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ORIGINAL ARTICLE

The Effect of Carvacrol on IL-1β and Nitric Oxide Levels on Lipopolysaccharide-induced Acute Renal Injury in Male Rats

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KEYWORDS

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ABSTRACT: Carvacrol is a phenolic monoterpenoid compound that has antibacterial, antifungal, anti-cancer, and anti-inflammatory effects. Lipopolysaccharide (LPS) is derived from the outer cell wall of gram-negative bacteria and is responsible for acute kidney injury. In this research, the protective effect of carvacrol on lipopolysaccharide-induced acute kidney injury was studied. For this purpose, 40 male Wistar rats (200-250 g) were used. Animals were randomly divided into 5 equal groups: 1) control, 2) LPS group, 3) LPS+carvacrol (25 mg kg^{-1}), 4) LPS+carvacrol (50 mg kg^{-1}) and 5) LPS+carvacrol (100 mg kg^{-1}). To induce acute renal injury, daily 1 mg kg^{-1} LPS for 2 weeks was injected intraperitoneally. Carvacrol was administered intraperitoneally daily for 30 minutes before LPS injection. LPS-induced kidney injury was evaluated by blood urea nitrogen (BUN), serum creatinine, and nitric oxide levels in kidney tissue by spectrophotometric methods. The level of the interleukin 1 beta was detected by ELISA in the kidney. Our results showed that LPS injection increased BUN, creatinine, nitric oxide, and IL-1β levels ($P < 0.001$). Pretreatment with carvacrol reduced BUN at 25 mg kg^{-1} ($P < 0.001$), 50 mg kg^{-1} ($P < 0.01$), and 100 mg kg^{-1} ($P < 0.001$) doses, nitric oxide at 25 mg kg^{-1} ($P < 0.05$), 50 mg kg^{-1}($P < 0.01$) and 100 mg kg^{-1}($P < 0.001$) doses, and IL-1β levels ($P < 0.001$) at all doses significantly but did not affect serum creatinine. These results indicate that carvacrol has an anti-inflammatory effect and protects kidneys against LPS by reducing pro-inflammatory mediators such as IL-1β and nitric oxide.

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INTRODUCTION

Septicemia is characterized by immune system hyperactivity during infection, resulting in vital organ dysfunction [1]. Above 50% of patients with sepsis suffer from acute kidney injury, which is a major difficulty of sepsis and endotoxemia [2]. Lipopolysaccharide (LPS) is the main constituent of the outer membrane of gram-negative bacteria. LPS injection is commonly used as a model of acute experimental kidney injury associated with sepsis [3]. Lipopolysaccharides are heat-resistant endotoxins and have long been recognized as a key factor in septic shock in humans. The presence of lipopolysaccharide in the bloodstream leads to the secretion of inflammatory cytokines, including alpha tumor necrosis factor (TNFα). Endotoxic shock is also triggered by TNFα and causes failure of vital organs such as the kidneys, liver, heart, and lungs [4]. Initially, LPS reacts with TLR4 receptors, and then by activating the NF-κB transcription factor, it increases the transcription of pro-inflammatory cytokines such as monocyte chemoattractant protein-1, TNFα and interleukin 1 beta (IL-1β) [5]. Furthermore, it also simultaneously produces abundant reactive oxygen species and causes oxidative stress [6]. Due to the high blood flow in the kidneys, this organ is exposed to high levels of pro-inflammatory agents. Therefore, the kidneys are sensitive to inflammatory responses. IL-1β is an important mediator in inflammation and involves in cellular proliferation, differentiation and apoptosis. This cytokine plays an important role in increasing cyclooxygenase-2 activity and nitric oxide levels. Nitric oxide is a small molecule that is synthesized by nitric oxide synthase in various cell types. This molecule plays a major role in immune responses [7]. Recent studies have shown that renal mesenchymal cells in response to IL-1β, TNFα and, LPS, increase nitric oxide through nitric oxide synthase (iNOS) activation [8]. Nitric oxide reduces ultrafiltration by decreasing glomerular hydrostatic pressure and involves renal failure development [9]. It should be noted that in the kidney, physiological levels of nitric oxide need for regulating renal hemodynamics and excretion of sodium and water. But, the excess synthesis of nitric oxide by cytokines or LPS may lead to glomerular dysfunction [10]. It has been determined that LPS not only increases nitric oxide synthesis but also increases harmful superoxide in the kidney and finally leading to kidney injury [11].

