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Leonurine Exerts Anti-Catabolic and Anti-Apoptotic Effects via Nuclear Factor kappa B (NF-κB) and Mitogen-Activated Protein Kinase (MAPK) Signaling Pathways in Chondrocytes

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

ABCG 1 Peng-Fei Hu*
ABCDEF 2 Fang-Fang Sun*
ACDE 3 Jing Qian

1 Department of Orthopedic Surgery, Second Affiliated Hospital, School of Medicine, Zhejiang University; Orthopedics Research Institute of Zhejiang University, Hangzhou, Zhejiang, P.R. China
2 Cancer Institute (Key Laboratory of Cancer Prevention and Intervention, China National Ministry of Education, Key Laboratory of Molecular Biology in Medical Sciences, Zhejiang Province, China), Second Affiliated Hospital, Zhejiang University School of Medicine, Hangzhou, Zhejiang, P.R. China
3 Pharmaceutical Informatics Institute, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou, Zhejiang, P.R. China

* Peng-Fei Hu and Fang-Fang Sun contributed equally to this study

Corresponding Author: Jing Qian, e-mail: jingqian@zju.edu.cn

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Background: Leonurine confers neuroprotection, inhibits myocardial apoptosis, ameliorates endothelial dysfunction, and shows anti-inflammatory effects, and may be beneficial for clinical applications. However, the effects of leonurine on chondrocytes remain unknown. Here, we investigated the protective role of leonurine in rat chondrocytes.

Material/Methods: To explore the potential therapeutic effect of leonurine against osteoarthritis (OA), rat chondrocytes were treated with IL-1β along with different concentrations of leonurine *in vitro*. The levels of matrix metalloproteinases (MMPs), ADAMTS, Bax, and Bcl-2 were measured by PCR, ELISA, and Western blotting. Caspase-3 activity in chondrocytes was determined using a caspase-3 activity assay. Western blotting was also performed to examine activation of the NF-κB and mitogen-activated protein kinase (MAPK) pathways to elucidate the likely regulatory mechanisms.

Results: Leonurine counteracted IL-1β-induced production of MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5. Leonurine treatment reduced both the mRNA and protein levels of Bax and increased the level of Bcl-2. Leonurine also inhibited the activity of caspase-3 in IL-1β-induced chondrocytes. Furthermore, the activation of MAPK and phosphorylation of p65 were suppressed by leonurine.

Conclusions: The results of this study indicate that leonurine exerts anti-catabolic and anti-apoptotic effects in chondrocytes *in vitro* via suppression of the NF-κB and MAPK signaling pathways.

MeSH Keywords: Matrix Metalloproteinases • Mitogen-Activated Protein Kinase Phosphatases • NF-kappa B • Osteoarthritis

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Background

Osteoarthritis (OA) is a very common degenerative disease in the ageing population, which occurs in the knees, hips, and interphalangeal joints. The disease is characterized by irreversible cartilage loss, osteophyte formation, progressive subchondral bone sclerosis, and synovial inflammation, which can cause pain, stiffness, and functional limitation of the affected joints [1]. Common risk factors for OA include older age, obesity, sedentary lifestyle, trauma, inherent genetic factors, and epigenetic alterations [2]. The homoeostasis between synthesis and degradation of extracellular matrix (ECM) is disrupted and leads to cartilage destruction. Matrix metalloproteinases (MMPs) are critical catabolic enzymes that are strongly connected to cartilage collagenolysis because they can digest collagen fibers and proteoglycans [3]. Aggrecan is cleaved by 'a disintegrin and metalloproteinase with thrombospondin motifs' (ADAMTS) enzymes, especially ADAMTS-4 and ADAMTS-5 [4]. Known as pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)-α are important mediators of cartilage degeneration that can stimulate the expression of MMPs, ADAMTS, and COX-2, as well as production of free radicals [5,6].

Leonurine, an active natural alkaloid compound isolated from plants belonging to the Herba leonuri family, has been used widely in the treatment of gynecological diseases such as dysmenorrhea, menoxenia, and gynecological dysfunctions [7,8]. Recent studies have also indicated that leonurine has several other biological activities, including neuroprotection, inhibition of myocardial apoptosis, amelioration of endothelial dysfunction, and anti-inflammatory effects [9–12]. The relationship between leonurine and chondrocytes is unclear. With regard to the musculoskeletal system, Li et al. reported that leonurine can attenuate the severity of arthritis by decreasing the expression of IL-1β, IL-6, IL-8, TNF-α, and MMPs in mice with CIA. They also demonstrated that leonurine inhibited the migration and invasion of RA fibroblast-like synoviocytes via the NF-κB and mitogen-activated protein kinase (MAPK) pathways [13]. A recent study showed that leonurine suppressed RANKL-induced osteoclastogenesis and actin ring formation by regulation of RANK-TRAF6, NF-κB, and PI3K/Akt signaling in a dose-dependent manner in RAW 264.7 cells and mouse bone marrow monocytes. Furthermore, leonurine administration has been shown to prevent bone loss caused by oestrogen deficiency in ovariectomised mice [14]. According to another recent study, performed by Yin et al., leonurine reduced the IL-1β-induced secretion of inflammatory cytokines, as well as enzymes such as MMP-3, MMP-13, and ADAMTS-5 in mouse chondrocytes [15]. At present, the association between leonurine and chondrocyte apoptosis remains unclear.

