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Thymoquinone modulates nitric oxide production and improves organ dysfunction of sepsis

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ABSTRACT

Aims: The present investigation was designed to evaluate the effect of thymoquinone in a septic animal model and to explore the role of nitric oxide (NO) in the process.
Main methods: To achieve this, mice (n = 12 per group) were treated in parallel with thymoquinone (0.75 mg/kg/day) and/or NG-nitro-L-arginine methyl ester (L-NAME; 400 μg/g/day) prior to sepsis induction with live *Escherichia coli*.
Key findings: Thymoquinone significantly improved renal and hepatic functions alone and in combination with L-NAME. This was associated with less NO production and lower oxidative stress in treated animals. Tumor necrosis factor-α concentration with thymoquinone and L-NAME were 36.27 ± 3.41 pg/ml and 56.55 ± 5.85 pg/ml, respectively, as opposed to 141.11 ± 6.46 pg/ml in septic controls. Similarly, Interleukin-1α, 2, 6 and 10 levels decreased significantly upon treatment with thymoquinone and L-NAME as compared with untreated septic animals. NF-κB and NF-κB-DNA binding activity in nuclear proteins were also significantly down-regulated. Vascular responsiveness studies in isolated mouse aortae demonstrated a reduced relaxation to acetylcholine exposure in septic mice treated with thymoquinone.
Significance: These findings suggest that thymoquinone prevents sequels of the multiple organ failure syndrome of sepsis by modulating the production of NO and its inflammatory sequela, and adjusting vascular responsiveness.

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1. Introduction

Sepsis or septic shock is a systemic inflammatory response to a microbial infection, which causes endothelial dysfunction, leading to a chain of events including microcirculatory deficiency, tissue hypoxia, apoptosis, multiple organ failure and death [1]. Mortality related to sepsis remains high and is the most common cause of death in the intensive care unit [2,3]. Various inflammatory mediators are released in the blood by different tissues including vascular cells during sepsis [4]. Overproduction of these mediators results in tissue damage, multiple organ dysfunctions and death [5].

Various microorganisms (*e.g.*, bacteria or fungi) can cause sepsis; in particular, Gram-negative bacteria are common etiologic pathogens and a key risk factor in the development of sepsis [6]. Lipopolysaccharide (LPS; endotoxin) is a major component of the outer membrane of Gram-negative bacteria and contributes majorly to the pathological consequences of sepsis through binding with specific toll-like receptors [7,8]. Many of the adverse effects of LPS depend on the activation of polymorphonuclear leukocytes, and the subsequent release of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) [9]. Initially, an appropriate host response to the infection occurs, which then may lead to an aberrant inflammatory cascade. Unfortunately, numerous therapeutic and intervention strategies proved to be hardly effective or even resulted in a further deterioration of the condition [10, 11].

The immunomodulatory therapies and clinical management protocols evaluated in sepsis include low dose corticosteroids [12], anti-TNF-α and anti-IL-1 antibodies [13,14], platelet activating factor (PAF) antagonists [15], antioxidants [16], adjusted nutrition and coagulation modulators [17]. However, the majority of these interventions showed little success in reducing the high mortality rate of septic patients. Some natural products exhibit anti-inflammatory, cytoprotective, and immunomodulatory properties among other biological activities [18]. Thymoquinone, the principal chemical constituent found in the volatile oil of the black seeds of *Nigella sativa*, exerts antioxidant actions and protects against organ damage [19]. The compound exerts a unique

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protective effect against sepsis in the mouse [20]. It also decreases the formation of reactive oxygen species and the presence of nitrotyrosine in the arterial wall of the rat [21]. Sepsis is associated with increased nitric oxide (NO) production. Reactive oxygen species (ROS) produced by NO interacting with superoxide ions are directly toxic host cells [22]. Therefore, the aim of the present study was to investigate the possible modulatory effect of thymoquinone on NO production *in vivo*. Furthermore, to evaluate changes in nuclear NF-κB (p65) expression and DNA binding activity in the course of treatment of septic animals.

2. Materials and methods

2.1. Chemicals and reagents

Thymoquinone, NG-nitro-L-arginine methyl ester (L-NAME), phenylephrine, acetylcholine and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). *Escherichia coli* (*E. coli*; ATCC-25992; 1.5×10^7 CFU/ml) were obtained from the Microbiology Laboratory, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia).

2.2. Animals and conditions

Male albino mice weighing ~25 g were obtained from the College of Pharmacy Animal Care and Use Facility at King Saud University. Animals used in the study were maintained in accordance with the recommendations of the "Guide for the Care and Use of Laboratory Animals" approved by the facility (NIH publications no. 80-23; 1996). They were housed in a temperature-controlled room with a 12-hour light/dark cycle and were allowed access to food and water *ad libitum* during the study except that the chow was removed 12 h prior to treatment.