Carvacrol is one of the phenolic and active compounds in plants such as thyme, marjoram, mint and, savory [12]. Carvacrol shows various properties such as antibacterial, antifungal, anti-cancer and vasodilator activity [13]. Apart from its antioxidant effect, the anti-inflammatory activity of carvacrol has been established in previous reports [14-16]. Moreover, it reduces the production of TNF-α, nitric oxide, prostaglandin, interleukin-1 beta and, interleukin-6 [17, 18]. Moreover, it has been shown that carvacrol inhibits the expression of cyclooxygenase -2 (COX-2) and reduced prostaglandins production [19].

Despite extensive research, there is no effective treatment strategy against acute kidney injury caused by sepsis [20]. In this research, the protective role of carvacrol in the kidney against intraperitoneal injection of LPS has been investigated.

MATERIALS AND METHODS

Animals and groups

40 male Wistar rats (200-250 g) were obtained from the Pasteur institute (Karaj, Iran). They were kept in a room with suitable conditions (12 hours of light and dark cycle, temperature 20-22 °C and, 40% humidity). Animals were randomly allocated into 5 equal groups: 1) control, 2) LPS, 3) LPS+carvacrol (25 mg kg^{-1}), 4) LPS+carvacrol (50 mg kg^{-1}) and, 5) LPS+carvacrol (100 mg kg^{-1}). Carvacrol doses were chosen based on our previous experiments [15]. To induce acute renal injury, LPS (1 mg kg^{-1}, daily) for 2 weeks was injected intraperitoneally [21]. Carvacrol was administered 30 minutes before the LPS injection.

Preparation of samples

Rats were anesthetized using ketamine (100 mg kg^{-1}) and xylazine (10 mg kg^{-1}) and blood samples were collected. After then the kidneys were removed from the body. Blood

samples were centrifuged at 3000 rpm for 10 minutes and serum samples were collected to assess urea and creatinine levels. The kidney tissue was then homogenized with phosphate buffer and centrifuged at 3000 rpm for 15 minutes. The supernatant was isolated and used for evaluating metabolites of nitric oxide or IL-1β level. The chemicals used for all measurements were purchased from Merck Company (Darmstadt, Germany) or Sigma–Aldrich (St. Louis, USA).

Evaluation of serum levels of BUN and creatinine

Serum urea and creatinine levels were assessed using a kit according to the instructions provided by Pars Azmoun Company [22].

Evaluation of NO metabolite levels

Nitrite was measured using the Griess method. Briefly, equal volumes of grease reagent [0.1% N (1-naphthyl) ethylenediamine dihydrochloride and 1% sulfanilamide in 3% H3PO4] were mixed with the sample and incubated to produce dye. Absorption was measured at 540 nm and nitrite concentration was determined using a sodium nitrite standards curve [23].

Evaluation of IL-1β in kidney tissue

Kermania Pars Gene Company (Iran) ELISA kit was used for IL-1β evaluation in the kidney tissue. The samples were placed in wells and incubated at room temperature. The wells were then washed and biotin antibodies were added. After adding Horseradish peroxidase, the wells were washed again. Incubation in the dark (15 min) was performed using TMB substrate (3,3%, 5,5′-Tetramethylbenzidine). After adding the stop solution, the adsorption was measured at 450 nm.

Statistical analysis

After normality analysis by the Kolmogorov-Smirnov test, experimental groups were compared by Student's T-test or one-way ANOVA followed by LSD post hoc test (SPSS software). The results are shown as Mean ± SE.

RESULTS

The effect of carvacrol on serum levels of BUN and creatinine

To examine the effects of carvacrol and LPS on kidney function, we investigate the level of serum BUN and creatinine in rats. Statistical comparison by T-student test showed a significant increase ($P < 0.001$) between the control and LPS group in serum BUN (Figure 1) and creatinine levels ($P < 0.001$, Figure 2). Statistical analysis by one-way ANOVA followed by LSD test showed that pretreatment with carvacrol at 25 mg kg^{-1}($P < 0.001$), 50 mg kg^{-1}($P < 0.01$) and, 100 mg kg^{-1}($P < 0.001$) significantly reduced BUN levels compared to the LPS group (Figure 1). Carvacrol did not affect creatinine levels in LPS-receiving groups (Figure 2).

