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The protective effect of thymoquinone against sepsis syndrome morbidity and mortality in mice

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ABSTRACT

Sepsis and septic shock are life threatening complications and most common cause of death in intensive care units. Thymoquinone, a constituent of *Nigella sativa* (black seed), holds exceptional promise as an anti-cancer and anti-inflammatory agent. No evidence has been published, however, whether this compound has a protective effect from sepsis-related morbidity, mortality and associated organ dysfunction. To examine this, two sets of mice ($n=12$ per group), with parallel control groups, were acutely treated with thymoquinone intraperitoneal injections of 1.0 and 2.0 mg/kg body weight, and were subsequently challenged with endotoxin Gram-negative bacteria (LPS O111:B4). In another set of experiments, thymoquinone was administered at doses of 0.75 and 1.0 mg/kg/day for three consecutive days prior to sepsis induction with live *Escherichia coli*. Survival of various groups was computed, and renal, hepatic and sepsis markers were quantified. Thymoquinone reduced mortality by 80–90% and improved both renal and hepatic biomarker profiles. The concentrations of IL-1α with 0.75 mg/kg thymoquinone dose was 310.8 ± 70.93 and 428.3 ± 71.32 pg/ml in the 1 mg/kg group as opposed to controls (1187.0 ± 278.64 pg/ ml; $P<0.05$). Likewise, IL-10 levels decreased significantly with 0.75 mg/kg thymoquinone treatment compared to controls (2885.0 ± 553.98 vs. 5505.2 ± 333.96 pg/ml; $P<0.01$). Mice treated with thymoquinone also exhibited relatively lower levels of TNF-α and IL-2 (P values $=0.1817$ and 0.0851, respectively). This study gives strength to the potential clinical relevance of thymoquinone in sepsis-related morbidity and mortality reduction and suggests that human studies should be performed.

1. Introduction

Sepsis, defined as the systemic host response to microorganisms, is a syndrome related to severe infections characterized by "Systemic Inflammatory Response Syndrome" (SIRS) and end-organ dysfunction away from the primary site of infection [1]. The normal host response to infection is complex, aiming to both identify and control pathogen invasion and to start immediate tissue repair. Both the cellular and humoral immune systems are activated, giving rise to a massive liberation of pro-inflammatory and anti-inflammatory mediators [2]. Exacerbation of these mechanisms can cause a chain of events that leads to endothelial injury, tissue hypo-perfusion, disseminated intravascular coagulation, and treatment- refractory shock, and eventually, progression to the multiple organ dysfunction syndrome (MODS) and, possibly, death [3].

While almost any microorganism can be associated with sepsis and septic shock, Gram-negative bacteria are common etiologic pathogens. Lipopolysaccharides (LPS) are normal components of the cell wall of Gram-negative bacteria and have been recognized for many years as key risk factors in the development of septic shock syndrome [4]. Many of the adverse effects of LPS in mammals are dependent on the activation of polymorphonuclear leukocytes (PMNs), and release of cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1). It has been shown that PMNs accumulate in tissues after LPS administration, and although they play an important role as a host defense mechanism, they are also responsible for mediating LPS-induced tissue injuries [5]. In addition, several lines of evidence have suggested that stimulatory inflammatory cells release a plethora of mediators including reactive oxygen species, proteolytic enzymes and products of lipid peroxidation.

Human septic shock-related morbidity and mortality remain unacceptably high, despite increasing knowledge about the pathophysiological pathways in sepsis and improved hospital care. SIRS is still one of the most prevalent causes of morbidity and mortality in the intensive care units (ICU) worldwide. It has been estimated that more than 750,000 sepsis cases occur in the United States every year, leading

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to approximately 220,000 deaths [6,7]. Similar incidences have also been reported in Europe and around the world [8]. Furthermore, the mortality rates of septic shock can be as high as 50% or up to 75% on longer follow-ups [9,10].

Clinical management usually begins with prompt recognition, determination of the probable infection site, early administration of antibiotics, low-dose corticosteroids, and resuscitation protocols [11]. Corticosteroids have demonstrated a modest decrease in mortality (absolute reduction of 10%) with moderate doses administered in sepsis and septic shock [12]. Furthermore, in attempts to find efficacious drugs that reduce mortality, a large number of immunomodulatory agents have been studied in experimental and clinical settings [13]. However, the vast majority of these trials showed little success in reducing the overwhelmingly high mortality rates of septic shock patients.

Thymoquinone is the main constituent of the volatile oil of *Nigella sativa* (black seed). This compound exists in tautomeric forms including the enol form, the keto form and mixtures (Fig. 1). The keto form being the major fraction (~90%) as evident by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC) analyses, and is responsible for the pharmacological properties of thymoquinone. It has been studied for its cardiovascular, respiratory, cytotoxic, anti-inflammatory, and immunomodulatory properties [14]. The goal of the current work was to evaluate the effect of thymoquinone on sepsis-related morbidity and mortality using an animal model.

