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Original Article

Cordycepin modulates inflammatory and catabolic gene expression in interleukin-1beta-induced human chondrocytes from advanced-stage osteoarthritis: an *in vitro* study

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Abstract: Cordycepin is widely used as for its various pharmacological activities, such as anti-inflammation, anti-angiogenesis, anti-aging, anti-tumor and anti-proliferation. However, the precise role of cordycepin on chondrocytes is not clear. In the present study, we examined the inhibitory effects of cordycepin on interleukin-1 beta (IL-1β)-induced glycosaminoglycan (GAG) release, nitric oxide production as well as gene expressions of inflammatory and catabolic mediators in human cartilage and chondrocytes. Cartilage explants and human chondrocytes were cultured in the absence or in the presence of IL-1β (10 ng/ml) and with or without cordycepin (5-100 μM). GAG content in the cartilage explants was measured by using the dimethylmethylene blue method and Safranin O staining. Nitric oxide level was determined by Griess reaction. Expressions of MMP-1, MMP-13, cathepsin K, cathepsin S, ADAMTS-4 (a disintegrin and metalloproteinase with thrombospondin motifs-4) and ADAMTS-5, inducible nitric oxide synthase (iNOS) and cyclooxgenase-2 (COX-2) were evaluated by real-time quantitative PCR. We found that cordycepin suppressed IL-1β-stimulated GAG release. Gene expressions of catabolic enzymes, including MMP-1, MMP-13, cathepsin K, cathepsin S, ADAMTS-4 and ADAMTS-5, were decreased by cordycepin in a dose-dependent manner. In addition, cordycepin inhibited IL-1β-induced COX-2 and iNOS expression at the transcript level as well as blocked NO production. Our results suggest that cordycepin may possess chondroprotective effect by preventing cartilage denegation and interfering inflammatory response in the pathogenesis of OA.

Keywords: Cordycepin, osteoarthritis, chondrocytes, interleukin-1 beta

Introduction

Cordycepin, also known as 3'-deoxyadenosine, is a nucleoside derivative isolated from Cordyceps militaris [1]. Various studies have focused on the pharmacological activities of cordycepin and revealed it exerted several properties, such as anti-inflammatory [2, 3], anti-angiogenesis [4], anti-aging [5], anti-tumor [4, 6], and anti-proliferation [7]. These evidences suggested that cordycepin have important roles in clinical application. Moreover, a recently study has found that cordycepin inhibited expression of MMP-1 and MMP-3 in IL-1β-induced rheumatoid arthritis (RA) synovial fibroblasts, indicating cordycepin may be a potential candidate to prevent inflammation of RA [8]. To date, nothing is reported about the role of cordycepin in osteoarthritis (OA).

OA is a chronic degenerative joint disease that is widely thought of as primarily affecting the cartilage with changes in subchondral bone, osteophyte formation, and synovial inflammation. A progressive degradation of extracellular matrix (ECM) components, including type II collagen and aggrecan, is a main pathological process in OA [9]. Excessive productions of catabolic enzymes play a major role in cartilage ECM breakdown. Traditionally, MMPs have been considered as the main factor in the degradation of cartilage [10, 11]. Among these, MMP-1 (collagenase 1) and MMP-13 are viewed as key catabolic enzymes since they degrade types I, II, III, IV, X, and XIV collagens, and aggrecan core protein [12-15]. Besides, another family called "aggrecanases" which cleaves the Glu373-Ala374 bond of the aggrecan core protein also is responsible for cartilage destruc-