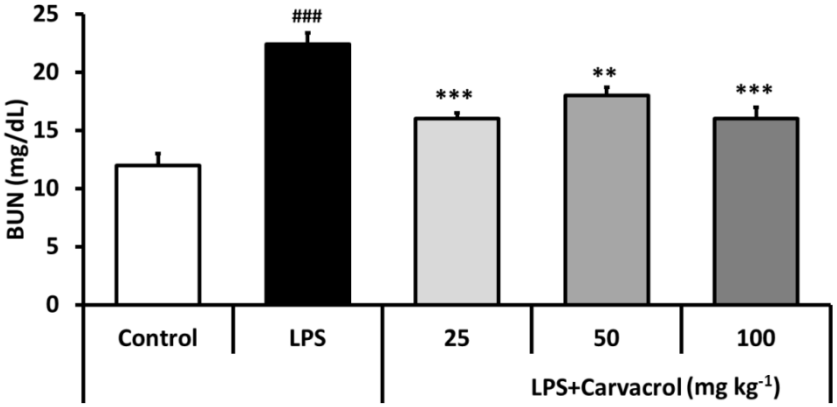

Figure 1. The Effect of carvacrol pretreatment on serum BUN levels in LPS-induced renal injury: LPS injection (1 mg kg^{-1}) increased serum BUN levels. Carvacrol treatment (in all three doses) decreased serum BUN levels. The results are shown as Mean ± SE.
(### $P < 0.001$ compared with control group and *** $< P < 0.001$ compared with LPS group, N=8)

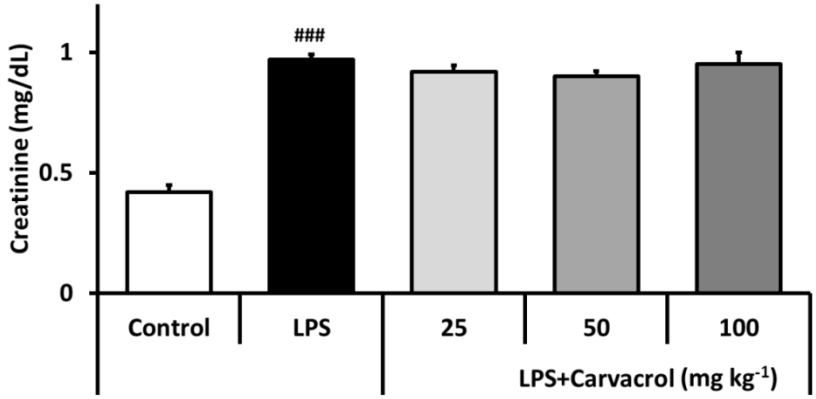

Figure 2. The effect of carvacrol pretreatment on serum creatinine levels in LPS-induced renal injury: LPS injection (1 mg kg^{-1}) increased serum creatinine levels. Carvacrol pretreatment could not alter serum creatinine levels in all three doses. The results are shown as Mean ± SE.
(### P <0.001 compared with the control group, N=8)

The effect of carvacrol on the amount of IL-1β and NO metabolites in kidney tissue

To examine the effects of carvacrol on LPS-induced inflammatory responses in rats, we investigated the levels of nitric oxide and IL-1β levels. Statistical comparison by T-student test showed that LPS injection has increased significantly (P <0.001) nitric oxide level in kidney tissue. Further statistical comparison by one-way ANOVA followed by LSD test, showed that carvacrol treatment has reduced the level of nitric oxide in kidney tissue at 25 mg kg^{-1}(P <0.05), 50 mg kg^{-1}(P <0.01) and, 100 mg kg^{-1}(P <0.001) doses compared to LPS group (Figure 3).

Also, statistical comparison by T-student showed a significant increase (P <0.001) between the control and LPS groups in the amount of IL-1β level in kidney tissue (Figure 3). Statistical comparison by one-way ANOVA followed by LSD test showed that administration of 25, 50 and, 100 mg kg^{-1}carvacrol significantly reduced (P <0.001) IL-1β level in kidney tissue compared to the LPS group (Figure 4). It seems that carvacrol protects kidneys against LPS-induced inflammation by reducing IL-1β and nitric oxide levels.

