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Betulinic acid attenuates renal oxidative stress and inflammation in experimental model of murine polymicrobial sepsis

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ABSTRACT

Sepsis is a common cause of acute kidney injury (AKI) and is associated with substantial morbidity and mortality. Objective of the study was to evaluate the effect of betulinic acid, a triterpenoid in sepsis-induced AKI using cecal ligation puncture (CLP) mouse model. Mice subjected to CLP developed histologic AKI at 18 h after CLP. There was an increase in renal proinflammatory response (nuclear factor-kappa B expression, tumor necrosis factor-alpha, interleukin (IL)-6 and IL-10), matrix metalloproteinase-9, plasma creatinine, renal neutrophil gelatinase-associated lipocalin and oxidant stress response (malondialdehyde, inducible nitric oxide synthase, total nitrite and superoxide); decrease in anti-oxidant levels (superoxide dismutase and catalase) at 18 h of CLP. However, BA pretreatment at the doses of 10 and 30 mg/kg prevented the CLP-induced kidney damage by restoring the aforementioned inflammatory mediators, oxidant and anti-oxidant imbalance. These evidences suggest that, the protective effects of BA on kidney are associated with defending action against inflammatory and oxidative stress response in CLP mice and BA could be potential therapeutic agent in sepsis-induced AKI.

1. Introduction

Sepsis is a condition characterized by the severe inflammatory response to infection and sepsis-induced acute kidney injury (AKI) is associated with 70% of mortality in sepsis patients (Bagshaw et al., 2007).

One of the important mechanisms in intra-abdominal sepsis-induced kidney damage is increase in oxidative stress and pro-inflammatory response (Doi et al., 2009). Oxidative stress in septic patients is thought to play an important role in the multiorgan failure associated with severe sepsis (Galley, 2010). Animal models have suggested that reactive oxygen species (ROS) and reactive nitrogen species (RNS) contribute to tubular epithelial injury during sepsis (Wu and Mayeux, 2007; Wu et al., 2007). Superoxide generated in cellular components contributes to increased oxidative stress during sepsis (Pathak et al., 2012). The deleterious effects of ROS are quenched, at least in part by antioxidants such as superoxide dismutase (SOD), an endogenous antioxidant that scavenges superoxide in conditions like endotoxaemia. Moreover, increased production of nitric oxide (NO) in the kidney via inducible NO synthase (iNOS) during sepsis (Heemskerk et al., 2006) reacts with superoxide to generate a potent oxidant, peroxynitrite. Free radicals produced during sepsis react with DNA, proteins, and lipids, leading to their degradation and thereby accelerating the loss of cell function and damage (Galley, 2010). Thus, treatment with antioxidants is considered effective in reducing mortality or preventing organ damage in septic animal models (Liaw et al., 2005). Further, some agents were found to have antioxidant activity and exert a protective effect in septic rats, e.g. melatonin (Wu et al., 2001) and tempol (Liaw et al., 2005). Lipid peroxidation mediated by oxygen free radicals is believed to be an important cause of destruction and damage to cell membranes. Septic kidney injury was also found to be associated with reduced SOD and catalase (CAT), the major anti-oxidant enzymes that fight the oxidative stress (Maurya et al., 2014).

In AKI, many cytokines are released by leukocytes and renal tubular cells in injured kidney and are important components of both initiation and extension of inflammation. Levels of cytokines,

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including interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), are significantly higher in sepsis-related acute renal failure patients compared to non-sepsis cases (Murugan et al., 2010).

Pro-inflammatory cytokine IL-6 as well as the anti-inflammatory cytokine IL-10 were significantly higher in nonsurvivors than in those patients who survived hospital discharge. Also, elevated plasma levels of IL-10 in sepsis syndrome are associated with poor survival (Friedman et al., 1997).

Paradoxically, elevated levels of circulating IL-10 in sepsis have been shown to correlate with adverse outcomes (Oberholzer et al., 2002). This is not inconsistent with the anti-inflammatory role of IL-10, which is normally produced in response to TNF-α and acts to diminish transcription and production of TNF-α, and other pro-inflammatory cytokines (van Der Poll et al., 1994).

In addition, significant correlations of neutrophil gelatinase-associated lipocalin (NGAL) with IL-6, IL-10 and MCP-1 were found exclusively after 24 h but not after 6 h. Late association of NGAL with IL-6, IL-10 and MCP-1 were triggered by TNF-α (Otto et al., 2013). Authors hypothesized that septic AKI, as remote organ failure, is mainly initiated by TNF-α (Otto et al., 2013).

Glomerular fibrin deposition is believed to occur in part due to enhanced induction of nuclear factor-kappaB (NF-κB) and TNF in the kidney (Yamamoto et al., 2002). Local renal TNF-α production promotes cytotoxic and inflammatory responses within the kidneys that trigger the generation of additional cytotoxic mediators. Plasma creatinine levels were elevated in AKI in cecal ligation puncture (CLP) mice model (Leelahavanichkul et al., 2008) indicating kidney damage. Additionally, studies showed that, NGAL is a highly sensitive, specific, and predictive, early biomarker for AKI in a wide range of different disease processes (Devarajan, 2007).