Table 1. Primers of targeted genes

Gene	GenBank Accession	Primer Sequences	Size (bp)
MMP-1	NM_002421	F: 5'-GGGAATAAGTACTGGGCTGTTCAG-3'	125
		R: 5'-CCTCAGAAAGAGCAGCATCGATATG-3'	
MMP-13	NM_002427	F: 5'-CTGGCCTGCTGGCTCATGCTT-3'	162
		R: 5'-GCAGGGTCCTTGGAGTGGTCA-3'	
Adamts-4	NM_005099	F: 5'-CCTGGCAAGGACTATGATGCTGA-3'	147
		R: 5'-GGGCGAGTGTTTGGTCTGG-3'	
Adamts-5	NM_007038	F: 5'-GCAGAACATCGACCAACTCTACTC-3'	112
		R: 5'-CCAGCAATGCCCACCGAAC-3'	
iNOS	L09210	F: 5'-CCTTACGAGGCGAAGAAGGACAG-3'	278
		R: 5'-CAGTTTGAGAGAGGAGGCTCCG-3'	
COX-2	NM_000963	F: 5'-GAGAGATGTATCCTCCCACAGTCA-3'	117
		R: 5'-GACCAGGCACCAGACCAAAG-3'	
Cathepsin k	NM_000396	F: 5'-CACTGGGAGCTATGGAAGAAGAC-3'	122
		R: 5'-GAGAAGCCTCAAGGTTATGGATGGA-3'	
Cathepsin S	NM_004079	F: 5'-GCTTGCTGGGCTTTCAGTGCTGT-3'	195
		R: 5'-GCGTCTGAGTCGATGCCCTTGTTAT-3'	
Hu GAPDH	NM_002046	F: 5'-CTGCTCCTCCTGTTCGACAGT-3'	100
		R: 5'-CCGTTGACTCCGACCTTCAC-3'	

tion, especially in the early stages [16]. ADAMTS-4 (also known as aggrecanase-1) and -5 (aggrecanase-2) are the most efficient aggrecanases [17, 18]. What's more, biochemical factors like cathepsin and plasmin are closely involved in the degenerative process [19, 20].

In the present study, we desired to know whether cordycepin exerts a potential beneficial role in the progression of OA. We used a model by culturing cartilage explants and human articular chondrocytes *in vitro*. Firstly, we examined effects of cordycepin on GAG release on IL-1β-stimulated cartilage explants cell viability on IL-1β-stimulated chondrocytes. Secondly, we evaluated the effects of cordycepin on the expression of the catabolic factors including MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K and cathepsin S, as well as inflammatory mediators, such as iNOS and COX-2. In addition, we also observed cordycepin on NO production.

Materials and methods

Primary cultures of human OA chondrocytes

Human OA cartilages were obtained from the femoral condyles of OA patients undergoing knee joint replacement in our hospital. Cartilage was washed three times in phosphate buffered saline (PBS) and finely minced into approximately 2 mm^3 pieces. The cartilage tissue was then subjected to a sequential digested with 0.2% pronase (0.5 h, Sigma, St. Louis, MO, USA), 0.2% collagenase (3 h, Sigma, St. Louis, MO, USA) at 37°C. After collection of individual cell by brief centrifugation, cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented containing antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies). Medium was replaced every 2 days, and cells were incubated at 37°C under a humidified 5% CO_2 atmosphere. All patients were from The Second Hospital of Medical College, Zhejiang University. The study was approved by the local ethic committee and written informed consent was obtained from each participant.

Assessment of cell proliferation

Chondrocyte proliferation was assessed in the presence of increasing concentrations of cordycepin (0, 5, 10, 25, 50, 100, 200, 500 μM) (Sigma, St. Louis, MO, USA), by the use of a MTT assay (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) (Sigma). Cell proliferation was examined at 24 h after addition of cordycepin. A concentration of cordycepin ranging caused no significant reduction in cell viability was viewed as non-cytotoxic and would use in subsequent experiments.

Cell treatment and mRNA expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K, cathepsin S, iNOS and COX-2 by real-time quantitative PCR

For real-time quantitative PCR, OA chondrocytes on a 6-wells plate at a density of 5×10^4

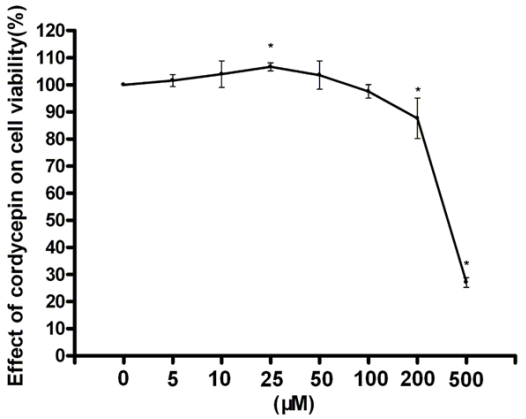


Figure 1. Effect of cordycepin on human chondrocytes viability. Cells were cultured in 96-well plates and incubated with various concentrations of cordycepin (0-500 μM) for 24 h. Cells incubated in a medium without cordycepin were regarded as 100%.