The present study was performed to evaluate the pharmacological effects of leonurine and the underlying potential mechanisms involved in IL-1β-induced apoptosis in chondrocytes. We used RT-PCR, ELISA, and Western blot analysis to examine the levels of MMPs, ADAMTS, Bcl-2, and Bax *in vitro*. The underlying regulatory mechanisms were also investigated by assessing involvement of the NF-κB and MAPK pathways.

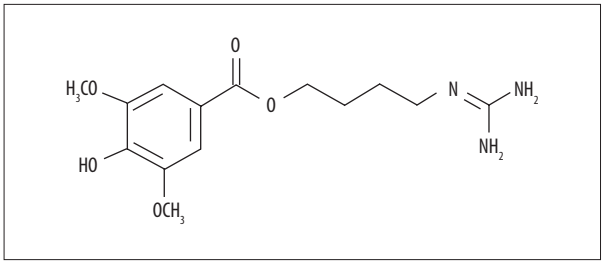

Figure 1. Chemical structure of leonurine.

Material and Methods

Cell culture and treatment

Articular cartilage was collected from 4-week-old Sprague-Dawley rats. We carefully isolated the knee joint and femoral head. Then, the cartilage was cut into pieces 1–3 mm^3 in size. After pronase and collagenase digestion, cells were cultured in complete cell medium in monolayers. Chondrocytes were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Chondrocytes from passage 1 were used in the next steps. All experiments were officially approved and supervised by the University of Zhejiang Institutional Animal Care and Use Committee.

Chondrocytes cultured in 96-well plates were incubated with different concentrations of leonurine for 24 h to detect cell viability by MTT assay. For chondrocyte inflammation and apoptosis involvement experiments, chondrocytes seeded in 6-well plates were pre-treated with leonurine for 3 h, followed by IL-1β (10 ng/ml) stimulation for 24 h. For signal pathway analysis, chondrocytes were pre-treated with leonurine for 3 h, followed by IL-1β (10 ng/ml) stimulation for 30 min.

Cell viability assay

Chondrocytes (5×10^3 cells/well) were seeded in 96-well plates and incubated with different concentrations of leonurine (Sigma Aldrich; Merck KGaA; Figure 1) for 24 h. Then, 20 µL MTT (5 mg/mL) was added to each well, followed by incubation for 4 h at 37°C. The culture medium was removed before adding an equal volume of DMSO. The absorbance was determined using a microplate reader. The range of concentrations that did not exhibit toxic effects was used in subsequent experiments.

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Table 1. Real-time PCR primers and conditions.

Gene	Genbank accession	Primer sequences (5' to 3')	Size (bp)	Annealing (°C)
GAPDH	NM_017008.4	GAAGGTCGGTGTGAACGGATTTG	127	60
		CATGTAGACCATGTAGTTGAGGTCA		
MMP-1	NM_001134530.1	GCTTAGCCTTCCTTTGCTGTTGC	136	60
		GACGTCTTCACCCAAGTTGTAGTAG		
MMP-13	NM_133530	CAACCCTGTTTACCTACCCACTTAT	85	60
		CTATGTCTGCCTTAGCTCCTGTC		
ADAMTS-4	NM_023959	GCCAGCAACCGAGGTCCCATA	118	60
		CCACCACCAGTGTCTCCACGAAT		
ADAMTS-5	NM_198761.1	GCCACGACCCTCAAGAACTTTT	90	60
		CAGGATGGCTGCATCGTAGT		
Bcl2	L14680	GGCTACGAGTGGGATACTGGAGAT	86	60
		CTCTCAGGCTGGAAGGAGAAGATG		
Bax	NM_017059	CCCCAGGACGCATCCACCAA	112	60
		GGGAGTCTGTATCCACATCAGCAA		

Quantitative real-time polymerase chain reaction

Chondrocytes (5×10^5 cells/well) were seeded in 6-well plates. Following retreatment with 5 or 20 μM leonurine for 3 h, IL-1β (10 ng/mL; Sigma Aldrich; Merck KGaA) was added for 24 h. According to the manufacturer's instructions, total RNA of chondrocytes was extracted using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and cDNA was produced by 45 cycles at 95°C for 15 s and 60°C for 30 s. The primer sequences and annealing temperatures are listed in Table 1. Relative gene expression was calculated based on the $2^{-\Delta\Delta Ct}$ method using GAPDH as a reference gene for normalization.

Measurement of MMP-1 and MMP-13 using ELISA

MMP-1 and MMP-13 protein-secreting levels in cell culture supernatants were measured by ELISA following the manufacturer's instructions. The ELISA kits for MMP-1 and MMP-13 were purchased from Sangon Biotech (Shanghai, China). All assays were done in duplicate.

