

***Cynomorium songaricum* extract promotes osteoblast differentiation and inhibits lipopolysaccharide induced apoptosis of osteoblast.**

Wei Xue^{1#}, Nina Dai^{2#}, Fei Feng¹, Fei Wang³, Linli Luo⁴, Jiangrong Huang^{5*}, Zhonghua Cheng^{1*}

¹Department of Orthopaedics, Huanggang Central Hospital, Huanggang, Hubei, PR China

²Department of Ultrasound, Taihe Hospital, Hubei University of Medicine, Shiyan, Hubei, PR China

³Department of Auxiliary, Huanggang Central Hospital, Huanggang, Hubei, PR China

⁴Department of Nursing, Huanggang Central Hospital, Huanggang, Hubei, PR China

⁵Department of Integrative Medicine, Medical School of Yangtze University, Jingzhou, Hubei, PR China

#These authors contributed equally to this work

Abstract

Bone development is a dynamic process involving the balance between osteoblasts formation and osteoblasts resorption. Lipopolysaccharide (LPS) can break this balance and cause bone injury. *Cynomorium songaricum* is a Chinese herb that could stimulate osteoblast proliferation and inhibit osteoclast formation. This study aimed to investigate the effects of CSE on the apoptosis and differentiation of osteoblast modulated by LPS. Osteoblastic MC3T3-E1 cell line was used as the experimental model and our results showed that CSE relieved LPS induced inhibition of MC3T3-E1 cell proliferation. Moreover, we found that alkaline phosphatase activity was markedly inhibited by LPS, but could be recovered by CSE. These data suggest that LPS inhibits osteoblast proliferation and differentiation, and CSE antagonizes these inhibitory effects of LPS on osteoblast proliferation and differentiation. Furthermore, we demonstrated that CSE inhibited LPS induced caspase-3 activity in MC3T3-E1 cells, and LPS induced upregulation of caspase-3 and Bax and downregulation of Bcl-2 in MC3T3-E1 cells. These data suggest that LPS induces osteoblast apoptosis while CSE protects osteoblast from LPS induced apoptosis. In conclusion, CSE promotes osteoblast differentiation and inhibits lipopolysaccharide induced apoptosis of osteoblast.

Keywords: Osteoblasts, *Cynomorium songaricum*, Lipopolysaccharide.

Accepted on May 29, 2017

Introduction

Bone development is a dynamic process that involves the balance between osteoblasts formation and osteoblasts resorption [1]. Disruption of this balance would contribute to rheumatoid arthritis, osteomyelitis, bacterial arthritis and infection of orthopedic implants [2]. Lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria and regulates immune and inflammatory responses [3]. LPS has the capacity to induce bone resorption *in vitro* and stimulates osteoblasts to secrete osteolytic factors [4]. Thus LPS could break the balance between osteoblasts formation and osteoblasts resorption and cause bone injury.

Cynomorium songaricum (CS) is a well-known root-parasitic plant and is distributed mainly in northwest China [5]. *Cynomorium songaricum* extract (CSE) has been widely used as Chinese herbal for the treatment of sexual dysfunction, renal disease, and lumbar weakness [6]. Moreover, CSE was shown

to promote cell proliferation and neuroblast differentiation in the dentate gyrus of mice by reducing serum corticosterone levels and increasing BDNF levels [7]. CSE also stimulated osteoblast proliferation and inhibited osteoclast formation [8].

In this study, we aimed to investigate the effect of CSE on the apoptosis and differentiation of osteoblastic cell line MC3T3-E1 stimulated with LPS.

Materials and Methods

Preparation of CSE

Cynomorium songaricum Rupr. was purchased from Kyung-dong market (Seoul, South Korea). The plants were authenticated by two oriental medical doctors and the voucher specimen was deposited in our laboratory (deposition number: 2012-028). The plants (100 g) were chopped and blended using a blender and soaked in 2 L of 80% ethanol and refluxed at

20°C for 2 h three times. The insoluble materials were removed through centrifugation at 10,000 Xg for 30 min, and the resulting supernatant was concentrated and freeze-dried to yield a powder (yield: 13.2%). Before each experiment, the dried extract was dissolved in distilled and deionized water [9].