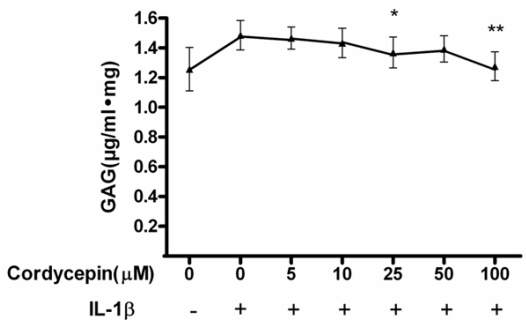


Figure 2. Effect of cordycepin on IL-1β-stimulated GAG release into the medium in OA cartilage explants cultured for 24 h. Data represent mean ± SD (n = 6). **P* < 0.05, ***P* < 0.01 vs. IL-1β group (cordycepin 0 μM + IL-1β 10 ng/ml).

cells/cm^2 were pre-treated with cordycepin (0, 5, 10, 25, 50, 100 μM) for 1 h and then incubated with IL-1β (10 ng/ml) for 24 h. At the end of the culture, cells were separated, centrifuged and stored at -80°C for next assays. The cells were pulverized in liquid nitrogen, and total RNA was isolated using the Trizol reagent (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer's instructions. After a treatment period of 20 min at 37°C with 1U of DNase I (Sigma-Aldrich) to prevent genomic DNA contamination, 1 μg of total RNA was reverse transcribed using 10 pmol random hexanucleotide primers (Promega, USA), 0.5 mM dNTPs and 200U of Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) at 37°C for 1 h. The reaction was terminated by incubation at 70°C for 10 min. The quantification of gene-expression levels for MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K, cathepsin S, iNOS and COX-2 were carried out by real-time quantitative PCR on an iCycler system (Bio-Rad, Laboratories, Hercules, CA, USA). iQTM SYBR Green supermix PCR kit (Bio-Rad) was used for real-time monitoring of amplification (5ng of template cDNA; 45 cycles: 95°C/15 s, 60°C/15 s) with appropriate primers (**Table 1**). A parallel amplification of oyster GAPDH transcript (NM_002046) was carried out (forward primer: 5'-CTG CTC CTC CTG TTC GAC AGT-3'; reverse primer: 5'-CCG TTG ACT CCG ACC TTC AC-3') to normalize the expression data of the target transcripts. The relative expression levels of targeted genes were calculated for 100 copies of the 18s housekeeping gene according to the formula: $n = 100 \times 2^{-(\Delta CT \text{ targeted gene} - \Delta CT \text{ GAPDH})}$.

Human cartilage explants culture

The cartilage explants were prepared according to the previous report [21]. Articular cartilages were obtained from the superficial zone of non-erosive and smooth keen joints of OA patients undergoing knee joint replacement in our hospital. Then the cartilage was cut into 6*6 mm^2 pieces and placed in a 24-well plate. Explants were cultured in 0.5 ml of DMEM supplemented containing antibiotic-antimycotic solution (100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B; Life Technologies). After pre-treated with cordycepin (0, 5, 10, 25, 50, 100 μM) (Sigma, St. Louis, MO, USA) for 1 h, plates were incubated in the absence or in the presence of human recombinant IL-1β (10 ng/ml, R&D system) at 37°C and 5% CO_2 for 24 h. At the end of the culture, cartilage explants were weighed, and culture supernatants were collected into individual Eppendorf tube and stored at -20°C until assaying.

Measurement of GAG release and Safranin O staining

The GAG release into culture supernatants from OA cartilage explants was assayed in 96-well plates using the dimethylmethylene blue (DMMB) method [22]. A standard curve for the DMMB assay was generated by chondroitin sulphate C (Sigma, St. Louis, MO, USA) at concentrations ranging from 0 to 100 mg/ml. 40 μL of each culture supernatants and standard were added in 96-well plates in duplicate, and

