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Original Research Article

Effect of Corynoline Isolated from *Corydalis bungeana* Turcz on Lipopolysaccharides-Induced Sepsis *In vivo* and *In vitro*

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Abstract

Purpose: *To investigate the protective effect of corynoline isolated from Corydalis bungeana Turcz on lipopolysaccharides (LPS)-induced sepsis, and determine the possible mechanism of anti-sepsis effect of the isolated corynoline.*
Methods: *Corynoline was extracted by column chromatography. LPS (100 ng/mL) was used to induce the release of TNF-α and IL-6 in RAW 264.7 cells, and the isolated corynoline was added. ELISA method was used to determine the levels of TNF-α and IL- 6. Furthermore, sepsis in mice was established by injection of LPS (2 mg/kg, i.v.), and the levels of TNF-α and IL-6 in plasma were determined by ELISA method. For survival rate test, LPS (15 mg/kg, i.v.) and heat-killed E. coli (1.0 ×10^{11} CFU/kg, i.v.) were used to establish sepsis in mice model, and the mice were observed in 7 days.*
Results: *The results indicate that corynoline significantly elevated the survival rate of septic mice induced by LPS and heat-killed E. coli, in a dose-dependent manner ($p < 0.05$). Corynoline decreased the release of TNF-α and IL-6 induced by LPS, in a dose-dependent manner ($p < 0.05$).*
Conclusion: *Treatment with corynoline significantly inhibits the mortality of LPS-induced septic mice, and the mechanism of action is probably related to the decrease of TNF-α and IL-6 release. Thus corynoline has the potential to be developed as an effective and safe drug for treating sepsis.*

Keywords: *Corynoline, Corydalis bungeana, Sepsis, Lipopolysaccharides, TNF-α, IL-6*

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INTRODUCTION

Sepsis, defined as the harmful or damaged systemic host response to infection induced by microorganisms, is a life-threatening disorder [1]. The incidence of sepsis has significantly increased over the last ten years, and remains a serious clinical problem [2,3]. Sepsis is a severe clinical syndrome with high mortality (ranges from 30% to 50%), resulting in multiple organ damage, multiple organ dysfunction syndrome (MODS), and septic shock [4,5]. Although major advances have been made in diagnosis and pathogenesis of sepsis, no satisfactory therapy has emerged. Therefore, it is imperative and urgent to find an effective therapy for the treatment or alleviation of sepsis.

Corydalis bungeana Turcz, which belongs to the Papaveraceae family, is a perennial herb found in many parts of the world, and has been traditionally used to treat inflammation, upper respiratory tract infections and influenza [6-7]. Corynoline is one of the major alkaloids of *C. bungeana*, and also has wide spectrum

pharmacological activities, such as anti-inflamation, sedative, anti-leptospira, and hepatoprotective. [8-9]. However, thus far there have been no reports on the effects of corynoline on LPS-induced sepsis in mice and its possible mechanisms of action.

In the present work, a large quantity of corynoline had been isolated from *C. bungeana*, and evaluated for is protective effects in LPS-induced sepsis in mice in order to determine a scientific basis, if any, for future application of corynoline as a prophylactic or therapeutic agent for sepsis.

EXPERIMENTAL

Plant material

Corydalis bungeana Turcz was purchased from the *Tong-ren-tang* Pharmaceutical Group and identified as the whole plant of *C. bungeana* by Professor Pingfei Fang, Department of Traditional Chinese Medicine, the Second Xiangya Hospital of Central South University, Changsha, China. A voucher specimen (S20120306#) was kept in the herbarium of Department of Traditional Chinese Medicine, the Second Xiangya Hospital of Central South University.

Experimental animals

Institute of Cancer Research (ICR) mice (20 ± 2 g) were kept on a 12 h light/dark cycle with free access to standard laboratory chow and water. Humidity was maintained at 50 % and the temperature at 25 °C. Each animal was used only once in the experiment. The experimental protocols were approved by the Animal Care and Use Committee of the Central South University (no. 2012-2769). The animals were handled according to the standard protocols for the use of laboratory animals [10].

Chemicals

Silica-gel was purchased from Qingdao Haiyang Chemical Co, Ltd (Qingdao, China).The DMEM and FBS were purchased from Invitrogen (Carlsbad, USA). Fibronectin and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and LPS were obtained from Sigma (St. Louis, USA). All other chemicals used in this study were of analytical reagent grade. Mouse TNF-α and IL-6 ELISA kits were purchased from Biosource International (Camarillo, CA, USA).

