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Pomegranate protects liver against cecal ligation and puncture-induced oxidative stress and inflammation in rats through TLR_4/NF-κB pathway inhibition

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ABSTRACT

Acute liver injury secondary to sepsis is a major challenge in intensive care unit. This study was designed to investigate potential protective effects of pomegranate against sepsis-induced acute liver injury in rats and possible underlying mechanisms. Pomegranate was orally given (800 mg/kg/day) for two weeks before sepsis induction by cecal ligation and puncture (CLP). Pomegranate improved survival and attenuated liver inflammatory response, likely related to downregulation of mRNA expression of toll like recptor-4, reduced nuclear translocation and DNA binding activity of proinflammatory transcription factor NF-κB subunit p65, decreased mRNA and protein expression of tumor necrosis factor-alpha and reduction in myeloperoxidase activity and mRNA expression. Pomegranate also decreased CLP-induced oxidative stress as reflected by decreased malondialdehyde content, and increased reduced glutathione level and superoxide dismutase activity. These results confirm the antiinflammatory and antioxidant effects of pomegranate in CLP-induced acute liver injury mediated through inhibiting TLR_4/NF-κB pathway, lipid peroxidation and neutrophil infiltration.

1. Introduction

Liver diseases remain one of the serious health problems throughout the world associated with a high rate of morbidity and mortality (Pushpavalli et al., 2010). The impairment of liver is multifactorial where it may result from chronic use of alcohol, viral or bacterial or protozoal infection, drugs and other xenobiotics (Farghali et al., 2009). Today, millions of people worldwide suffer from various hepatic disorders. Among these hepatic disorders is acute hepatitis which is the most prevalent and occurs at many countries (Manna et al., 2007).

Sepsis is a major clinical challenge in intensive care units causing acute liver injury. Cecal ligation and puncture (CLP) induced liver injury is a well-established model because it closely resembles the progression and characteristics of human sepsis in which liver injury is mediated through an inflammatory pathway (Dejager et al., 2011; Kobayashi et al., 2001).

Liver plays a central role in bacterial phagocytosis and clearance. The invading bacteria and their products are firstly captured and cleared by liver cells, which release several proinflammatory mediators, such as tumor necrosis factor- (TNF-) α, interleukin- (IL-) 1, IL-6, interferon-γ, IL-8, nitric oxide, and reactive oxygen species (ROS), which cause liver injury (Dhainaut et al., 2001; Yan et al., 2014). It has been previously reported that attenuation of liver injury and restoration of liver function lowers morbidity and mortality rates in patients with sepsis (Yan et al., 2014).

Acute liver injury induced by sepsis is largely initiated through activation of toll like receptors (TLR_4) by bacterial products (e.g., LPS or lipoteicohoic acid or peptidoglycan) or cytokines (e.g., TNF-α or IL-1), which leads to activation of transcription factor-nuclear factor-kappa B (NF-κB), resulting in enhanced transcription of

Abbreviations: CLP, cecal ligation and puncture; GSH, reduced glutathione; LDH, lactate dehydrogenase; MDA, malondialdehyde; MPO, myeloperoxidase; κB, nuclear factor-κB; SOD, superoxide dismutase; TLR, toll-like receptor-4, tumor necrosis factor-α.

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genes responsible for the expression of proinflammatory cytokines such as TNF-α, chemokines, adhesion molecules, apoptotic factors, and other mediators of the inflammatory response associated with sepsis (Abraham, 2003; Liu et al., 2015).

NF-κB is a major player in regulating the expression of many immunoregulatory mediators implicated in oxidative stress and consequently in sepsis (Sakaguchi and Furusawa, 2006). Once NF-κB p65 is activated through phosphorylation of its inhibitory protein kappa B- alpha (IκB)-α by Ikappa B kinase (IKK), NF-κB p65 translocates from the cytoplasm to the nucleus. In nucleus, NF-κB p65 attaches to κB binding sites and triggers the transcription of proinflammatory cytokines such as TNF-α and IL-1β (Huang et al., 2015).

Additionally, oxidative stress is another mechanism involved in the development of sepsis-induced acute liver injury (Macdonald et al., 2003; Yaylak et al., 2008). Previous study reported that activated neutrophils are a potential source of ROS which induce tissue damage directly or indirectly (Haegens et al., 2009; Yaylak et al., 2008).

Pomegranate (Punica granatum, Punicaceae) is an ancient and highly distinctive fruit with various pharmacological and biological activities. It has been reported to be active in the treatment of inflammation, parasitic and microbial infections, respiratory complications, and cancer (Viladomiu et al., 2013). The unique antioxidant tannins and flavonoids contained in pomegranate have recently drawn the attention of many scientists. Previous studies showed that pomegranate has anti-inflammatory activity as shown in models of skin inflammation (Khan et al., 2012), gastritis (Colombo, 2013) and rheumatoid arthritis (Shukla et al., 2008), and anti-oxidant activity as shown in gentamicin-induced nephrotoxicity model (Cekmen et al., 2013). Anti-inflammatory and anti-oxidant effects of pomegranate were found to be NF-kB dependent (Colombo, 2013; Dikmen et al., 2011).

