Tropical Journal of Pharmaceutical Research June 2022; 21 (6): 1183-1188
ISSN: 1596-5996 (print); 1596-9827 (electronic)

Available online at http://www.tjpr.org
http://dx.doi.org/10.4314/tjpr.v21i6.7

Original Research Article

Protective effects of β-eudesmol against septic liver injury via inhibition of NF-κB signaling

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Sent for review: 4 March 2022 *Revised accepted: 13 May 2022*

Abstract

Purpose: *To investigate the role of β-eudesmol in septic liver injury in mice.*
Methods: *Mice were intraperitoneally injected with 50 or 100 mg/kg β-eudesmol, and then subjected to cecal ligation and puncture for the establishment of a septic model 2 h later. Haematoxylin and eosin staining was used to evaluate histopathological changes in the liver tissues. Terminal deoxynucleotidyl transferase (TdT) dUTP Nick-End Labeling (TUNEL) staining and enzyme-linked immunosorbent assay (ELISA) were employed to determine liver damage, while inflammation and oxidative stress were evaluated by ELISA.*
Results: *Liver tissues of septic mice showed infiltration of inflammatory cells, vacuolar degeneration and obscure nucleus. However, treatment with β-eudesmol ameliorated the histopathological changes ($p < 0.01$). Moreover, β-eudesmol also reduced hepatocyte apoptosis, and decreased the levels of biomarkers for liver damage. The up-regulation of TNF-α, IL-1β, IL-6 in septic mice were significantly down-regulated by β-eudesmol ($p < 0.01$), but increased the levels of superoxide dismutase (SOD) and glutathione (GSH) and decreased malondialdehyde (MDA) and myeloperoxidase (MPO) in order to protect the mice against sepsis. In addition, β-eudesmol attenuated the cecal ligation and puncture-induced up-regulation of p-p65 in mice ($p < 0.01$).*
Conclusion: *β-Eudesmol exerts anti-inflammatory and anti-oxidant effects in septic mice by inactivating NF-κB signaling, and thus may be useful as a potential agent in the management of sepsis.*

Keywords: *β-Eudesmol, Inflammation, Oxidative stress, Cecal ligation and puncture, Sepsis, Liver injury, NF-κB*

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INTRODUCTION

Sepsis is caused by infection, and contributes to life-threatening organ dysfunction, including liver damage, lung injury, brain injury, and cardiac dysfunction [1]. Sepsis, with an incidence of 0.3 % to 1.03 %, is the most common cause of death in intensive care units [2]. The liver, as an important organ in homeostasis, immunity, metabolism, and detoxification, is prone to sepsis-induced damage [3]. Early goal-directed resuscitation, such as fluid resuscitation, infection source control, and antibiotic therapy, are currently used in the management of septic liver injury [3]. However, novel strategies are urgently needed for the improvement of

prognosis in septic patients. Apoptosis, cellular hypoxia, oxidative stress and inflammatory responses have been regarded as mechanisms that underscore the etiology of septic liver injury [4]. Agents with anti-apoptotic, anti-inflammatory and anti-oxidant capacities effectively attenuated the outcome of patients with sepsis [5]. Strategies that can prevent apoptosis, inflammation and oxidative stress might show promising effects against septic liver injury.

β-eudesmol, a sesquiterpene isolated from the rhizome of *Atractylodes lancea*, exhibits anti-oxidant, anti-bacterial and anti-inflammatory effects [6]. β-eudesmol also suppresses tumor cell proliferation, metastasis and drug resistance [7]. β-eudesmol reduced the expression of IL-6 and protected against mast cell-mediated inflammatory diseases [8]. β-eudesmol also functioned as a ROS scavenger in order to reduce oxidative stress in dermal fibroblasts [9]. However, the role of β-eudesmol in septic liver injury has not been extensively elucidated for now.

In this study, the effects of β-eudesmol on apoptosis, inflammation and oxidative stress on septic mice were investigated.