Preparation of corynoline from *C. bungeana*

The dried and powdered whole plant of *C. bungeana* (20 kg) was extracted trice under reflux (each extraction period lasted 2 h) with 70 % EtOH. The concentrated extract was dissolved in 2 % HCl (pH 3.5) and partitioned with ether. The pH of the aqueous solution was re-adjusted with ammonia water to 9.0 and extracted with $CHCl_3$. The $CHCl_3$ extract was chromatographed on silica gel (100-200 mesh) using $CHCl_3$/MeOH solvent system of increasing polarity (20:1, 15:1, 10:1, 5:1, and 2:1) as eluent to obtain five fractions A-E. Fraction C was further separated by repeated silica gel (200 -3 00 mesh) column chromatography, and eluted with cyclohexane / acetone, the yield was 3.2 g corynoline.

Characterization of corynoline by nuclear magnetic resonance (NMR)

The isolated chemical compound was identified by NMR. The NMR spectra were recorded on a Bruker AVANCE-600 MHz with TMS as internal standard and $CDCl_3$ as solvents.

Cell culture and cell viability assay

The RAW264.7 cells, a murine macrophage cell line, were cultured in plastic dishes containing Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % heat-inactivated fetal bovine serum (FBS). The RAW 264.7 cells were plated at a density of 4×10^5 and pre-incubated for 24 h in a CO_2 incubator (5 % CO_2) at 37 °C. Then RAW264.7 cells were cultured with Corynoline (0, 10, 20, 40, 80, 160, 320 μg/mL) in the presence of 100 ng/mL lipopolysaccharide (LPS) for 24 h at 37 °C. After that, the cells were washed twice with phosphate-buffered saline (PBS) and incubated with 100 μL of 0.5 mg/mL MTT for 2 h to measure the cell viability. The medium was then discarded, and 100 μL dimethyl sulfoxide (DMSO) was added. After 30-min incubation, absorbance at 570 nm was read using a microplate reader.

Evaluation of inhibition of TNF-α and IL-6 release induced by LPS *in vitro*

RAW264.7 (1.5×10^6) were grown in a 48-well plate and incubated for 4 h. corynoline (20, 40, 80 μg/mL) added immediately after addition of 100 ng/mL LPS. After incubation for another 4 h, the supernatants were collected to assess TNF-α and IL-6 levels using ELISA kits.

Plasma TNF-α and IL-6 levels in LPS-induced sepsis mice

A total of 96 ICR mice were randomly divided into four groups (n = 24). Group 1 was given LPS (2 mg /kg) while groups 2, 3 and 4 were respectively given 10, 20 and 40 mg/kg of corynoline respectively, followed by injection with LPS (2 mg/kg). Four mice were sacrificed at 0, 2, 4, 8, 12, and 24 h after inception of the experiment, and blood samples collected from their heart (n = 4). TNF-α and IL-6 levels were determined by using ELISA kits.

Survival analysis in mouse model of sepsis

For survival analysis, mice were randomly divided into four groups, control group and corynoline-treated groups (10, 20, 40 mg/kg). Mice were injected *i.v.* with 15 mg/kg LPS and heat-killed *E. coli* (*EC*, 1.0 ×10^{11} CFU/kg). The survival rate of mice was observed up to 7 days, and each group were 20 mice. The general conditions and mice mortalities were observed for 7 days.

Statistical analysis

All data were presented as mean ± SD. The Chi-square test was used to analyze the significance of rat mortality differences among groups. All other differences in means between two groups were analyzed with two-tailed Student's t-test. Results were considered to be statistically significant at a level of $p < 0.05$.

RESULTS

Structural characteristics of corynoline isolated from *C. bungeana*

The ^{1}H-NMR and ^{13}C-NMR spectra for corynoline are shown in Table 1. They indicate good agreement with literature data for corynoline [7,11]. The structure of the compound is shown in Figure 1.