2. Materials and methods

2.1. Animals and reagents

Male Albino mice weighing ~25 g were obtained from College of Pharmacy Animal Care and Use Facility, king Saud University (Riyadh, Saudi Arabia). 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. They were housed in a temperature-controlled room with a 12-h light/dark cycle and were allowed free water and food ad libitum during the study except that the chow was pulled 12 h prior to animals' dosing.

Thymoquinone and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich Inc. (St. Louis, MO, USA). Endotoxin (LPS *Escherichia coli* O111:B4) was acquired from List Biological Laboratories Inc. (Campbell, CA, USA), and *E. coli* (ATCC-25992) was obtained from the Microbiology Laboratory, College of Pharmacy, King Saud University (Riyadh, Saudi Arabia). All used chemicals were of the highest grade available.

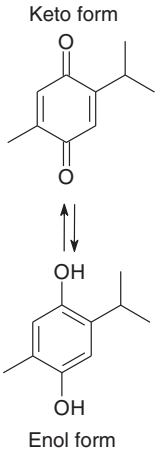

Fig. 1. The chemical structure of thymoquinone in its tautomeric keto and enol forms. The keto form being the major fraction (~90%) and is responsible for the pharmacological properties of thymoquinone.

2.2. Sepsis model

Two sets of mice ($n=12$ per group) were treated with an intraperitoneal (IP) dose of thymoquinone of 1.0 and 2.0 mg/kg body weight; a vehicle control group (10% DMSO) was also run in parallel. Four hours after thymoquinone administration, mice were challenged with LPS *E. coli* O111:B4 (2.0 mg/kg IP); mortality of groups was computed for 4 days. To further assess the thymoquinone efficacy in sepsis, two groups of animals (twelve mice each) were treated with an IP dose of 0.75 and 1.0 mg/kg/day thymoquinone for three consecutive days; control groups were also used. Four hours after thymoquinone treatment, all groups were challenged with an IP dose of 2 μl/g *E. coli* (ATCC-25992; 1.5×10^7 CFU/ml) prepared in normal saline. Mortality in the groups was thereafter followed for 4 days.

2.3. Biochemical and immunological analyses

In another set of thymoquinone treatment experiments, after 6 h of *E. coli* challenge [15] as described above, animals ($n=12$ per group) were lightly anesthetized with ether and blood samples were collected, centrifuged and the resultant plasma were stored at −80 °C and used within 48. The levels of TNF-α, IL-1α, IL-2 and IL-10 were determined using an ELISA technique according to manufacturer's instructions (Amersham Biosciences Inc; Piscataway NJ, USA). Relevant biochemical parameters including serum creatinine, blood urea nitrogen (BUN), alanine aminotransferase (ALT), albumin, and lactate dehydrogenase (LDH), and creatine kinase (CK) were also assessed using colorimetric methods (Randox Laboratories Ltd; Crumlin, Co, Antrim, UK).

2.4. Statistical analysis

Variables are presented as mean ± SEM. All variables were log transformed prior to analysis. Analysis of variance with Dunnett post-hoc testing was carried out to determine differences in immunological and biochemical parameters between controls and thymoquinone treated groups. Survival (primary endpoint) was calculated using Kaplan–Meier analysis and log rank test. Significance was set at a P value less than 0.05. All analyses were performed using GraphPad Prism version 4.03 for Windows (San Diego, CA, USA).

3. Results

Several sets of preliminary experiments were performed on different days to determine the effective dose of thymoquinone in mice and a range of 0.75–2 mg/kg IP was found to be optimum. Thymoquinone treatment reduced mortality in mice following LPS and Live *E. coli* challenge by 80–90% (Fig. 2A and B). Table 1 shows differences in the biochemical and immunological parameters between treatment groups. There was also a significant protection of the kidney function in the treated groups measured by serum creatinine (0.64 ± 0.07 vs. 0.40 ± 0.02 mg/dl, $P<0.05$) and the hepatic function measured by ALT (161.6 ± 30.01 vs. 60.0 ± 12.87 mg/dl, $P<0.05$) at 1.0 mg/kg dose as opposed to controls. Furthermore, there was an important preservation of the oncotic pressure exerted by plasma total protein, and plasma albumin (6.4 ± 0.43 vs. 5.0 ± 0.35 g/dl, and 3.2 ± 0.09 vs. 2.7 ± 0.22 g/dl, respectively, $P<0.05$). A reduction in total CK, which is a biomarker of tissue injury, was also observed (618.7 ± 100.44 vs. 996.3 ± 119.12 U/l, $P<0.05$). Mice that were treated with thymoquinone also had higher levels of plasma glucose, triglyceride and calcium, and lower phosphorus concentrations as opposed to the *E. coli* control group (Table 1).