Caspase-3 activity

Caspase-3 activity was evaluated using a Caspase-3 Cellular Activity Assay Kit (Cell Signaling Technology, Danvers, MA, USA). Activated caspase-3 cleaves the Ac-DEVD-AMC (N-acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin) between DEVD and AMC, generating highly fluorescent AMC that can be detected using a fluorescence reader. According to the manufacturer's protocol, chondrocytes were collected and lysed using cell lysis buffer in the presence of synthetic fluorescent substrates at 37°C. Caspase-3 activity was detected at 405 nm on a microplate reader.

Western blotting

Chondrocytes from different groups were lysed with RIPA buffer (Cell Signaling Technology, Danvers, MA, USA). Then, the Bradford dye assay was performed to assess protein concentrations. A total of 2 μg protein was added per lane and separated by 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). Proteins were then transferred onto nitrocellulose membranes and probed with primary antibodies against ADAMTS-4 and ADAMTS-5 (Abcam, Cambridge, MA, USA), NF-κB p65, p-NF-κB p65, JNK p-JNK, ERK, p-ERK, p38, and p-p38 (Cell Signaling Technology) overnight at 4°C. After washing, the membranes were applied to secondary antibodies for 1 h at room temperature. Immunoreactive bands were visualised using an ECL substrate (Fude Biological Technology, Hangzhou, China) and exposed to Kodak X-Omat film (Kodak, Rochester, NY, USA).

Statistical analysis

All tests were performed at least 3 times, and values are presented as the means ±SD. One-way analysis of variance was done using SPSS (v19.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism 7 (La Jolla, CA, USA) software. Comparisons between groups were performed using the post hoc Tukey's test. $P<0.05$ was regarded as statistical significance.

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Results

Effects of leonurine on cell viability

The classical MTT assay was used to assess the effects of leonurine on cell viability. Leonurine at concentrations of 0 to 20 μM exhibited no significant effect on cell viability, whereas treatment with 30 and 40 μM leonurine markedly reduced cell viability (Figure 2). Therefore, leonurine concentrations of 5 and 20 μM (low and high doses, respectively) were used for further experiments.

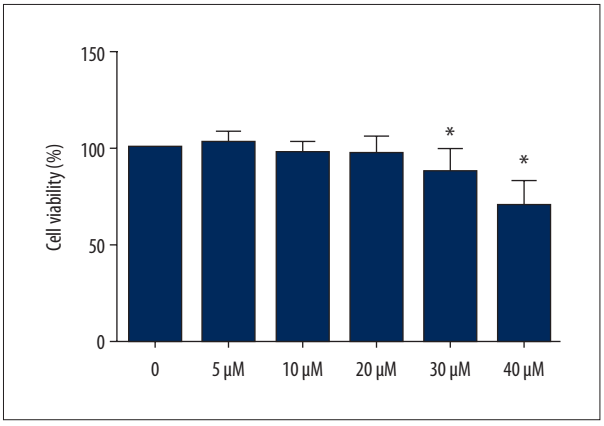

Figure 2. Effects of leonurine on cell viability. Chondrocytes were treated with various concentrations of leonurine (5–40 μM) for 24 h. Chondrocytes incubated in medium without leonurine were used as controls and were considered to be 100% viable. * P<0.05 *vs.* control group.

Leonurine inhibits IL-1β-induced MMP and ADAMTS expression in chondrocytes

The function of leonurine in regulating MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5 expression in chondrocytes were assayed by RT-PCR, ELISA, and Western blotting. We found