2.3. Sepsis model and drug administration

The animals were divided into six different groups ($n = 12$). Group I served as vehicle (10% DMSO) control. Group II was treated with an intraperitoneal (*i.p.*) dose of 0.75 mg/kg/day thymoquinone prepared in 10% DMSO for three consecutive days. Group III served as *E. coli* control. Group IV served as sepsis group and treated with an *i.p.* dose of 0.75 mg/kg/day thymoquinone for three consecutive days. Group V was treated with an *i.p.* dose of 0.75 mg/kg/day thymoquinone for three consecutive days and on day three received L-NAME (400 μg/g body weight, prepared in normal saline) 2 h after thymoquinone dosing. Group VI was treated with an *i.p.* dose of L-NAME (400 μg/g/day) for three consecutive days. All groups except I and II were challenged with an *i.p.* dose of *E. coli* 2 μl/g 4 h after the last administration of thymoquinone or L-NAME.

2.4. Biochemical and immunological analyses

After 6 h of *E. coli* challenge [20], animals were sacrificed under diethyl ether anesthesia (1.9%) exposure in a closed chamber and blood samples were collected from the heart in sterile heparinized tubes, and liver and lung tissues harvested. The resultant plasma samples were stored at −80 °C for biochemical and inflammatory mediator analyses. Relevant biochemical parameters including serum creatinine (SC), blood urea nitrogen (BUN), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), albumin and creatine kinase (CK) were assessed using colorimetric methods according to manufacturer's instructions (Human Diagnostic Worldwide; Wiesbaden, Germany) to quantitate the amount of a particular analyte in the assay by measuring the amount of the chromogenic reaction product at a characteristic wavelength [23,24].

The levels of TNF-α, IL-1α, IL-2, IL-6 and IL-10 were determined using an ELISA technique according to manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Briefly, the assay employs the quantitative immunoassay technique. A monoclonal antibody specific for mouse TNF-α was pre-coated onto a microplate. Standard control and samples were pipetted into the wells and any mouse TNF-α present bound by the immobilized antibody. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for mouse TNF-α was added into the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution was then added to the wells. The enzyme reaction yielded blue product that turns yellow upon the addition of a stop solution. The intensity of the color measured was in proportion to the amount of mouse TNF-α bound initially Determination of the optical density of each well was measured within 30 min, using a microplate reader. The sample values were then read off the standard curve. Similar procedures were performed for IL-1α, IL-2, IL-6 and IL-10 using their corresponding antibodies.

2.5. Nitric oxide level determination

The levels of NO stable products, nitrite and nitrate (NOx) were measured in plasma using the Griess reaction [25]. Briefly, 100 μl of sodium nitrite or samples was added in to the well of a 96-microtiter plate. Subsequently, 100 μl of freshly prepared Griess reagent (1:1 ratio, mixture of 0.1% of N-1-napthylethylenediamine in water and 1% of sulfanilamide in 5% phosphoric acid) was added to each well and the plate was incubated in the dark at room temperature for 20 min. The intensity of the color developed was read at 540 nm using a Multiskan® 96-well ELISA Plate Reader (MTX Lab Systems Inc., Vienna, VA, USA).

2.6. Oxidative stress determination

Oxidative stress was assessed by measuring non-protein sulfhydryl (NPSH) level, and thiobarbituric acid reactive substance (TBARS) content as a marker of lipid peroxidation. TBARS is expressed as nanomoles of malondialdehyde (MDA) per gram of protein using a molar extinction coefficient for MDA of M^{-1} cm^{-1} [26,27]. Briefly, the hepatic and lung tissues were homogenized in ice-cold normal saline containing 0.02 mmol/l EDTA. The tissue homogenates were mixed with distilled water and 50% (w/v) trichloroacetic acid (TCA). Samples were centrifuged at 2500 $\times g$ and the supernatant was mixed with Tris buffer (pH 8) and 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB). Absorbance was measured within 5 min of addition of DTNB at 412 nm to determine NPSH levels. For MDA determination, tissue homogenates were incubated at 37 °C for 3 h in a metabolic shaker. Then, 1 ml of 10% aqueous TCA was added and samples were centrifuged at 2500 $\times g$ for 10 min. The supernatant was removed and mixed with 1 ml of 0.67% 2-thiobarbituric acid, boiled for 10 min and then the mixture was cooled and diluted with 1 ml distilled water. The resulting absorbance was measured at the wavelength of 532 nm at room temperature against blank reference. The concentration of MDA was read from a standard calibration curve plotted using 1,1,3,3′-tetraethoxypropane.

2.7. NF-κB(p65) expression and DNA binding analysis

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood by Ficoll density gradient centrifugation as described previously [28]. The cells were purified using lymphocyte separation medium (ICN Biomedical Inc., Aurora, OH, USA). Following centrifugation (1500 $\times g$, at room temperature for 30 min), PBMC located at the interface were harvested and washed two times with complete RPMI-1640 medium (Gibco Lab., Grand Island, NY, USA) and then resuspended in the medium. PBMC at a concentration of (1×10^6 cells/ml) from different experimental groups were homogenized in ice-cold RIPA buffer containing 1% protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Nuclear extracts were prepared by using NE-PER nuclear extraction kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's protocol and used for analysis of NF-κB (p65) protein expression as well as NF-κB-DNA binding assay by ELISA

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