Since inflammation and oxidative stress induced by sepsis are major pathways causing acute liver injury, therefore we hypothesized that pomegranate extract can ameliorate acute liver injury-induced by CLP through its antiinflammatory and antioxidant effects in rats.

2. Materials and methods

2.1. Chemicals

Hexadecyltrimethyl ammonium bromide was purchased from Bio Basic Canada INC, (Ontario, Canada). Ellman's reagent [5,50-dithio-bis(2-nitrobenzoic acid)], tris (hydroxymethyl) aminomethane, trichloroacetic acid (TCA), 1,1′,3,3′-tetramethoxypropane, reduced glutathione, thiobarbituric acid (TBA), pyrogallol, 3,3′,5,5′-tetramethylbenzidine (TMB) and pentobarbital sodium were purchased from Sigma-Aldrich chemical Co. (St. Louis, MO, USA).

2.2. Pomegranate extract

Standardized pomegranate fruit extract (POM) in capsule form was provided by Verdure Science Inc. (POMELLA, Noblesville, IN, USA). It has been previously reported that this extract was high-performance liquid chromatography (HPLC)-standardized using validated methods and standards to 30% of the major ellagitannins (punicalagin α, and punicalagin β) and approximately 5% ellagic acid as shown in the HPLC-photodiode array profile. Other ingredients were also characterized and the extract was clarified to be *Escherichia coli*, *Salmonella*, *Staphylococcus Aureus*, and *Enterobacteriaceae* free as previously reported (Sadik and Shaker, 2013).

2.3. Experimental animals

Male Sprague Dawley rats, aging 6–8 weeks (200 ± 20) g, were purchased from "Egyptian Organization for Biological Products and Vaccines" Giza, Egypt. Research protocol has been approved by the "Research Ethics Committee" of Faculty of Pharmacy, Mansoura University, Egypt which are in accordance with "Principles of laboratory Animal Care" (NIH publication No. 85–23, revised 1985).

2.4. Experimental design

Animals were divided into 4 groups, each of 18 rats. **Group 1:** Sham-operated group; **Group 2:** POM group; Rats receiving 800 mg/kg/day POM orally for 14 days and then treated as sham group; **Group 3:** CLP group and **Group 4:** POM + CLP group, Rats receiving 800 mg/kg/day POM for 14 days and then treated as CLP group. This dose was chosen based on results obtained in pilot experiments using lower doses of POM (400, 600 mg/kg/day) which failed to improve mortality and other parameters (data not shown) whereas 800 mg/kg/day was found to be optimal dose.

After 24 h, mortality rate was assessed, then live rats were anaesthetized. Blood samples were withdrawn via orbital sinus and allowed to clot for 30 min at room temperature, followed by centrifugation at 2000 g for 10 min at 4 °C. Serum samples were collected and stored at −80° C for assessment of liver function. Portions of liver tissues were rapidly removed, snap frozen in liquid nitrogen, and stored at −80 °C until subsequent analysis. Another portion of liver was placed immediately in 10% neutral buffered formalin for immunohistochemical and histopathological examination.

2.5. Induction of CLP

Polymicrobial sepsis was induced by CLP according to previously described method (Camara-Lemarroy et al., 2015) with some modification. Rats were anesthetized with thiopental sodium (40 mg/kg, i.p.) and placed over heating pads to maintain body temperature. The abdomen was gently shaved and prepared with a 10% povidone-iodine solution. Small midline incision was made to expose cecum. Cecum was ligated below the ileocecal valve without causing bowel obstruction. The cecum was then subjected to a single through and through perforation with a 16-gauge needle distal to point of ligation. The cecum was then gently squeezed to extrude some fecal contents, and repositioned. The abdominal muscles was closed by applying simple interrupted sutures with 4–0 silk suture. The skin layer were then closed using subcuticular running suture with 4–0 silk suture. Rats were resuscitated with 1 mL of warmed ringer solution subcutaneously. Sham-operated animals were subjected to laparotomy intestinal manipulation and resuscitation procedures; however, the cecum was neither ligated nor punctured and used as control.

2.6. Assessment of liver function

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma aminotransferase (γ-GT) and total bilirubin were determined using commercial kits from (Biodiagnostics, Badr, Egypt).

2.7. Preparation of liver homogenate

Liver tissue samples were weighed and homogenized (1:10, w/v) in phosphate buffered saline (pH 7.4) in an ice bath using Omni-125 hand held homogenizer (Omni international, USA). The homogenate was centrifuged at 2000*g*, 4 °C for 15 min.