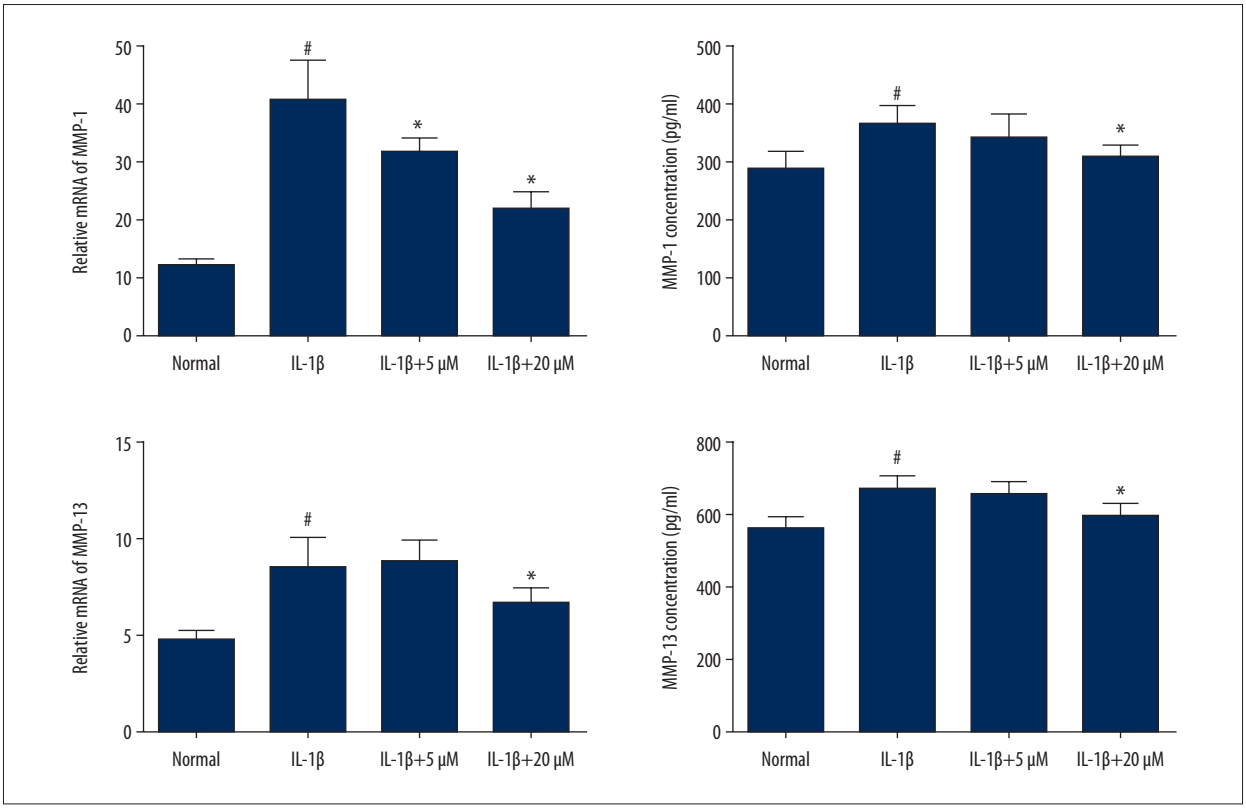

Figure 3. Leonurine inhibits IL-1β-induced expression of MMP-1 and MMP-13, as determined by RT-PCR and ELISA. Chondrocytes were pre-treated with different concentrations of leonurine (5 or 20 μM) for 3 h, followed by IL-1β (10 ng/mL) stimulation for 24 h. Each column represents the mean ±SD. Leonurine (20 μM) strongly decreased IL-1β-induced MMP-1 and MMP-13 production. * P<0.05 *vs.* IL-1β group. # P<0.05 *vs.* normal group.

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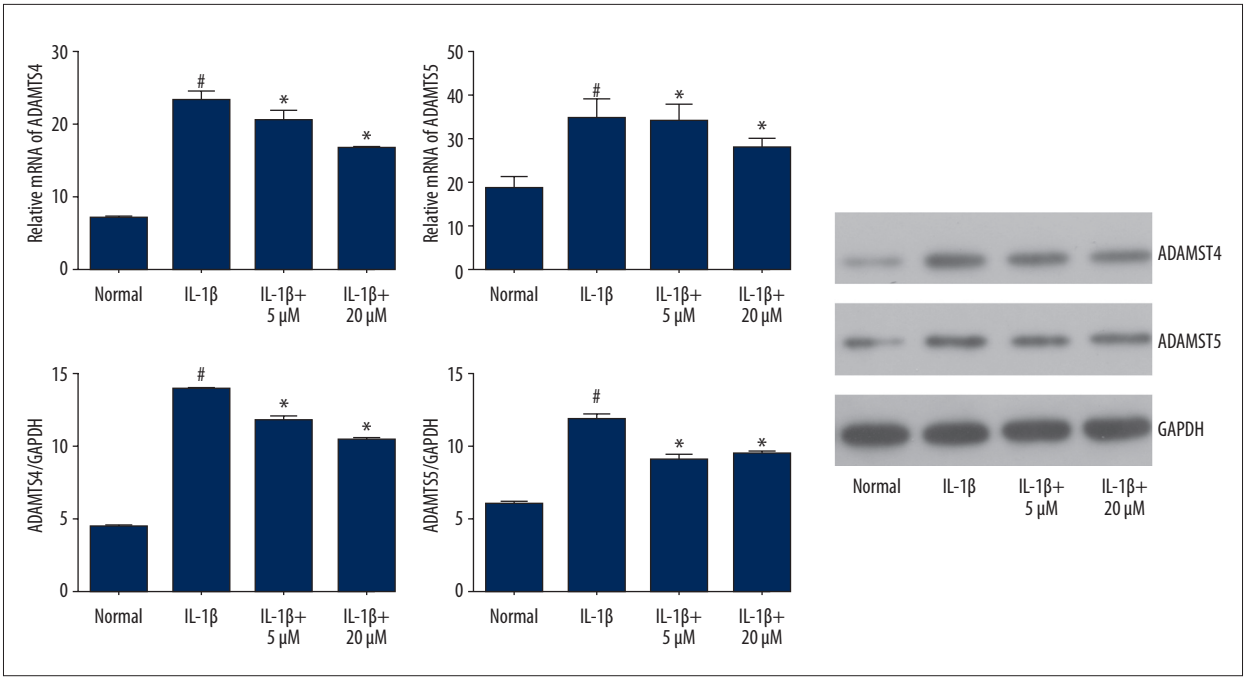