Studies showed that antioxidants enhance the host defense response to microbial sepsis in mice (Zarjou and Agarwal, 2011). Betulinic acid (BA) is a naturally occurring pentacyclic triterpenoid, safe and non-toxic at doses up to 500 mg/kg body weight in mice (Alakurtti et al., 2006). BA is reported to have anti-inflammatory and anti-oxidant activities in many experimental studies, including sepsis with no observed antibacterial activity (Takada and Aggarwal, 2003; Fontanay et al., 2008; Ekşioğlu-Demiralp et al., 2010; Viji et al., 2011; Nader and Baraka, 2012; Lingaraju et al., 2015). Further, in our previous study, BA improved the survival rate and prevented lung injury in the CLP mice (Lingaraju et al., 2015). However, its potential is not yet tested in the sepsis-induced AKI which is a major concern of fatality in critically ill patients. In this study, we examined whether BA exerted a protective effect in sepsis animals. Also, we investigated possible mechanisms by which BA ameliorated CLP-induced AKI in this model which may further add to improvement in survival.

2. Materials and methods

2.1. Experimental animals

Healthy male Swiss albino mice (25–30 g) were procured from Laboratory Animal Resource Section of the Institute and were kept in polypropylene cages in ambient environment (room temperature 22 ± 2 °C; relative humidity 55–60%; 12:12 h light: dark cycle) and maintained on a balanced ration. Fresh drinking water was offered to animals daily *ad libitum*. The experiments were carried out in accordance with the guidelines of Animal Ethics Committee, IVRI, Izatnagar.

2.2. Kits and chemicals

BA, thiobarbituric acid, trichloroacetic acid, 5,5′-dithiobis-2-nitrobenzoic acid and hydrogen peroxide were purchased from Sigma Chemicals Co., St. Louis, USA. ELISA kits were procured from Genetix Biotech Asia Pvt. Ltd. All the essential chemicals for conventional biochemical parameter estimation were purchased from Sigma and Sisco Research Laboratories Pvt. Ltd.

2.3. Experimental design

Mice were divided into five groups consisting six mice in each group and pretreated with respective agents intraperitoneally for three days before surgery and surgery was done on third day after one hour of last dosing. Groups were as follows: (a) sham-operated mice pretreated with vehicle, (b) CLP mice pretreated with vehicle, (c) CLP mice pretreated with 3 mg/kg BA, (d) CLP mice pretreated with 10 mg/kg BA and (e) CLP mice pretreated with 30 mg/kg BA. The doses were selected based on the results of survival rates in the previous study (Lingaraju et al., 2015).

2.4. CLP model of sepsis

Polymicrobial sepsis was induced by CLP surgical procedure (Rittirsch et al., 2009). Adult male swiss albino mice were anaesthetized with intraperitoneal injection of 100 mg/kg ketamine and 10 mg/kg xylazine. A midline laparotomy was performed, the cecum was exteriorized, a 5–0 silk ligature was placed 5 mm from the cecal tip. The caecum was punctured twice with a 21-gauge needle and gently squeezed to extrude a 1 mm column of faecal material. In sham operated animals, the caecum was exposed but was neither ligated nor punctured. Then, the abdominal incision was closed in two layers, and 1 mL of saline was given intraperitoneally to all mice after surgery.

2.5. Plasma collection

Animals were lightly anesthetized with ether after 18 h of CLP surgery and approximately 1.5 mL of blood was collected from the retroorbital sinus puncture into EDTA coated 2 ml vials, centrifuged (4000 rpm for 10 min in cooling centrifuge) and the plasma was separated into another 2 mL microcentrifuge tubes, stored with mammalian cocktail protease inhibitor at −80 °C for further analysis.

2.6. Kidney homogenate preparation

Mice of the entire group were killed at 18 h after sham or CLP; kidneys of the mice were stored in 10% formalin or were removed, weighed, snap-frozen in liquid nitrogen, homogenized in 50 mM/L phosphate buffer with mammalian cocktail protease inhibitor (pH 7.4) at 4 °C to make 5% tissue homogenate. The homogenates were centrifuged at 1500 g for 10 min in a cooling centrifuge at 4 °C. The resulting supernatants were stored at −80 °C for further analysis.

2.7. Mean survival time

Mean survival time was derived from the obtained observations of the previous 120 h survival study which was carried out as a separate experiment where 15 animals per group were used (Lingaraju et al., 2015).

2.8. Estimation of oxidative and anti-oxidative parameters in kidney

The kidney homogenate was used for estimation of various biochemical, oxidant and anti-oxidant parameters by various methods. Lipid peroxidation in terms of malondialdehyde (MDA) was estimated by the method described by Rehman (1984). Nitrite was estimated by method described previously (Green et al., 2004). Superoxide anion generation estimation as method