EXPERIMENTAL

Septic mice model

A total of 24 male C57BL/6 mice (Shanghai Silaike Experimental Animal Co. Ltd, Shanghai, China)) were housed in cages with controlled humidity and temperature. This study was approved by the Ethics Committee of The First Affiliated Hospital of Wenzhou Medical University (approval no. wydw2016–0224), and conducted in accordance with National Institutes of Health Laboratory Animal Care and Use Guidelines [10]. Mice were divided into four groups: sham (N = 6), CLP (cecal ligation and puncture; N = 6), CLP with 50 mg/kg β-eudesmol (N = 6) and CLP with 100 mg/kg β-eudesmol (N = 6).

Mice in the CLP groups were anesthetized by 1 % pentobarbital solution, and then subjected to midline abdominal incision. The half of the distal end of cecum was ligated, and the cecum was perforated using sterile needles. Saline (0.1 mL) was subcutaneously injected for fluid resuscitation, and the abdominal wall was sutured. Bowel and laparotomy manipulation was performed on mice in the sham group without perforation and ligation, and β-eudesmol (Sigma-Aldrich, Santa Clara, CA, USA) was intraperitoneally injected into the mice 2 h before the CLP operation. The liver tissues were harvested 12 h after operations for functional analysis.

Haematoxylin and eosin staining

Liver tissues were immersed in 4 % paraformaldehyde, and then embedded in paraffin. Tissues were sliced into sections (4 μm thick), and then placed on slides. The sections were subjected to deparaffin using xylene and rehydration using descending graded alcohol. Sections were stained with haematoxylin-eosin (Sigma-Aldrich), and observed under light microscopy (Olympus, Tokyo, Japan).

TUNEL staining

The deparaffinized and rehydrated liver sections were treated with with Proteinase K (Sigma-Aldrich). The sections were then incubated with TUNEL reaction mixture of One Step TUNEL Apoptosis Assay Kit (Beyotime, Beijing, China). DAPI was subsequently used, and the sections were observed under a fluorescence microscope (Olympus). The number of TUNEL positive cells were calculated using ImageJ software.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Liver tissues were lysed in TRIzol kit (Life Technologies, Carlsbad, CA, USA). The isolated RNAs (1 μg) were then synthesized to cDNAs using Multiscribe™ Reverse transcription Kit (Applied Biosystems, Foster City, CA, USA). The mRNA expression of TNF-α, IL-1β, IL-6 were determined by PreTaq II kit (Takara, Dalian, Liaoning, China) with the primers shown in Table 1. GAPDH was used as internal control.

Table 1: Primers used in PCR

Gene	Forward	Reverse
GAPDH	5'-GCAAAGTGGAGATTGTTGCC-3'	5'-TGGAAGATGGTGATGGGCTT-3'
TNF-α	5'-CCACCACGCTCTTCTGTCTA-3'	5'-GGTCTGGGCCATAGAACTGA-3'
IL-1β	5'-CTTTGAAGTTGACGGACCCC-3'	5'-GCTTCTCCACAGCCACAATG-3'
Il-6	5'-CCTCTGGTCTTCTGGAGTACC-3'	5'-GGAGAGCATTGGAAATTGGGG-3'

Enzyme-linked immunosorbent assay (ELISA)

Liver tissues were lysed in RIPA buffer (Beyotime), and the level of AST, ALT, MDA, MPO, SOD, and GSH were determined by ELISA

kits (Thermo Fisher Scientific, Waltham, MA, USA). Serum levels of TNF-α, IL-1β, IL-6 were also assessed by the ELISA kits (Thermo Fisher Scientific).

Western blot

Proteins isolated from the liver tissues were separated by 10 % SDS-PAGE, and transferred onto nitrocellulose membranes. The membranes were blocked in 5 % bovine serum albumin, and probed with specific antibodies: anti-β-actin (1: 2000), anti-p-IκBα and anti-IκBα (1: 3000), anti-p-p65 and anti-p65 (1: 4000). The membranes were then washed and incubated with horseradish peroxidase-conjugated secondary antibody (1: 5000). Immunoreactivities were visualized using enhanced chemiluminescence (Sigma-Aldrich). All the antibodies were acquired from Abcam.