Figure 4. Leonurine decreased IL-1β-induced expression of ADAMTS-4 and ADAMTS-5, as determined by RT-PCR and Western blotting. Each column represents the mean ±SD. Leonurine (5 and 20 μM) significantly decreased IL-1β-induced ADAMTS-4 production. Only the high dose (20 μM) of leonurine inhibited expression of ADAMTS-5. * P<0.05 *vs.* IL-1β group. # P<0.05 *vs.* normal group.

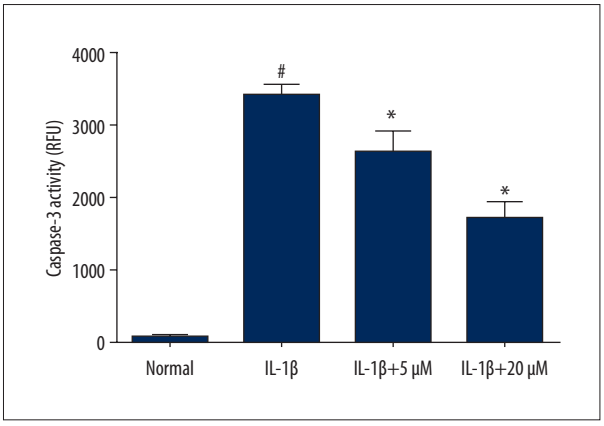

Figure 5. Leonurine decreased caspase-3 activity. After treatment with IL-1β, caspase-3 activity was significantly increased compared to the control group (p<0.05). Leonurine (5 and 20 μM) inhibited IL-1β-induced caspase-3 activity. * p<0.05 *vs.* IL-1β group. # p<0.05 *vs.* control group.

that IL-1β markedly increased MMP-1, MMP-13 (Figure 3), ADAMTS-4, and ADAMTS-5 (Figure 4) gene expression compared with the control group. However, 20 μM leonurine exhibited a better anti-catabolic effect in comparison to the 5 μM group. These findings were unanimous in the results of ELISA and Western blotting.

Leonurine inhibits IL-1β-induced apoptosis by suppressing caspase-3 activity

The activity of caspase-3 was evaluated using a caspase-3 assay kit. Relative fluorescent units (RFU) were used to represented the caspase-3 activity. Caspase-3 activity increased significantly following treatment of IL-1β (10 ng/mL). However, the addition of leonurine (5 and 20 μM) reduced IL-1β-induced activation of caspase-3 in a dose-dependent manner (Figure 5).

Leonurine suppresses the apoptotic pathway mediated by Bcl-2 and Bax

To detect the protective role of leonurine in the mitochondrial apoptosis pathway in IL-1β-induced chondrocyte, levels of the Bcl-2 and Bax were determined by qRT-PCR (Figure 6A) and Western blotting (Figure 6B). According to results shown in Figure 6, the expression of anti-apoptotic factor Bcl-2 was down-regulated at mRNA and protein levels, while the expression of

