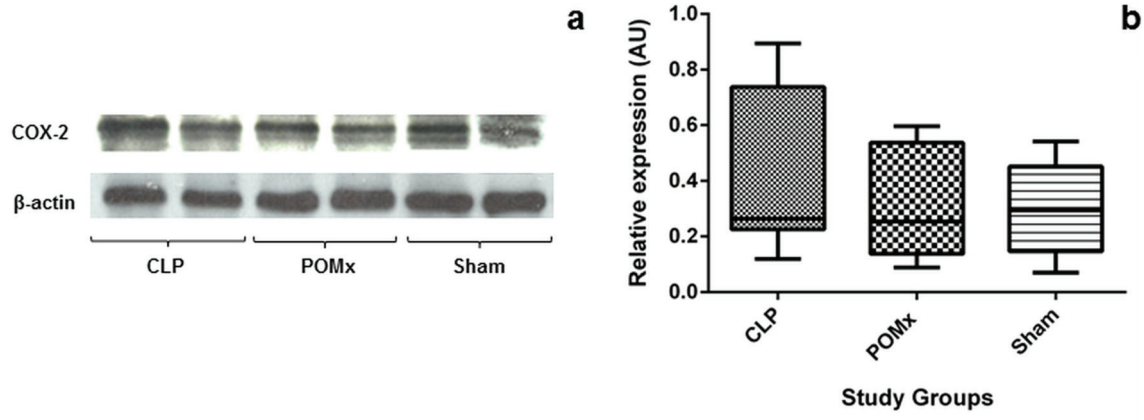

Figure 7. The effect of pomegranate polyphenol liquid extract on western blot analysis of cyclooxygenase 2 (COX-2) in liver tissue of experimental groups. Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each. Values are expressed as median ± Interquartile range and error bars represent minimum to maximum values (b).

Table 1. The effect of pomegranate polyphenol liquid extract on the severity of organ failure, studied by biochemical analyses. Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed of pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery)

	CLP (n = 8)	POMx (n = 8)	Sham (n = 8)	p-value [a]
BUN (mg/dl)	67.00 (58.25)	79.50 (134.05)	45.00 (31.25)	N.S.[b]
Creatinine (mg/dl)	0.65 (0.38)	0.65 (0.40)	0.60 (0.33)	N.S.
SGOT (U/L)	392.5 (304.3)	298.0 (137.0)	302.5 (145.2)	N.S.
SGPT (U/L)	106.0 (88.3)	89.5 (41.8)	65.5 (42.5)	N.S.
LDH (U/L)	1528 (1544)	935 (1014)	535 (659)	N.S.
Bilirubin (mg/dl)	0.20 (0.08)	0.20 (0.15)	0.15 (0.10)	N.S.

[a] Kruskal–Wallis one-way analysis of variance; [b] N.S. not significant; BUN: Blood urea nitrogen; SGOT: serum glutamic oxaloacetic transaminase; SGPT: serum glutamate-pyruvate transaminase; LDH: Lactate dehydrogenase.

NO production [45], chronic pomegranate consumption was shown to increase endothelial NOS (eNOS) expression and plasma NOx [46]. eNOS knockout mice were shown to be resistant to septic shock since NO from the eNOS is necessary to obtain maximal iNOS expression and activity [47]. In this context, we measured pre-septic serum NOx to evaluate if higher pre-septic NOx make the animals prone to higher severity and mortality. The results showed that neither pre-septic NOx nor post-septic NOx or iNOS were different between CLP and POMx groups. Since pre-septic NOx was not different between groups, this hypothesis seems unlikely. However, further studies with eNOS expression assessment are needed.

The remaining question is why inflammatory cytokines were not different between CLP and POMx groups despite higher TBARS levels in latter group and why no changes were detected in lung tissue. To answer, timing of event sequence and also the severity of disease model used in this study should be taken in mind. The CLP model used in this study was a moderate severity CLP model [17] which had about 50% mortality and the lethality began at the second day of disease [16], so the animals were not in late sepsis at the time of sampling. Considering time profile of oxidative and inflammatory changes in CLP model, oxidative stress and increase in MDA content has been shown to happen in onset of disease and precede the increase of inflammatory cytokines which happen later [48]. Regarding organ involvement in CLP model, it has been shown that low severe CLP models may not develop lung injury [49].