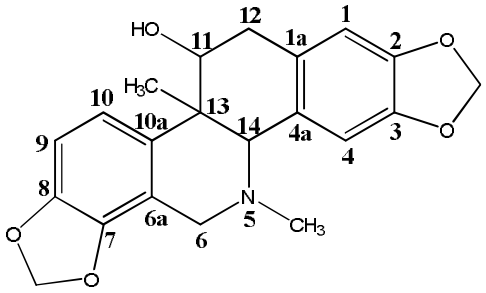

Figure 1: Structure of the corynoline isolated from *C. bungeana*

Cytotoxic effect of corynoline on RAW 264.7cells

To exclude the possibility that the inhibitory effects of corynoline on the pro-inflammatory cytokines production were due to the cytotoxcity of corynoline, MTT assay was performed, and when the RAW 264.7 cells were treated with corynoline and LPS, there was no obvious change in cell viability (Figure 2A).

Effects of corynoline on LPS-induced TNF-α, IL-6 levels

Pro-inflammatory cytokines, such as TNF-α, IL-6, play important roles in the sepsis process. In the results of our present study, corynoline at doses of 20, 40 and 80 μg/mL significantly suppressed the expressions of TNF-α and IL-6 induced by LPS in RAW 264.7 ($p < 0.05$) (Figure 2B and C).

Effect of corynoline on plasma TNF-α and IL-6 levels in mice sepsis

As can be seen in Figure 3, after LPS injection, the contents of TNF-α and IL-6 were increased, and the TNF-α and IL-6 levels peaked 4 h and 8 h after LPS injection. However, the TNF-α and IL-6 levels were significant lower than that in

Table 1: ^{1}H NMR (600 Hz) and ^{13}C NMR (150 Hz) data for corynoline in $CDCl_3$ (δ, ppm)

No.	δ_H (*J*)	δ_C	No.	δ_H (*J*)	δ_C
1	6.74 (1H, s)	107.7	**9**	6.91 (1H, d, *J*=8.1)	110.2
2		145.1	**10**	7.04 (1H, d, *J*=8.0)	118.9
3		145.2	**11**	4.07 (1H, s)	76.3
4	6.76 (1H, s)	112.6	**12**	3.28 (2H, m)	36.6
6	3.57, 4.15 (2H, ABq, *J*=15.0)	54.2	**13**		40.9
7		142.7	**14**	3.43 (1H, s)	70.1
8		48.1	**1a**		125.1
5-N-CH_3	2.34 (3H, s)	43.0	**4a**		127.9
13-CH_3	1.24 (3H, s)	23.3	**6a**		116.8
2-O-CH_2	6.12 (4H, m).	101.0	**10a**		136.1
7-O-CH_2		101.4			

group of LPS used alone at each time points after LPS injection ($p < 0.05$), in a dose-dependent manner.

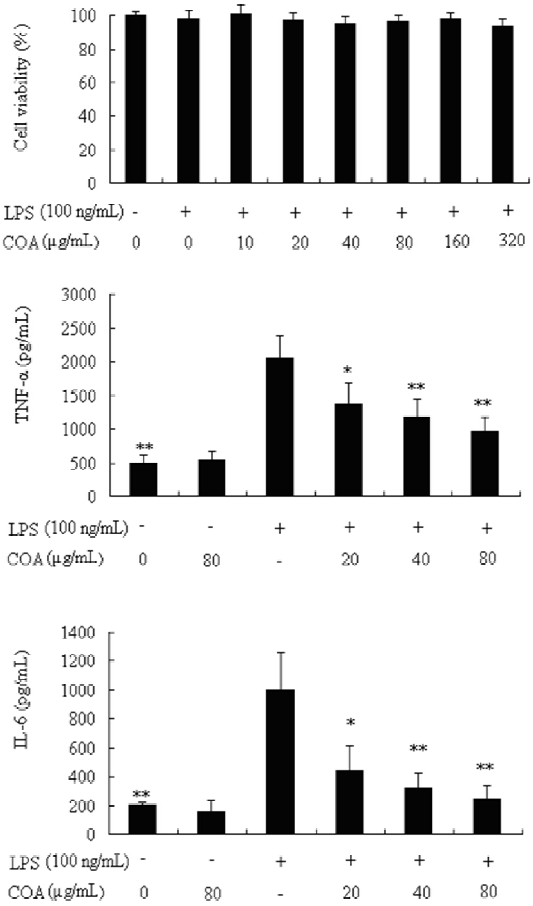

Figure 2: Effect of corynoline on TNF-α and IL-6 in RAW 264.7 cells induced by LPS. Data are shown and expressed as mean ± SD. (n=3). COA means corynoline. Asterisks indicated significant difference from LPS used alone. *$p < 0.05$, **$p < 0.01$.