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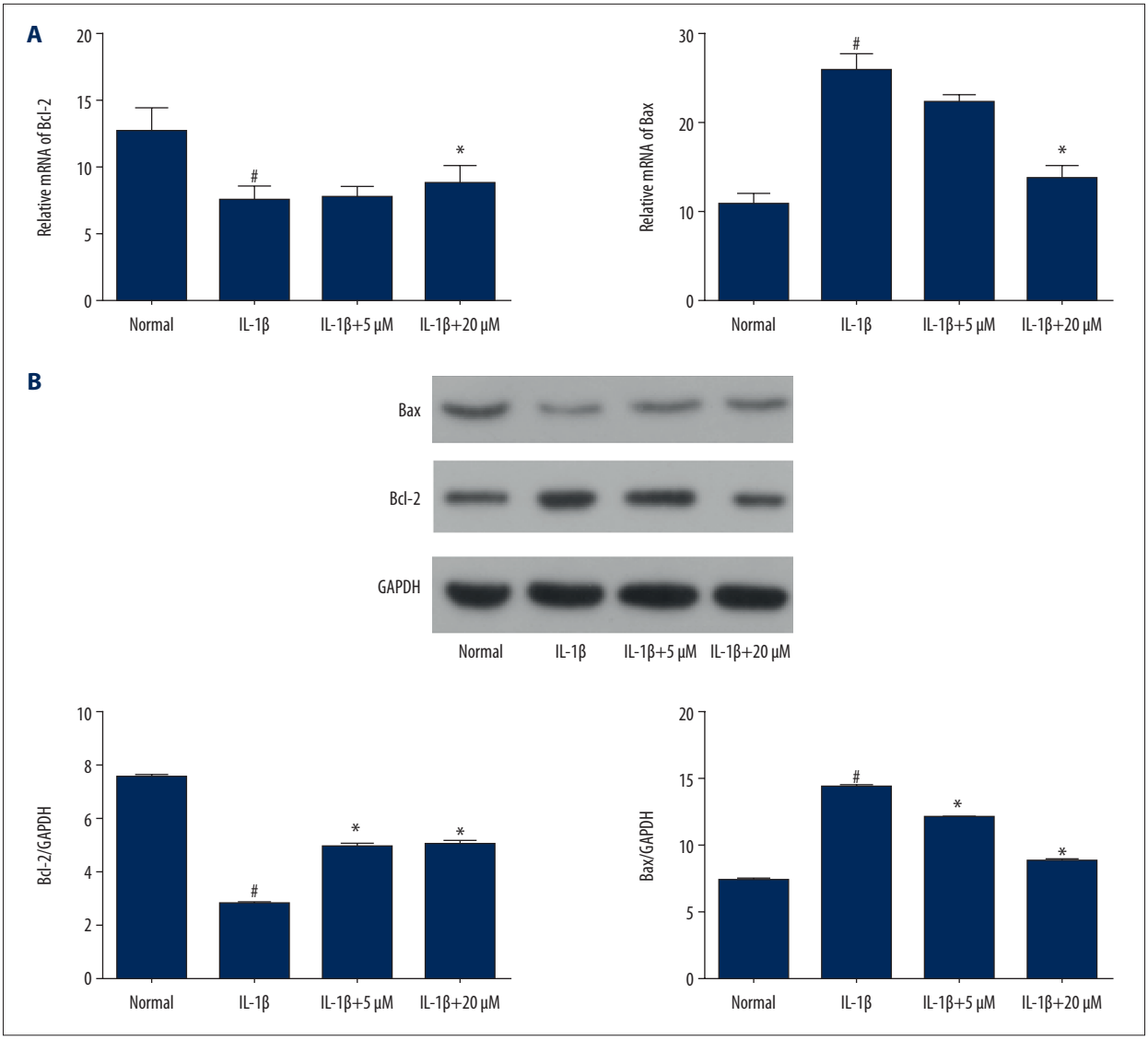

Figure 6. Leonurine downregulated Bcl-2/Bax gene and protein expression, as detrmined by qRT-PCR (**A**) and Western blot (**B**). After treatment of chondrocytes with IL-1β alone, Bax gene and protein levels increased significantly, while Bcl-2 expression decreased. Leonurine significantly downregulated Bax production and upregulated Bcl-2 expression in IL-1β-induced rat chondrocytes. * p<0.05 *vs.* IL-1β group. # p<0.05 *vs.* control group.

pro-apoptotic factor Bax was significantly increased in IL-1β-induced chondrocytes. With pre-treatment with leonurine at concentrations of 5 and 20 μM, Bcl-2 expression was markedly higher than that in the IL-1β group, and Bax expression was lower.

Leonurine inhibits MAPK and NF-κB activation in chondrocytes

To further explore the potential mechanism underlying the protective effect of leonurine in IL-1β-induced chondrocytes, we evaluated the activation of MAPK families and NF-κB. Western blotting was performed to determine the phosphorylation of p38, ERK, JNK, and NF-κB p65 (Figure 7). IL-1β treatment significantly induced activation of the MAPK pathway by promoting p-p38, p-ERK, and p-JNK protein expression. However, a high dose of leonurine reduced the phosphorylate activation of p38, ERK, and JNK induced by IL-1β. With regard to the NF-κB pathway, IL-1β stimulation resulted in phosphorylation of p65, and this effect was also inhibited by leonurine.

Discussion

Recent studies have suggested that active ingredients obtained from natural products have potential for the treatment