To our best knowledge, this is the first study investigating the effect of long term consumption of high dose pomegranate extract on peritoneal neutrophil myeloperoxidase in CLP model of sepsis. However, our study had some limitations; lack of dose-response experimental groups is the major limitation. Indeed, an additional arm to the study, where a rat model of a cancer coupled to this treatment was assessed for survival, could have strengthen the validity of our findings.

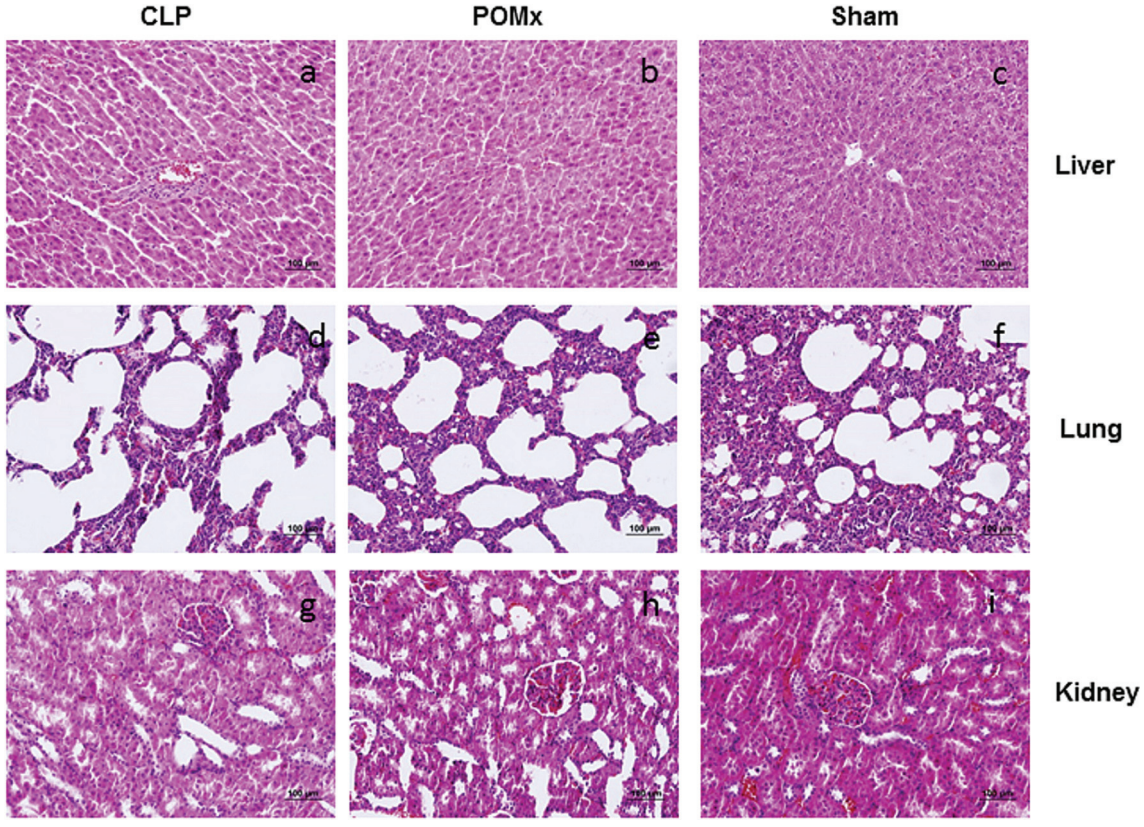

Figure 8. The effect of pomegranate polyphenol liquid extract on histological examination (H&E staining, X40) of liver, lung and kidney tissues of experimental groups after cecal ligation and perforation surgery. Experimental groups included: Sham (animals underwent the sham surgery without any other intervention), CLP (animals underwent cecal ligation and perforation surgery without any other intervention) and POMx (animals consumed pomegranate polyphenol liquid extract, 250 mg of pomegranate fruit extract/kg/day, in their drinking water four weeks before cecal ligation and perforation surgery); n = 8 each.

Conclusion

In conclusion, high dose POMx supplementation before sepsis induction resulted in impaired activity of peritoneal neutrophil MPO, a key enzyme for controlling infection at first hours of sepsis initiation, and consequent higher oxidative stress as judged by decreased serum TAS and increased liver TBARS levels. These findings indicate that caution should be made in using high dose pomegranate products. The main message of current study is that such useful compounds as antioxidants including pomegranate juice which have beneficial effect on general health status may have detrimental effects if misused or used in high doses.

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Conflict of interest
On behalf of all authors, the corresponding author states that there is no conflict of interest.

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