Statistical analysis

All the data were expressed as mean ± SEM, and analyzed by Student's t-test or one-way analysis of variance (ANOVA) using SPSS software. A *p*-value of < 0.05 was considered statistically significant.

RESULTS

β-Eudesmol ameliorated histopathological changes in septic mice

To induce septic mice, cecal ligation and puncture was performed. Mice in the sham group showed a clear morphological structure of liver lobules, neat architecture and normal size of liver cells (Figure 1 A). However, the septic mice showed an infiltration of inflammatory cells and obscure nucleus in the liver tissues (Figure 1 A). Treatment with β-eudesmol ameliorated the histopathological changes of septic mice (Figure 1 A). Moreover, β-eudesmol attenuated cecal ligation and puncture-induced hepatic cell apoptosis in a dosage dependent way (Figure 1 B). The up-regulation of hepatic injury biomarkers, ALT (Figure 1 C) and AST (Figure 1 D), in the septic mice were also down-regulated by β-eudesmol, revealing the protective effect of β-eudesmol against septic liver injury.

β-Eudesmol alleviated inflammation in septic mice

Serum levels of TNF-α, IL-1β, IL-6 were enhanced in the septic mice (Figure 2 A). β-eudesmol reduced TNF-α, IL-1β, IL-6 in septic mice (Figure 2 A). Moreover, β-eudesmol also attenuated cecal ligation and puncture-induced increase of TNF-α, IL-1β, IL-6 mRNAs in a dosage dependent way (Figure 2 B), indicating the anti-inflammatory effect of β-eudesmol against septic liver injury.

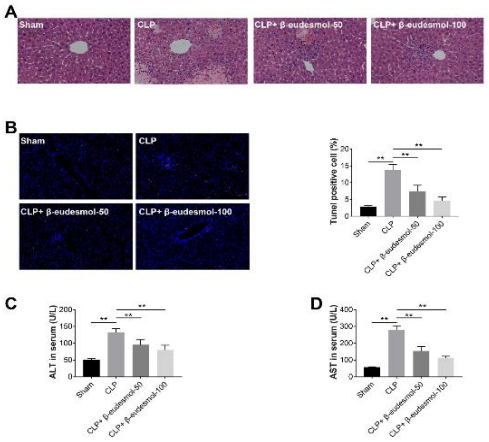

Figure 1: β-eudesmol alleviated histopathological changes in septic mice. (A) Treatment with β-eudesmol ameliorated the histopathological changes in the liver tissues of septic mice, as demonstrated by the decrease in infiltration of inflammatory cells, vacuolar degeneration and obscure nucleus. (B) Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of TUNEL positive cells in liver tissues of mice in a dosage dependent way. (C) Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of ALT in liver tissues of mice in a dosage dependent way. (D) Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of AST in liver tissues of mice in a dosage dependent way. ***P* < 0.01

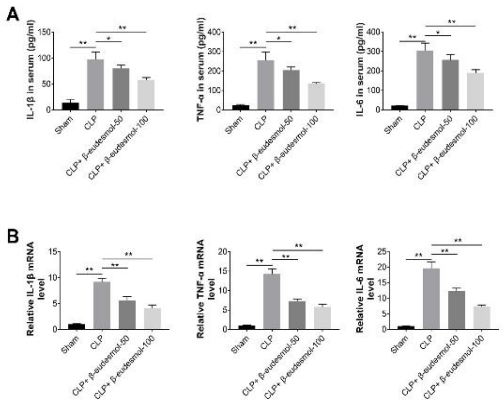

Figure 2: β-eudesmol alleviated inflammation in septic mice. (A) Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of serum levels of TNF-α, IL-1β, IL-6 in liver tissues of mice in a dosage dependent way. (B) Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of TNF-α, IL-1β, IL-6 mRNAs in liver tissues of mice in a dosage dependent way. * $p < 0.05$, ** $p < 0.01$

β-Eudesmol alleviated oxidative stress in septic mice

Cecal ligation and puncture induced the upregulation of MDA (Figure 3 A) and MPO (Figure 3 B) in liver tissues of mice. However, β-eudesmol reduced the levels of MDA (Figure 3 A) and MPO (Figure 3 B) in septic mice. Additionally, β-eudesmol weakened cecal ligation and puncture-induced decrease of SOD (Figure 3 C) and GSH (Figure 3 D) in mice, revealing the antioxidant effect of β-eudesmol against septic liver injury.