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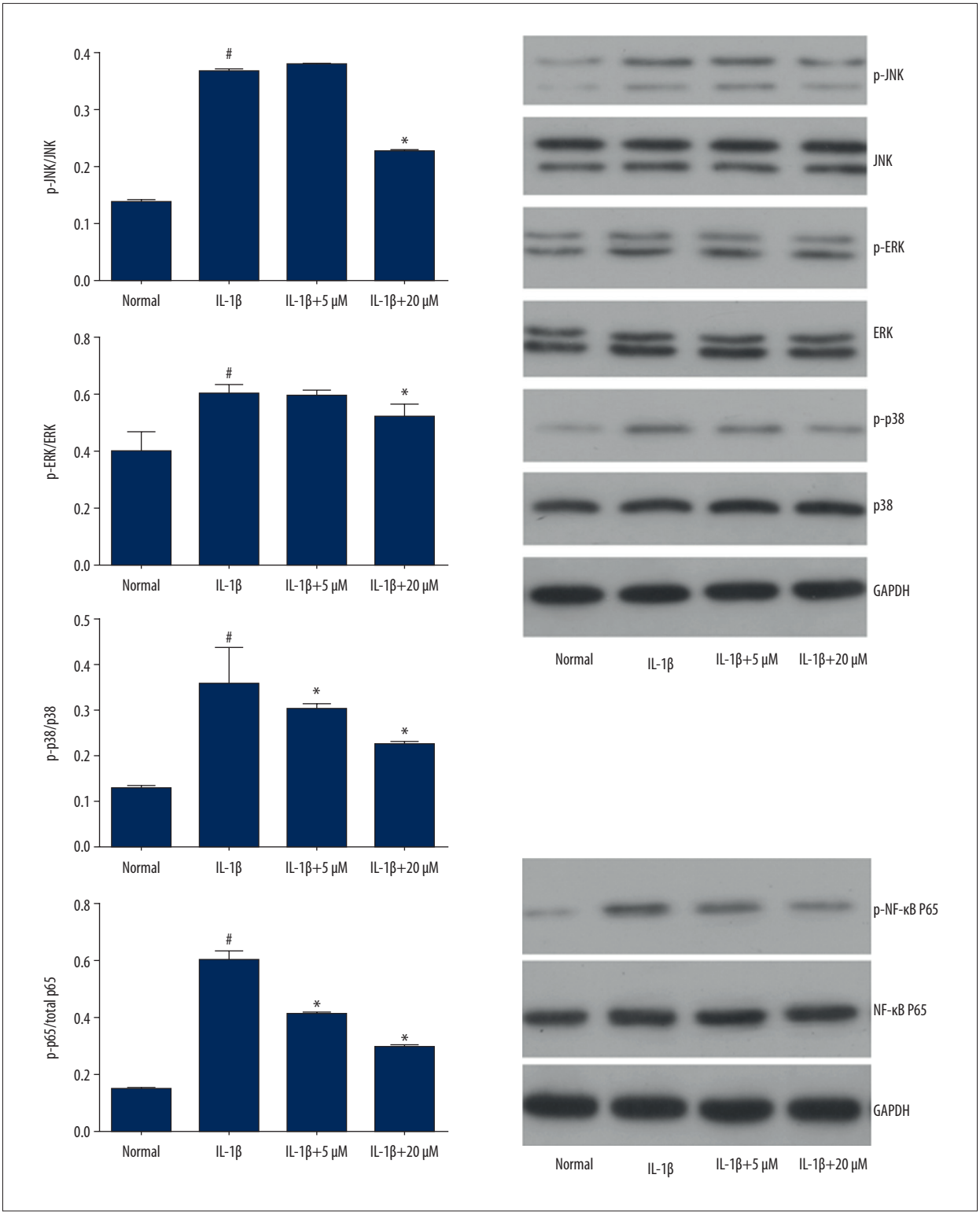

Figure 7. Leonurine counteracted MAPK and NF-κB activation induced by IL-1β in chondrocytes. Leonurine (20 μM) decreased the phosphorylation levels of p38, ERK, and JNK induced by IL-1β. In addition, leonurine (5 and 20 μM) attenuated IL-1β-induced activation of phosphor-NF-κB (p65). GAPDH was used as a loading control in Western blotting. * P<0.05 *vs.* IL-1β group. # P<0.05 *vs.* normal group.

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of OA because of their clear composition and good safety characteristics. For example, curcumin was shown to inhibit matrix degradation in chondrocytes by decreasing IL-1β, TNF-α, MMP-1/3/13, and ADAMTS-5 secretion, and upregulating chondroprotective factors [16]. Leonurine was reported to reduce synovial inflammation and cartilage damage in RA. However, the precise role of leonurine in chondrocytes has not been fully elucidated. ECM degradation and local inflammation play key roles in the disease progression of OA. We found that leonurine exerts anti-catabolic and anti-apoptotic effects on IL-1β-induced chondrocytes. Our results indicate that at concentrations of 5 and 20 μM, leonurine inhibited IL-1β-stimulated expression of MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5 at the gene and protein levels. In addition, we also determined the anti-apoptotic role of leonurine by detecting the Bax/Bcl-2/caspase-3 pathway. Furthermore, leonurine inhibited IL-1β-activated MAPK and the NF-κB pathway. These findings suggest that leonurine is useful as a new therapeutic agent to prevent or slow the progression of OA.

OA is now viewed as a chronic inflammatory disease caused by multiple inflammatory factors [17]. The imbalance of biosynthesis and degradation of ECM induce cartilage erosion and promote OA progression. During the development of OA, many proteolytic enzymes, including ADAMTS and MMPs, play central roles in the degradation of collagen II and aggrecan, which are the main components of the ECM [18,19]. MMP-1 and MMP-13 are classical collagenases involved in collagen degradation [20]. MMP-13 is the most potent collagenase associated with the catabolism of type II collagen [21]. The ADAMTS family, especially ADAMTS-4 and ADAMTS-5, also plays important roles in ECM breakdown and turnover. Suppression of ADAMTS-4 and ADAMTS-5 was reported to attenuate the degradation of aggrecan in TNF-α-stimulated normal cartilage [22]. Our data indicate that leonurine significantly inhibited the IL-1β-induced gene and protein expression of molecules involved in matrix degradation, including MMP-1, MMP-13, ADAMTS-4, and ADAMTS-5, which strongly suggests that leonurine has potential as an anti-catabolic drug.