Cell culture

Osteoblastic cell line MC3T3-E1 was obtained from ATCC and grown in DMEM supplemented with 10% (v/v) Fetal Bovine Serum (FBS) and 1% (v/v) penicillin-streptomycin solution at 37°C in 5% CO₂ humidified air. To stimulate differentiation, MC3T3-E1 cells at a density of 5×10^4 cells/cm² were cultured in osteogenic differentiation medium (DMEM with 10% FBS, 10 mM HEPES, 50 µg/ml L-ascorbic acid and 5 mM β-GP) for 2 d. On differentiation day 3, the cells were pretreated with or without CSE for 2 h and then treated with or without LPS for indicated period.

MTT assay

The cell growth was measured by MTT assay. Cells were seeded at a density of 5×10^3 cells/well in 96-well plates and cultured for indicated period. At each time interval, the medium was replaced with fresh cell culture medium containing 0.5 mg/mL MTT and incubated for 4 h. Then 150 µl DMSO were added into each well and shaken for 10 min until the crystal was fully dissolved, the plates were measured at 490 nm by a Multiskan MS ELSA reader (xMark Microplate Absorbance Spectrophotometer, BioRad, USA). The relative cell number was normalized by the absorbance from the control cells.

ALP and caspase-3 activity assay

MC3T3-E1 cells were seeded at 5×10^5 cells/cm² in 100 mm culture dishes and cultured in osteogenic differentiation medium for 2 d. On differentiation d 3, following pretreatment with 0, 25 and 50 µM CSE for 2 h, the cells were treated with 0, 100 and 1,000 ng/ml LPS for 1 and 2 d. The cells were detached aided by a cell scraper in Phosphate-Buffered Saline (PBS), and then centrifuged at 16,000 Xg for 5 min. Cell pellets were placed in 300 µl of lysis buffer (Cytobuster protein extraction reagent; Novagen, Darmstadt, Germany) supplemented with protease inhibitor cocktail. ALP activity in the lysates was determined by the measurement of p-nitrophenyl phosphate (pNPP) using a commercial kit (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's instructions. Relative ALP activity was defined as micromoles of pNPP hydrolyzed per min per 1 g of total protein.

Caspase-3 activity in the lysates was determined by using caspase-3 activity assay kit (Roche) according to the manufacturer's instructions. Relative caspase-3 activity was defined as micromoles of caspase-3-specific substrate cleaved per min per 1 g of total protein.

Western blot analysis

The total proteins were extracted from the cells and subjected to Western blot analysis following the standard protocol. The primary antibodies used were: Bax (1:1500, Santa Cruz, sc-70408), Bcl-2 (1:1500, Santa Cruz, sc-7382), caspase 3 (1:1000, Santa Cruz, sc-70498), and β-actin (1:1000, Santa Cruz, sc-47778). Horseradish Peroxidase (HRP) labeled secondary antibodies were applied and Enhanced Chemiluminescence (ECL) kit (Pierce, Rockford, IL, USA) was used according to the manufacturer's instructions. The integrated density mean grey value of the band was analyzed using ImageJ software.

Statistical analysis

Dates are expressed as means ± SE. The differences of means between multiple groups was assessed with the two tailed Student's t-test (for 2 groups) and the analysis of variance (ANOVA, for >2 groups). P<0.05 was considered statistically significant.