Effect of corynoline on plasma TNF-α and IL-6 levels in mice sepsis

As can be seen in Figure 3, after LPS injection, the contents of TNF-α and IL-6 were increased, and the TNF-α and IL-6 levels peaked 4 h and 8 h after LPS injection. However, the TNF-α and IL-6 levels were significant lower than that in group of LPS used alone at each time points after LPS injection ($p < 0.05$), in a dose-dependent manner.

Corynoline protects mice challenged by lethal dose of LPS and heat-killed *E. coli*

As shown in Figure 4A, 80 % of the mice challenged with LPS died within 48 h. However, the survival rate of mice treated with corynoline was significantly improved, in a dose-dependent manner ($p < 0.05$). In addition, corynoline at the dosage of 40 mg/kg exhibited the best protective effect with a survival rate of 80%. Furthermore, the sepsis model induced by lethal dose of heat-killed *E. coli* also demonstrated that the corynoline have a notable protective effect against sepsis ($p<0.05$). (Figure 4)

DISCUSSION

Natural products isolated from plants are the important resource for discovering new, effective and safe drugs [12,13]. Currently, lot of active monomers, which showed potential anti-septic effects, had been found from folk or traditional medicinal plants [4,14,15].

LPS is a common trigger of sepsis, and LPS stimulates various cells to release lots of cytokines such as TNF-α and IL-6. Therefore, LPS-induced sepsis model is one of the most used and effective methods used to screen anti-septic drugs [16]. In our present investigation, we tested the protective effect of corynoline on sepsis induced in mice by a lethal dosage of LPS, and found that corynoline has a good protective effect in a dose-dependent manner. Additionally, since sepsis is commonly induced by bacteria-released LPS, a sepsis model using heat-killed bacteria is a good approximation of clinical sepsis. In our present study, the sepsis mice induced by heat-killed *E. coli* was established, and the protective effect of corynoline on sepsis was further evaluate; the results revealed that the corynoline showed good protective effect on sepsis mice.

Sepsis remains one of the leading causes of mortality in intensive care units (ICU), and was defined as the systemic host responses to severe infections and various organisms, viruses and fungi [4,17,18]. Host responses to severe infection result in the over expressions of pro-inflammatory mediators such as TNF-α and IL-6. TNF-α is one of the key mediators in the initiating of systemic inflammatory response, and IL-6 is considered as an important later mediator. Furthermore, both of the two mediators were involved in the activation of cytokine cascade in sepsis [19,20]. In our present investigation, we examined the effect of corynoline on expressions of TNF-α and IL-6 *in vivo* and *in vitro*. Our results demonstrate that corynoline decreases the release of TNF-α and IL-6 induced by LPS in a dose-dependent manner, *in vivo* and *in vitro*, which is likely to be the mechanism of protective effect of corynoline in mice sepsis.