Inflammatory factors also play crucial roles in the pathogenesis of OA. Certain cytokines, such as IL-1β and TNF-α, have been shown to induce the release of NO and PGE2. As a classical pro-inflammatory cytokine, Interleukin-1β (IL-1β) is released by activated synoviocytes, chondrocytes, and monocytes. In our study, IL-1β (10 ng/mL) significantly increased catabolic factor expression, consistent with previous reports [23]. Moreover, IL-1β has been also used as a chondrocyte apoptosis-inducing agent [24]. Accumulating evidence indicates that chondrocyte apoptosis exerts an important role in the pathophysiological process of OA [25]. The increased number of apoptotic chondrocytes leads to an imbalance of anabolism and catabolism and causes the degeneration of cartilage [26]. In addition, apoptotic chondrocytes secrete higher levels of pivotal pro-apoptotic factors and pro-inflammatory factors, which could aggravate the erosion of articular cartilage [27]. The interaction between chondrocyte apoptosis and autophagy has become an important area of research [28]. Cleaved caspase-3 is an important executioner caspase in cellular apoptosis [29]. The Bcl-2 family members, including anti-apoptotic members (Bcl-2, Bcl-Xl, Bcl-w, and Mcl-1) and pro-apoptotic members (Bax and Bak), play important roles in mitochondrial apoptotic signaling via regulating the permeability of mitochondrial membrane [30].

In the present study, pre-treatment with leonurine was shown to directly increase the gene and protein levels of Bcl-2 and inhibit those of Bax, and IL-1β-induced caspase-3 activity was also reduced after the addition of leonurine. Our results suggest that leonurine attenuates IL-1β-induced apoptosis via the Bax/Bcl-2/caspase-3 pathway rat chondrocytes. A previous study has demonstrated that leonurine inhibits cell apoptosis in a rat model of acute myocardial infarction via the PI3K/AKT/GSK3β signaling pathway [31]. Leonurine was also reported to exert a neuroprotective effect by decreasing cell apoptosis in oxygen-glucose deprivation (OGD)-induced PC12 cells [32]. Leonurine appears to have potential therapeutic value in OA-related chondrocytes apoptosis and may be an innovative therapeutic candidate in the prevention and treatment of OA.

The mechanism underlying apoptosis and catabolism in chondrocytes exposed to leonurine remains unclear. The MAPK pathway, consisting of ERK, p38, and JNK, is the chief pathway involved in chondrocyte inflammation, MMP regulation, and apoptosis [33,34]. In human OA chondrocytes, TNF-α stimulated the expression of MMP-13 through regulation of ERK, p38, and JNK MAP kinases [35]. Similar results indicated that IL-1-induced production of MMPs involves various pathways, including those of p38, JNK, and NF-κB [36]. With regard to osteoclasts, leonurine exerts anti-osteoporotic properties in inhibition of the MAPK signaling pathway [14]. Our Western blotting data also indicated that leonurine decreased the IL-1β-induced phosphorylation of ERK, p38, and JNK. Similar to its parent chemical, gallic acid, which was demonstrated to interact closely with the MAPK signaling pathway, leonurine protected chondrocytes against IL-1β-induced inflammation by inhibiting 1 or more components of the MAPK pathway [37–39]. As ERK, p38, and JNK were all affected, leonurine may have an effect on an upstream factor in the MAPK pathway, such as TRAF6. Further studies are required to identify this upstream site of action of leonurine. Furthermore, the NF-κB transcription family can effectively regulate the expression of numerous pro-inflammatory cytokines and proteases in OA, including MMPs, iNOS, and COX-2 [40]. After stimulation of NF-κB, the phosphorylated p65 unit translocates from the cytoplasm to the nucleus, where it induces the expression of various catabolic and inflammatory genes. The present study shows that

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leonurine suppressed phosphorylation of the p65 unit in IL-1β-treated cells, which is consistent with a previous study performed by Yin and Lei [15]. In addition, previous studies have shown that leonurine can impede the translocation of the p65 unit into the nucleus by inhibiting IκBα phosphorylation in BV-2 cells, concordant with our *in vitro* observations [41]. However, more research is required to further explore the efficacy and mechanism of action of leonurine. Our study indicates that the anti-catabolic and anti-apoptotic effects of leonurine that prevent cartilage degeneration and inflammation are associated with inhibition of the NF-κB and MAPK signaling pathways.

Conclusions

Leonurine exerted anti-catabolic and anti-apoptotic effects in chondrocytes *in vivo* via suppression of the NF-κB and MAPK signaling pathways. Our results suggest that leonurine has value in prevention and treatment of OA.

Conflicts of interest

None.

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