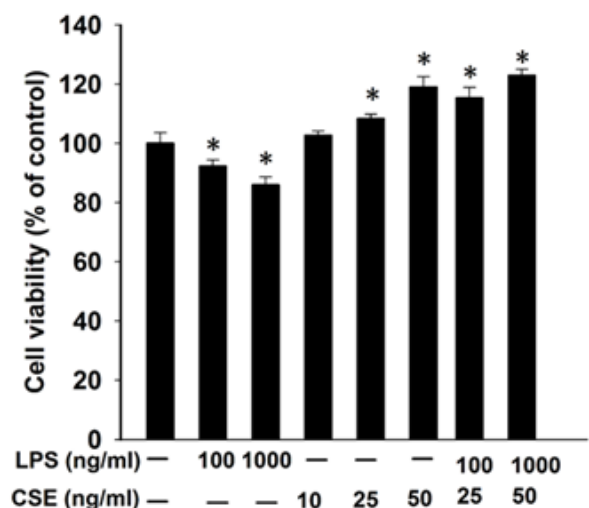


Figure 1. Effects of CSE and LPS on the proliferation of MC3T3-E1 cells. MC3T3-E1 cells were treated with LPS or CSE alone. In addition, the cells were pretreated with 25 or 50 µM CSE for 2 h, and then treated with 100 and 1,000 ng/ml LPS in the presence of CSE for 24 h. Cell viability was detected by MTT assay. *P<0.05 vs. control untreated (n=3).

Results

CSE relieves the inhibition of MC3T3-E1 cell proliferation by LPS

First we performed MTT assay to examine the proliferation of MC3T3-E1 cells. The results showed that LPS inhibited the proliferation of MC3T3-E1 cells in a dose dependent manner. In contrast, CSE increased the proliferation of MC3T3-E1 cells in a dose dependent manner. Furthermore, pretreatment with CSE could relieve the inhibition of the proliferation of

MC3T3-E1 cells by LPS in a dose dependent manner (Figure 1).

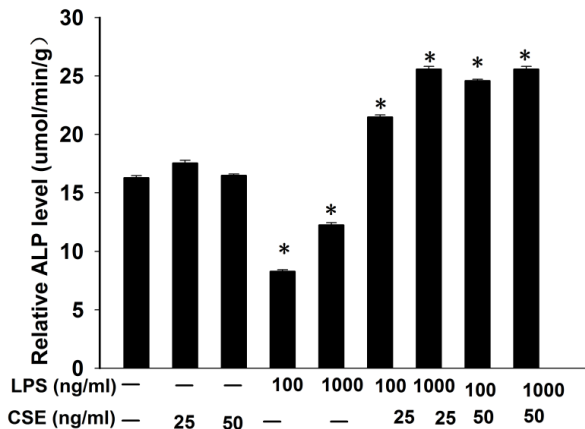


Figure 2. Effects of CSE and LPS on ALP activity in MC3T3-E1 cells. MC3T3-E1 cells were treated with LPS or CSE alone. In addition, the cells were pretreated with 25 or 50 μ M CSE for 2 h, and then treated with 100 and 1,000 ng/ml LPS in the presence of CSE for 24 h. ALP activity was detected by ALP activity assay. * $P < 0.05$ vs. control untreated ($n = 3$).

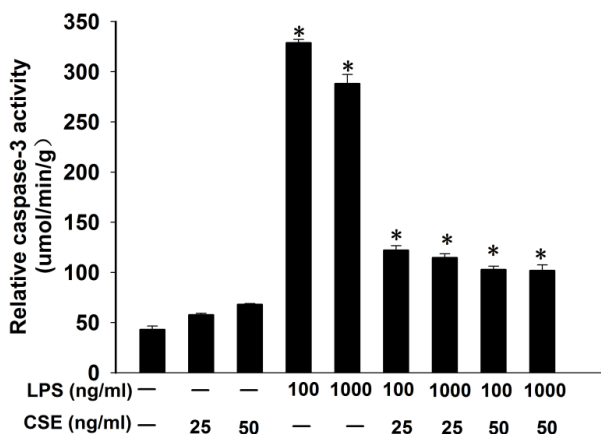


Figure 3. Effects of CSE and LPS on caspase-3 activity in MC3T3-E1 cells. MC3T3-E1 cells were treated with LPS or CSE alone. In addition, the cells were pretreated with 25 or 50 μ M CSE for 2 h, and then treated with 100 and 1,000 ng/ml LPS in the presence of CSE for 24 h. Caspase-3 activity was detected by caspase-3 activity assay. * $P < 0.05$ vs. Control untreated ($n = 3$).