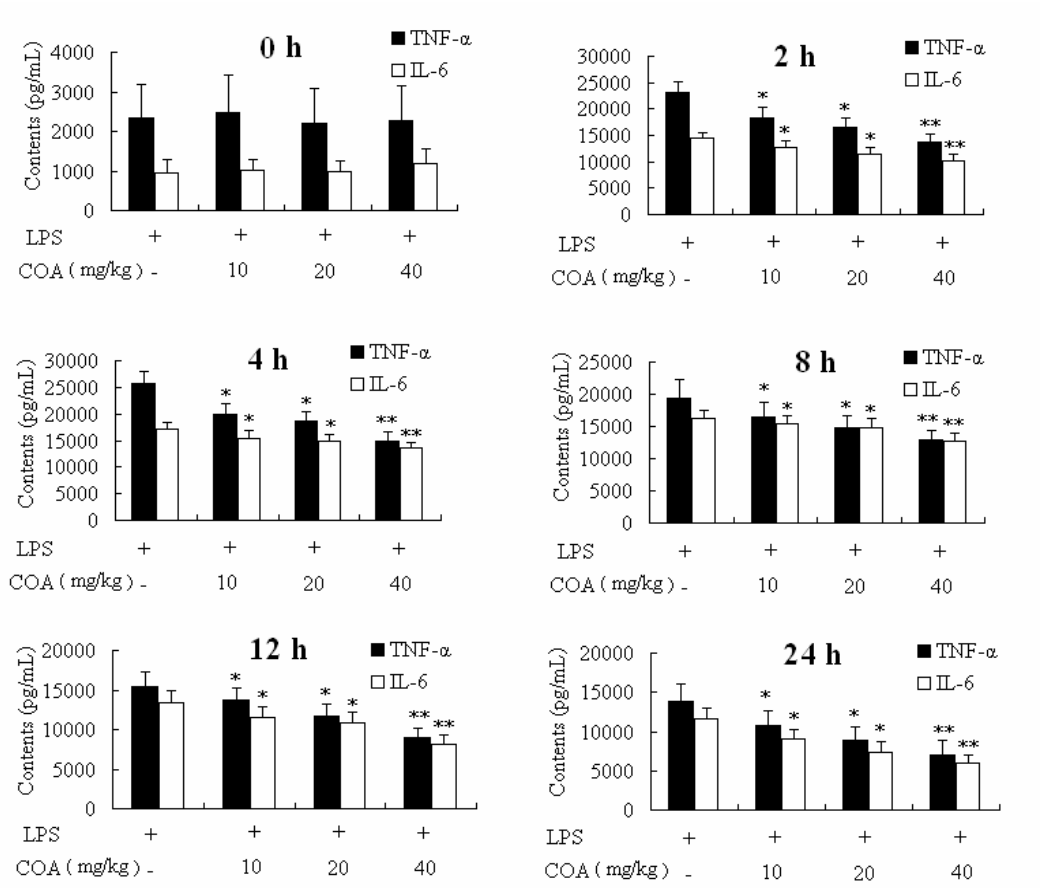

Figure 3: Effect of corynoline on TNF-α and IL-6 in plasma of LPS-induced sepsis mice. Data are shown and expressed as mean ± SD. (n = 4). COA means corynoline. Asterisks indicated significant difference from LPS used alone; *$p < 0.05$, **$p < 0.01$.

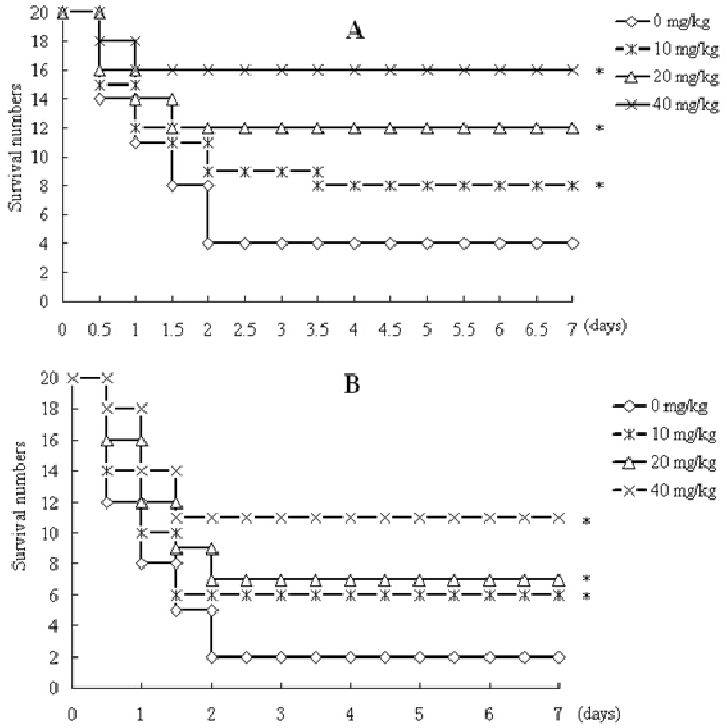

Figure 4: Effect of corynoline on the survival rate of mice challenged with lethal dose of LPS and heat-killed *E. coli*. Mice were randomly divided into four groups (n = 20) The mice were observed for 7 days. Asterisks indicated significant difference from LPS or heat-killed *E. coli* used alone; *$p < 0.05$, **$p < 0.01$

CONCLUSION

Treatment with corynoline can significantly inhibit the mortality of LPS-induced septic mice and the mechanism is probably related to the decrease of TNF-α and IL-6 releases. The results of our present investigation suggest that corynoline can be developed as an effective and clinically safe drug for treating sepsis.

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