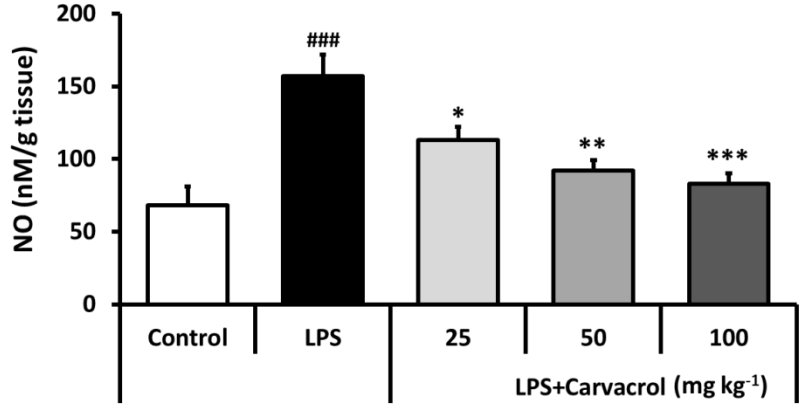

Figure 3. The effect of carvacrol pretreatment on nitric oxide (NO) levels in LPS-induced kidney injury: LPS injection (1 mg kg^{-1}) increased nitric oxide levels. Carvacrol pretreatment reduced serum nitric oxide levels at all three doses in the kidney tissue. The results are shown as Mean ± SE.
(### P <0.001 compared with control group and * P <0.05, ** P <0 01, *** P <0.001 compared with LPS group, N=8)

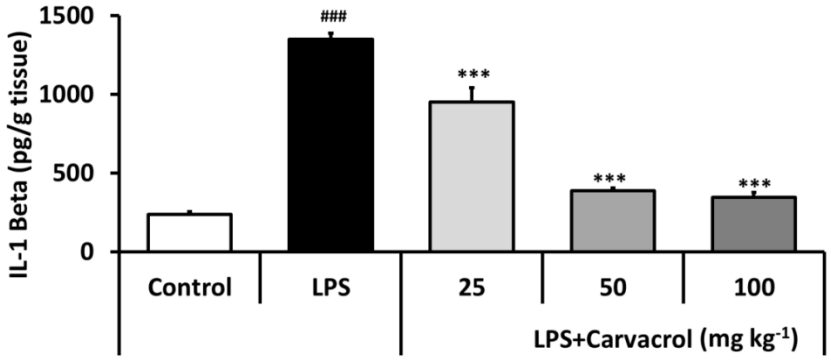

Figure 4. The effect of carvacrol pretreatment on IL-1β levels in LPS-induced renal injury: LPS injection (1 mg kg^{-1}) increased IL-1β levels. Carvacrol pretreatment reduced IL-1β levels at all three doses in the kidney tissue. The results are shown as Mean ± SE.
(### P <0.001 compared with control group and *** <P <0.001 compared with LPS group, N=8)

DISCUSSION

In the present investigation, aiming to recognize the mechanisms by which carvacrol protects renal tissue and prompts its anti-inflammatory effects, the production of important pro-inflammatory mediators has been assessed. Herein, our results show that carvacrol protects kidneys against LPS-induced inflammation by reducing IL-1β and nitric oxide levels and prevents LPS-induced BUN elevation. However, carvacrol did not significantly reduce creatinine, which is consistent with some reports [10, 24].

In patients with septicemic conditions, fast renal dysfunction is occurred due to inflammation or subsequent oxidative stress. In the animal model, LPS is used to induce sepsis by intraperitoneal injection [3]. LPS triggers the innate immune response and causes acute renal inflammation. Renal vasoconstriction is considered as a primary cause of acute renal failure following sepsis. LPS rapidly induces renal vasoconstriction and creates ischemic tissue injury [25]. In addition, there are suggestions for the involvement of the innate immune in LPS-induced acute kidney injury. It has been demonstrated that LPS binds to TLR4 receptors in immune cells. By activating these receptors, various factors such as pro-inflammatory cytokines (IL-1, TNF-α, IL-6, NO, and PGE2) are produced. These factors destroy tubular epithelial cells and impair renal microcirculation [26]. Busulati et al. showed that proximal tubular cells are up to 100 times more sensitive to LPS. This area plays an important role in the renal excretion of urea and creatinine [27]. The diagnosis of acute kidney injury depends mainly on clinical manifestations, such as urine volume, blood creatinine and, BUN.