CSE promoted LPS induced ALP activity in MC3T3-E1 cells

Next we performed ALP activity assay to examine the differentiation of MC3T3-E1 cells. The results showed that LPS inhibited ALP activity in MC3T3-E1 cells in a dose dependent manner. CSE had no significant effect on ALP activity in MC3T3-E1 cells, but pretreatment with CSE could significantly increase LPS induced ALP activity in MC3T3-E1 cells (Figure 2).

CSE inhibited LPS induced caspase-3 activity in MC3T3-E1 cells

Caspase-3 activity assay showed that LPS induced caspase-3 activity in MC3T3-E1 cells. CSE had no significant effect on caspase-3 activity in MC3T3-E1 cells, but pretreatment with CSE could significantly decrease LPS induced caspase-3 activity in MC3T3-E1 cells (Figure 3).

CSE modulated protein expression of Bax, Bcl-2 and caspase-3 in MC3T3-E1 cells

LPS treatment led to increased protein expression levels of caspase-3 and Bax and decreased expression of Bcl-2 in MC3T3-E1 cells. However, pretreatment with CSE could inhibit LPS induced upregulation of caspase-3 and Bax and downregulation of Bcl-2 in MC3T3-E1 cells (Figure 4).

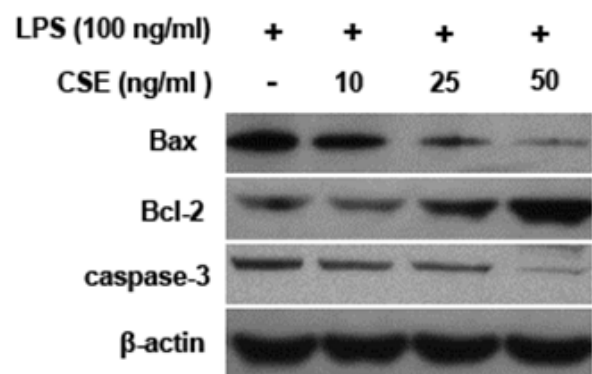


Figure 4. Effects of CSE and LPS on protein expression of Bax, Bcl-2 and caspase-3 in MC3T3-E1 cells. MC3T3-E1 cells were treated with LPS or/and CSE as indicated for 24 h. Protein expression was detected by Western blot analysis. β -actin was loading control.

Discussion

Bone injury is hard to cure because of bacteria induced activation of inflammatory responses [10]. LPS is a component of cell membrane of gram-negative bacteria and could break the balance between osteoblasts formation and osteoblasts resorption [11]. *Cynomorium songaricum* is a Chinese herb which has been used for the treatment of osteoporosis [12]. In this study, we found that LPS significantly inhibited MC3T3-E1 cell proliferation, consistent with previous study [13]. However, CSE relieved LPS induced inhibition of MC3T3-E1 cell proliferation. ALP is an essential marker in osteoblast differentiation [14]. We found that ALP activity was markedly inhibited by LPS, but could be recovered by CSE. These data suggest that LPS inhibits osteoblast proliferation and differentiation, and CSE antagonizes these inhibitory effects of LPS on osteoblast proliferation and differentiation.

LPS can induce cell apoptosis *via* extrinsic pathway, and this may contribute to bacteria induced bone destruction [15]. We examined the effect of CSE on the apoptosis of MC3T3-E1 cells stimulated by LPS. The results showed that caspase-3 activity was significantly increased in MC3T3-E1 cells treated

with LPS, but CSE inhibited LPS induced caspase-3 activity in MC3T3-E1 cells. Moreover, LPS treatment led to the upregulation of pro-apoptotic factors such as caspase-3 and Bax and the downregulation of anti-apoptotic factor Bcl-2 in MC3T3-E1 cells. However, CSE could decrease the expression of caspase-3 and Bax and increase the expression of Bcl-2 in MC3T3-E1 cells. These data suggest that LPS induces osteoblast apoptosis while CSE protects osteoblast from LPS induced apoptosis.

