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Leonurine inhibits IL-1β induced inflammation in murine chondrocytes and ameliorates murine osteoarthritis

Wenhua Yin*, Ying Lei

Department of Orthopaedics, Yuebei People's Hospital Affiliated to Medical College of Shantou University, Shaoguan 512026, China

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ABSTRACT

Osteoarthritis (OA) is a chronic degenerative joint disease characterized by cartilage degradation, subchondral bone sclerosis and synovitis. Leonurine, an active component extracted from the leaves of Herba leonuri, has been reported to possess various potent biological effects such as anti-oxidant, anti-apoptosis, and anti-inflammatory. However, the therapeutic benefits of leonurine on OA have not been reported. This study aimed to evaluate the therapeutic effect of leonurine on chondrocytes and in murine OA models. Murine chondrocytes were pre-treated with leonurine (5, 10, and 20 μM) for 2 h and then stimulated with IL-1β for 24 h. Production of NO, PGE2, IL-6, TNF-α, MMP-3, MMP-13, and ADAMTS-5 was assessed with the Griess reagent and ELISAs. The mRNA expression of COX-2, iNOS, MMP-3, MMP-13, ADAMTS-5, aggrecan, and collagen-II was tested with real-time polymerase chain reaction. The protein expression of iNOS, COX-2 and NF-κB-related signaling molecules was measured with western blotting. In this study, leonurine visibly inhibited the IL-1β-induced production of NO, PGE2, IL-6 and TNF-α; and decreased the expression of iNOS, COX-2, MMP-3, MMP-13 and ADAMTS-5 in chondrocytes. Furthermore, leonurine significantly suppressed IL-1β-stimulated NF-κB activation. In addition, treatment with leonurine not only prevented cartilage destruction and subchondral bone thickening, but also alleviated synovitis in a murine OA model. Taken together, these results suggest that leonurine may be a potential therapeutic agent in OA treatment.

1. Introduction

Osteoarthritis (OA) is a chronic degenerative disease caused by joint disorder and occurs worldwide [1]. The main characteristic of OA is loss of articular cartilage and synovial inflammation, leading to joint stiffness, swelling, pain and loss of mobility [2]. There are no effective disease-modifying drugs currently available until the end stage of disease necessitating arthroplasty [3,4]. This approach, however, neither addresses the morbidity associated with early disease nor the limitations of joint replacement surgery, which include persistent pain, absence of function impairment and the finite lifespan of prostheses [5–7].

The etiology of OA is multifactorial and the exact pathogenesis has not been fully elucidated, but a rapid growth study showed that inflammation is closely correlated with OA processes [8]. Inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) play vital roles in OA progression [9,10]. Elevated IL-1β levels have been found in synovial fluid and cartilage tissue of OA patients [11]. This alters chondrocyte anabolism through suppression of collagen synthesis and proteoglycan production while enhancing catabolic factors including matrix metalloproteinases (MMPs) and other inflammatory mediators such as IL-6, prostaglandin E2 (PGE2) and nitric oxide (NO) synthesis which promote OA [12]. Therefore, exploring therapies that inhibit these inflammatory mediators is essential for clinical prevention and treatment of OA.

Herba leonuri (HL) is a traditional Chinese herbal medicine that has been widely used for many years to treat various disorders and diseases, including menstrual disorders, metrorrhagia, dysmenorrhea and some other diseases [13–15]. Leonurine, the major alkaloid purified from HL, has attracted considerable interest due to its anti-oxidant, anti-apoptosis, anti-inflammatory, and anti-bacterial biological activities [16,17]. Although there is much research into leonurine, the therapeutic benefits of leonurine on OA remain unknown. Therefore, in this study, the potential therapeutic effects and the underlying mechanisms of leonurine on OA pathogenesis were investigated both *in vitro* and *in vivo*. Our results indicate that leonurine attenuates the progression of OA in part by inhibiting the activation of NF-κB pathways.

* Corresponding author at: Yuebei People's Hospital Affiliated to Medical College of Shantou University, Huimin South Road 133, Shaoguan, Guangdong, China.
E-mail address: yinwenhuasohu@sohu.com (W. Yin).

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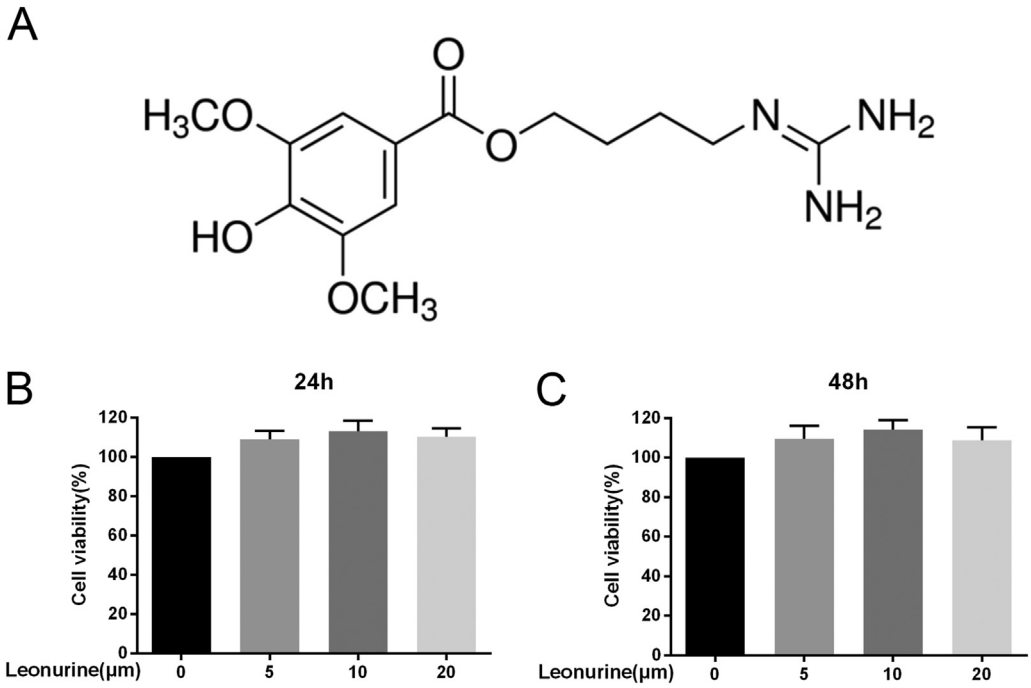