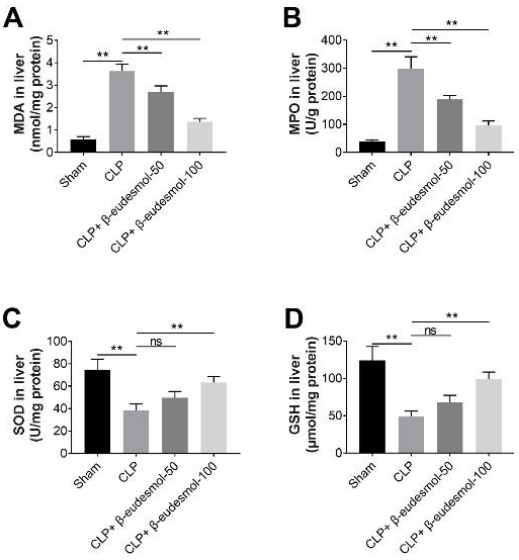

Figure 3: β-eudesmol alleviated oxidative stress in septic mice. Treatment with β-eudesmol attenuated cecal ligation and puncture-induced increase of MDA in liver tissues of mice in a dosage dependent way (A), attenuated cecal ligation and puncture-induced increase of MPO in liver tissues of mice in a dosage dependent way (B), attenuated cecal ligation and puncture-induced decrease of SOD in liver tissues of mice in a dosage dependent way (C), and attenuated cecal ligation and puncture-induced decrease of GSH in liver tissues of mice in a dosage dependent way (D). $^{**}P < 0.01$; "ns" indicates not significant ($p > 0.05$)

β-Eudesmol alleviated the activation of NF-κB signaling in septic mice

Protein expression of the negative regulator of NF-κB, IκBα was down-regulated, while p-IκBα was up-regulated in the liver tissues of septic mice (Figure 4). The expression of p-p65 was increased in septic mice (Figure 4). However, β-eudesmol increased IκBα, and decreased p-IκBα so as to inhibit the phosphorylation of p65 in the septic mice (Figure 4), demonstrating the suppressive effect of β-eudesmol against NF-κB signaling in septic liver injury.

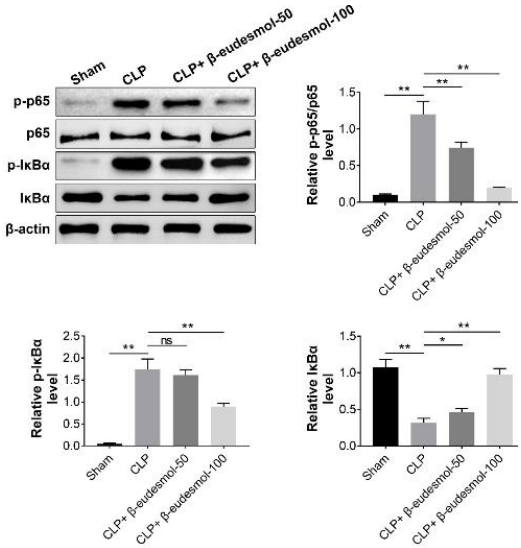

Figure 4: β-eudesmol alleviated activation of NF-κB signaling in septic mice. Treatment with β-eudesmol attenuated cecal ligation and puncture-induced decrease of IκBα, increase of p-IκBα and p-p65 in liver tissues of mice in a dosage dependent way. $^{*}P < 0.05$, $^{**}p < 0.01$. "ns" indicates not significant ($p > 0.05$

DISCUSSION

Medical plants have been widely used in the management of sepsis, through the regulation of immune responses to infection [11]. This study found that β-eudesmol, as a natural sesquiterpene isolated from rhizome of *Atractylodes lancea*, protected against sepsis-induced liver injury.