In conclusion, our study suggests that CSE promotes osteoblast differentiation and inhibits lipopolysaccharide induced apoptosis of osteoblast. CSE could be used to treat bacteria induced bone injury.

Conflict of Interest

None declared.

Acknowledgement

This study was supported by basic disciplines of research and development fund of the department of science and technology (No. 2014JCY004) and scientific research foundation for talented scholars of the personnel department of Yangtze University (No. 801250010132).

References

- Bae HW, Patel VV, Sardar ZM. Transient local bone remodeling effects of rhbmp-2 in an ovine interbody spine fusion model. *J Bone Joint Surg Am* 2016; 98: 2061-2070.
- Azeena S, Subhapradha N, Selvamurugan N. Antibacterial activity of agricultural waste derived wollastonite doped with copper for bone tissue engineering. *Mater Sci Eng C Mater Biol Appl* 2017; 71: 1156-1165.
- Teramachi J, Inagaki Y, Shinohara H. PKR regulates LPS-induced osteoclast formation and bone destruction in vitro and in vivo. *Oral Dis* 2016.
- Zhang Y, Yan M, Yu QF. Puerarin prevents LPS-induced osteoclast formation and bone loss via inhibition of AKT activation. *Biol Pharm Bull* 2016; 39: 2028-2035.
- Tuvaanjav S, Shuqin H, Komata M. Isolation and antiviral activity of water-soluble *Cynomorium songaricum* Rupr. polysaccharides. *J Asian Nat Prod Res* 2016; 18: 159-171.
- Liu HP, Chang RF, Wu YS. The Yang-Tonifying herbal medicine *Cynomorium songaricum* extends lifespan and delays aging in drosophila. *Evid Based Complement Alternat Med* 2012; 2012: 735481.
- Yoo DY, Choi JH, Kim W. *Cynomorium songaricum* extract enhances novel object recognition, cell proliferation and neuroblast differentiation in the mice via improving hippocampal environment. *BMC Complement Altern Med* 2014; 14: 5.
- Yin J, Tezuka Y, Kouda K. Antiosteoporotic activity of the water extract of *Dioscorea spongiosa*. *Biol Pharm Bull* 2004; 27: 583-586.
- Yoo DY, Choi JH, Kim W. *Cynomorium songaricum* extract enhances novel object recognition, cell proliferation and neuroblast differentiation in the mice via improving hippocampal environment. *BMC Complement Altern Med* 2014; 14: 5.
- Lapérière O, Cloitre A. Interleukin-33 and RANK-L Interplay in the Alveolar Bone Loss Associated to Periodontitis. *PLoS One* 2016; 11: e0168080.
- Kanno Y, Ishisaki A, Miyashita M. The blocking of uPAR suppresses lipopolysaccharide-induced inflammatory osteoclastogenesis and the resultant bone loss through attenuation of integrin β 3/Akt pathway. *Immun Inflamm Dis* 2016; 4: 338-349.
- Cui Z, Guo Z, Miao J, Wang Z, Li Q. The genus *Cynomorium* in China: an ethnopharmacological and phytochemical review. *J Ethnopharmacol* 2013; 147: 1-15.
- Liu YH, Huang D, Li ZJ. Toll-like receptor-4-dependence of the lipopolysaccharide-mediated inhibition of osteoblast differentiation. *Genet Mol Res* 2016; 15.
- Lin FX, Du SX, Liu DZ. Naringin promotes osteogenic differentiation of bone marrow stromal cells by up-regulating Foxc2 expression via the IHH signaling pathway. *Am J Transl Res* 2016; 8: 5098-5107.
- Fragoso IT, Ribeiro EL, Gomes FO. Diethylcarbamazine attenuates LPS-induced acute lung injury in mice by apoptosis of inflammatory cells. *Pharmacol Rep* 2016; 69: 81-89.

*Correspondence to

Jiangrong Huang

Department of Integrative Medicine

Medical School of Yangtze University

PR China

Zhonghua Cheng

Department of Orthopaedics

Huanggang Central Hospital

PR China