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Betulinic acid negates oxidative lung injury in surgical sepsis model

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ABSTRACT

Background: Sepsis commonly progresses to acute lung injury and is associated with high morbidity and mortality. Septic acute lung injury is characterized by severe oxidative stress response, remained refractory to present therapies, and new therapies need to be developed to improve further clinical outcomes. We determined the effect of betulinic acid (BA) on oxidative lung injury in mice using cecal ligation and puncture (CLP) model.

Materials and methods: Five groups of mice (six in each group) received three pretreatments at 24-h interval before surgery. Surgery was done 1 h after last dosing. Sham and CLP control group mice received vehicle. BA was administered to other three groups of mice at 3, 10, and 30 mg/kg dose. Lung and plasma samples were collected for analysis by sacrificing the mice at 18 h of surgery.

Results: Compared with sham, CLP significantly increased total protein, nitrite, malondialdehyde, isoprostane, superoxide, protein carbonyl, oxidative stress index, inducible nitric oxide synthase protein, and histopathologic changes and reduced the superoxide dismutase, catalase activity, and total thiol levels in lungs and plasma, which were restored by BA pretreatment.

Conclusions: BA pretreatment decreased the levels of oxidants, increased the levels of antioxidants in lungs and plasma thereby reducing the oxidative lung injury in CLP mice. Additionally, BA was found to scavenge the superoxide and nitric oxide radical *in vitro*. Thus, BA is suggested to be effective in treatment of oxidative lung injury in sepsis.

1. Introduction

In spite of decades of advances in critical care and management of sepsis, it is still considered to be the most common cause of death in intensive care units [1]. Morbidity and mortality in sepsis are largely associated with multiple organ failure, and the lung injury is most commonly speculated [2].

Oxidative stress plays an important role by promoting and mediating the pathogenesis of sepsis, which makes it as a potential target for the treatment of critically ill patients [3–5]. Oxidative stress is the result of overproduction of reactive

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oxygen species (ROS) and depletion of antioxidants. Oxidative stress promotes injury and death of cells and is well described in sepsis patients [3,6]. Neutrophils, the first line of defense against infection, play an important role in pathogenesis of acute lung injury (ALI) by contributing to ROS and reactive nitrogen species production [7].

Overproduction of nitric oxide (NO) by inducible nitric oxide synthase (iNOS) is responsible for the pulmonary oxidative and nitrosative stress and plays an important role in the pathophysiology of ALI [8]. iNOS is reported to contribute for microvascular dysfunction, which is well documented in sepsis patients. Microvascular dysfunction is associated with increased mortality [9] and is characterized by impaired barrier function with increased permeability leading to extravascular leak of protein-rich edema fluid [10].

Sepsis research relies mainly on animal models [11]. The cecal ligation and puncture (CLP) model is the most commonly used tool for studying the process of sepsis as it reproduces the human disease. This model can be used in preclinical trial to test the efficacy of therapeutic agents for the treatment of sepsis [12]. Furthermore, in this model, lung stress is a common feature [13].

Betulinic acid (3β, hydroxy-lup-20 (29)-en-28-oic acid; BA) is a naturally occurring pentacyclic triterpenoid, exists naturally in many kinds of food. It has been reported to have many biological functions such as anticancer, antiretroviral, antimalarial, and anti-inflammatory properties while causing minimal toxicity to unaffected cells in various experimental models [14]. Moreover, recently BA has been shown to have protective effect in mice and rats by reducing oxidative stress in many experimental models [15–17]. However, the effect of BA on murine polymicrobial sepsis is yet unknown. As reviewed, with the aforementioned scenario of sepsis and its associated organ damage, we hypothesized that the oxidative stress in blood (plasma) and lung is critical, which mainly contribute to lung injury and mortality in sepsis patients and reduction of the same could be a potential therapeutic target in septic ALI. Therefore, looking in to the advantageous effects of BA, we sought to evaluate pro-oxidant and anti-oxidant disturbances in murine polymicrobial sepsis and possible protective effects of BA on the same with special reference to lung.

2. Material and methods

2.1. Experimental animals

Healthy male Swiss albino mice (25–30 g) were procured from Laboratory Animal Resource Section of the Institute. These animals 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 obtained from the Feed Technology Unit of the Institute. Fresh drinking water was offered to animals daily *ad libitum*. Mice were fasted for 12 h before experiment and had free access to water during this period and subsequently. The experiments were carried out in accordance with the guidelines of Animal Ethics Committee, Indian Veterinary Research Institute, Izatnagar. The mice were acclimatized for 1 wk before use.

2.2. Drug, reagents and kits

BA, thiobarbituric acid, trichloroacetic acid, and hydrogen peroxide were purchased from Sigma Chemicals Co, St. Louis. Dimethyl sulfoxide was purchased from Genetix Biotech Asia Pvt Ltd, Delhi. Enzyme immunoassay kits were procured from Genetix Biotech Asia Pvt Ltd. All the necessary chemicals for conventional biochemical parameter methods were purchased from Sigma Chemicals, Genetix Asia Pvt. Ltd, and Hi Media Laboratories Pvt Ltd, Mumbai.

2.3. Animal grouping and drug administration

The animals were initially divided into five groups of six animals each. Animals of all group received three pretreatments on day 1, 2, and 3 at 24-h interval by intraperitoneal route as noted in the following. In groups 1 and 2 (sham and CLP control, respectively), the mice received vehicle (5% dimethyl sulfoxide in peanut oil). In groups 3, 4, and 5, the mice received BA (dissolved in vehicle) at the dose rate of 3, 10, and 30 mg/kg body weight, respectively. One hour after the last dose on day 3, the CLP was done in all the animals of all the groups except group 1 where the mice underwent sham operation (CLP procedure and sham operation are explained in following Section 2.4).

2.4. Sepsis induction by CLP surgical procedure

Polymicrobial sepsis was induced by CLP surgical procedure [18]. Animals were anesthetized by an intraperitoneal injection of a mixture of 10 mg/kg xylazine and 100 mg/kg ketamine hydrochloride. The abdomen was gently shaved and cleaned with antiseptic sterile swab. One-centimeter midline incision was made on the anterior abdomen, and the cecum was exposed and ligated with silk thread 4-0 distal to the ileocecal valve, without causing intestinal obstruction. The cecum was punctured twice with 21-ga needle (0.80 mm) in the midline, with a 0.5 cm distance between the punctures, and the part of the cecum with less larger vessels was preferred to puncture to avoid hemorrhage. Then, the cecum was squeezed to extrude some fecal contents to ascertain patency of the puncture site and to assure the presence of bacteria in the peritoneum. The cecum was repositioned, incision was closed with a 4-0 suture material (chromic catgut), and each mouse received 1 mL of warmed sterile lactated Ringer solution subcutaneously for fluid resuscitation. The animals were allowed free access to food and water after CLP. Sham-operated mice underwent the same procedure with the exception that the cecum was neither ligated nor punctured.

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 retro-orbital sinus puncture into EDTA-coated vials. The blood containing vials were centrifuged at 4°C (4000 rpm for 10 min). The plasma was collected in to another 2-mL microcentrifuge tubes, stored with mammalian cocktail protease inhibitor at −80°C for further analysis.