Fig. 1. Effects of leonurine on chondrocyte viability.
Chondrocytes were cultured with increasing concentrations of leonurine (0, 5, 10 or 20 μM) for 24 and 48 h. The cell viability was determined with the CCK-8 assay. (A) Chemical structure of leonurine and (B, C) effect of leonurine on *in vitro* cell cytotoxicity. The values are the mean ± SD, $n = 6$.

2. Materials and methods

2.1. Chemicals and reagents

Leonurine (the chemical structure is shown in Fig. 1A), recombinant human IL-1β, collagenase type II, dimethylsulfoxide (DMSO), and Safranin O/Fast Green were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo (Kumamo, Japan). Primary antibodies against COX-2, iNOS, and collagen-II were purchased from Abcam (Cambridge, MA, USA). Primary antibodies against p65, p-p65, IκBα, and p-IκBα were purchased from Cell Signaling Technology (Beverly, MA, USA). TRIzol reagent was purchased from Invitrogen (Carlsbad, CA, USA). PrimeScript RT reagent Kit and SYBRR Premix Ex Taq II (Tli RNaseH Plus) kit were purchased from TaKaRa (Dalian, China). Griess reagent was purchased from Beyotime Institute of Biotechnology (Shanghai, China). ELISA kits of PGE2, IL-6, TNF-α, MMP-3, MMP-13 and ADAMTS-5 were purchased from R & D systems (Minneapolis, MN, USA).

2.2. Primary chondrocyte isolation and culture

Primary chondrocytes were isolated following a procedure as described previously [18]. Briefly, rib cartilage from newborn mice was dissected under a stereo light microscope to harvest primary chondrocytes. After trypsin digestion for 30 min, primary chondrocytes were separated and purified, and digested in 0.1% collagenase type II with 10% FBS, 100 U/mL penicillin, and 100 mg/ml streptomycin sulfate at 37 °C for 4–6 h. Primary chondrocytes were resuspended and seeded in a 24-well plate and cultured in DMEM/F12 with 10% FBS, 100 U/mL penicillin, and 100 mg/ml streptomycin sulfate at 37 °C with 5% CO2. The medium was changed every 2–3 days. Cells were passaged when at 80–90% confluence using 0.25% trypsin-EDTA solution. Only passages 1 to 3 were used in our study to avoid phenotype loss.

2.3. Cell viability

The effect of leonurine on the viability of chondrocytes was determined with a CCK-8 assay according to the manufacturer's instructions. Cells were cultured in 96-well plates (5×10^3/well) for 24 h and then pre-treated with different concentrations (0, 5, 10 or 20 μM) of leonurine for 24 h and 48 h. Subsequently, the cells were incubated with 10 μL CCK-8 solution at 37 °C for 4 h. The absorbance at 450 nm was measured with a microplate reader (Leica Microsystems, Germany).

2.4. Measurement of NO, PGE2, IL-6, TNF-α, MMP-3, MMP-13, and ADAMTS-5

Chondrocytes were cultured in DMEM/F12 at a density of 3×10^5 cells/mL in 6-well plates and pretreated with leonurine (5, 10 or 20 μM) 2 h and then stimulated with IL-1β (10 ng/mL) for 24 h. The NO levels in the culture medium were determined with the Griess reaction as previously described [19]. The level of PGE2, IL-6, TNF-α, MMP-3, MMP-13, and ADAMTS-5 in the culture medium was determined with commercial ELISA kits according to the manufacturers' instructions. All assays were performed in duplicate.

2.5. Quantitative real-time polymerase chain reaction

Total RNA samples were isolated from chondrocytes by using TRIzol reagent according to the manufacturer's instructions. The total RNA products were immediately transcribed by reverse transcription (RT) into cDNA by using a PrimeScript RT reagent Kit with gDNA Eraser. Polymerase chain reaction (PCR) amplification was performed in a Chromo4 Four-Color Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) by using the SYBRR Premix Ex Taq II (Tli RNaseH Plus) kit. Data were analyzed using the $2^{-\Delta\Delta CT}$ method [20]. Primer sequences for each gene used in this study are shown in Table 1.

2.6. Western blot analysis

Proteins isolated from 6-well plates were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene membranes (Sigma Chemical Co). Membranes were probed with the primary antibodies against INOS (1:1000), COX-2 (1:1000), p65 (1:1000), p-p65 (1:1000), IκB (1:1000), p-IκB (1:1000) and β-actin (1:3000), and with a second antibody (1:1000). PVDF membranes were incubated with primary antibodies overnight at 4 °C and washed three