Our results indicated that LPS injection significantly increased BUN and creatinine levels. The BUN is an indicator of the serum urea and its amount depends on protein intake, protein catabolism, body hydration, hepatic urea synthesis and, renal urea excretion. Most urea is excreted by the kidneys in a process that begins with glomerular filtration. Usually, 40% of the filtered urea is reabsorbed. BUN levels are elevated by urea production, fever, infection, and glucocorticoids. Reports indicate that LPS injection significantly increases BUN and creatinine levels by reducing filtration or inducing tubular injury [20]. Further, LPS increases pro-inflammatory cytokines, particularly IL-1β and nitric oxide, which play a key role in acute renal injury. Fu et al. showed that inhibition of TLR4 receptors could reduce LPS-induced kidney injury [28]. On the other hand, studies have shown that TNF-α and IL-1β are responsible for extensive renal tubular lesions and cause renal inflammation [29]. Therefore, treatment with an anti-inflammatory drug will be useful against acute kidney injury caused by sepsis [30]. Although nonsteroidal anti-inflammatory drugs are widely used for inflammation treatment, inhibition of cytokines may exhibit another strategy for the treatment of inflammation. Studies have

shown that pretreatment with carvacrol reduces inflammation not only by inhibiting TNF-α secretion but also by preventing from the migration of immune cells which involve in the production of pro-inflammatory agents, including nitric oxide and prostaglandins. In addition, carvacrol reduces nitric oxide production in macrophages by reducing the enzyme nitric oxide synthase [17]. Nitric oxide reacts with superoxide anions and produces peroxynitrite (-) ONOO. Peroxynitrite breaks down carbohydrates, oxidizes fats, breaks down DNA, and causes cell death [31]. Reports have also shown that carvacrol not only attenuates pro-inflammatory cytokines but also elevates anti-inflammatory cytokines. Ozer et al. reported that carvacrol reduces BUN, creatinine, and inflammatory cytokines such as TNF-α, IL-1β, and improves mesenteric blood flow [18]. Additionally, it has been shown that carvacrol inhibits COX-2 expression and reduced prostaglandin production [16]. It is well established that systemic administration of LPS enhances COX-2 activity. Carvacrol reduces the LPS-enhanced activity of COX-2 [32]. Therefore, it seems that carvacrol reduces inflammation t by reducing IL-1b, nitric oxide and, COX-2 activity which leads to kidney's protection from inflammation.

However, carvacrol did not significantly reduce creatinine, which is consistent with several reports (10, 23). Creatinine is produced during processes of energy production in muscles. The amount of creatinine determines the ability of the kidneys in filtering waste products from the blood. Healthy kidneys remove it from the blood by filtration and secretion. Therefore, it is known as an indicator of renal filtration. Serum creatinine is associated not only with glomerular filtration rate (GFR) but also with muscle mass, age, sex, and, protein-rich meals [33]. It has been shown that the production of creatinine increases with the loss of muscle fibers [34]. LPS significantly increases expression of the pro-inflammatory cytokines such as IL-1β, IL-6 and, TNF-α in skeletal muscle cells [35]. Much evidence suggests that in patients with sepsis or experimental animals, muscle cells, especially myofibrils, are sensitive to the effects of sepsis. Pro-inflammatory cytokines cause myofibrils proteolysis and elevate creatinine in the blood [36]. Therefore, it seems that the lack of the carvacrol effects on creatinine levels probably is related to the rise of the creatinine release from muscles. Of course, another possibility is related to glomerular filtration rate reduction by LPS. It was reported that LPS reduces creatinine excretion by affecting the heart and lowering blood pressure. In this regard, it has been shown that long-term injection of LPS reduces blood pressure due to the production of pro-inflammatory cytokines in heart cells which leads to myocardial dysfunction [37].

In conclusion, our results showed that LPS injection significantly increased creatinine, BUN, IL-1β and, nitric oxide levels. But carvacrol pretreatment reduced levels of IL-1β, nitric oxide and, BUN. Therefore, carvacrol has an anti-inflammatory effect and can protect this organ from injury caused by LPS.

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ETHICAL CONSIDERATION

All procedures were performed following the ethical standards of the National Research Committee and the Helsinki Declaration (1964 and its later amendments). Furthermore, instructors of ethics committee of Mashhad University of Medical Sciences controlled animal caring and all experimental procedures. All rats received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory animals" All efforts were made to minimize the number of animals used and their suffering.

Conflict of interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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