Cecum is full of bacteria, and the puncture of cecum leads to the translocation of bacteria into the blood, polymicrobial peritonitis, multi-organ dysfunction, septic shock, and ultimately death [12]. Therefore, cecal ligation and puncture was widely used as a procedure for the establishment of *in vivo* septic model [12].

In this study, cecal ligation and puncture induced histopathological changes in the liver tissues of mice, with infiltration of inflammatory cells, vacuolar degeneration and obscure nucleus. Moreover, hepatic cell apoptosis, as well as levels of ALT and AST, were also upregulated in mice following cecal ligation and puncture. These results confirmed the septic liver injury model in mice. A previous study has shown that sesquiterpenoid components of *Atractylodes rhizomes*, including hinesol, atractylon, and β-eudesmol, protected rat hepatocytes against carbon-tetrachloride and galactosoamine-induced cytotoxicity [13]. Here, treatment with β-Eudesmol also ameliorated histopathological changes in liver tissues of septic mice, reduced

hepatic cell apoptosis and down-regulated levels of ALT and AST, thus exerting hepatoprotective effects against septic liver injury.

During the progression of sepsis, severe infection induces an immune response in the liver to scavenge the pathogen or toxins [14]. However, the dysfunction of the immune response in liver promotes excess secretion of pro-inflammatory factors, and leads to multiple organ dysfunction and even death [14]. Moreover, the accumulation of ROS was associated with dysregulated immune response in the development of septic liver injury [15]. Accumulation of ROS induced oxidative stress and promoted the liver damage [16]. Oxidative stress also promoted the release of pro-inflammatory factors in hepatocytes and Kupffer cells, and induced infiltration of neutrophils and lymphocytes to augment the liver damage [16]. Suppression of oxidative stress and inflammation ameliorated septic liver damage [17]. β-eudesmol reduced hydrogen peroxide-induced inflammation and oxidative stress in dermal fibroblasts [9]. Here, β-eudesmol also reduced levels of TNF-α, IL-1β, IL-6, down-regulated MDA and MPO, and up-regulated SOD and GSH in septic mice, thus exhibiting anti-inflammatory and anti-oxidative effects against septic liver injury.

NF-κB, essential for the secretion of pro-inflammatory factors, was activated in cecal ligation and puncture-induced septic rats [18]. Inhibition of NF-κB signaling attenuated cecal ligation and puncture-induced septic liver injury [17]. β-eudesmol suppressed the activation of NF-κB signaling in hydrogen peroxide-induced dermal fibroblasts in order to reduce inflammation and oxidative stress [9]. Here, β-eudesmol increased the protein expression of IκBα, and decreased p-IκBα so as to inhibit the phosphorylation of p65 in the septic mice. Therefore, β-eudesmol exerted anti-inflammatory and anti-oxidative effects against septic liver injury through the inactivation of NF-κB signaling.

CONCLUSION

β-Eudesmol ameliorates histopathological changes in liver tissues of septic mice, suppresses cecal ligation and puncture-induced oxidative stress and inflammation in mice through inactivation of NF-κB signaling. Thus, β-eudesmol might be a promising strategy for the prevention of septic liver injury. However, clinical trials of β-eudesmol in septic patients should be investigated in further research.

DECLARATIONS

Acknowledgement

This work was supported by The Science and Technology Program of Wenzhou Municipality (Grant no. 2021Y1377 to one of the authors, Junjian Li).

Competing interests

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Qigang Xu and Junjian Li designed the study and carried them out, Zhe Chen supervised the data collection, analyzed the data, interpreted the data, Yefan Mao and Chonglin Tao prepared the manuscript for publication and reviewed the draft of the manuscript. All authors read and approved the manuscript.

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