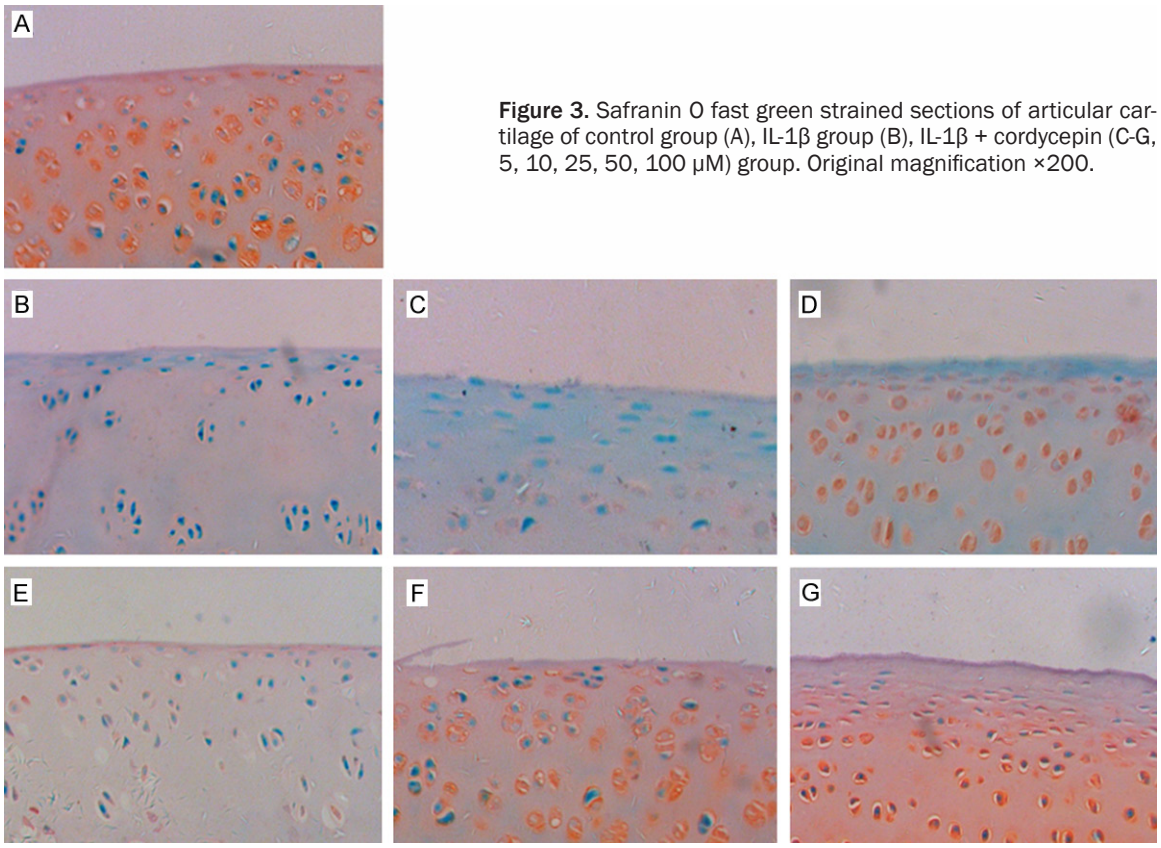


Figure 3. Safranin O fast green strained sections of articular cartilage of control group (A), IL-1β group (B), IL-1β + cordycepin (C-G, 5, 10, 25, 50, 100 μM) group. Original magnification ×200.

then 200 μL DMMB solution was added to each well and the absorption read at 490 nm after 3 min. Cartilage explants were weighed and results were expressed as amount of GAG released per milligram of tissue.

Meanwhile, cartilage explants were fixed in 10% buffered formalin, decalcified in hydrochloric acid, and then embedded into paraffin. After being sectioned at 5 μm, GAG was stained with Safranin O-fast green (1% Safranin O counterstained with 0.75% hematoxylin and then 1% fast green; Sigma).

Determination of NO production in supernatants of cartilage explants

NO was determined by Griess reaction, a classical way to assay the presence of NO derived product, nitrite, in supernatants as described [23]. Culture supernatants and standard (100 μl) were mixed with 100 μl Griess reagent and the absorbance was measured at 550 nm after 10 min of reaction at room temperature.

Statistical analysis

All experiments were performed in triplicate. Results were expressed as mean ± standard deviation (SD) of three experiments. Statistical analyses were performed with software, SPSS 12.0 for Windows. Evaluation of the data was based on Paired-Samples T test. Statistical significance was set at $P < 0.05$.

Results

Effect of cordycepin on cell viability of human OA chondrocytes

Effect of cordycepin on human OA chondrocytes viability was examined at concentrations of 0, 5, 10, 25, 50, 100, 200, 500 μM after 24 h of culture (**Figure 1**). According to the results of MTT assay, a concentration of cordycepin ranging from 5 to 100 μM did not shown significant toxicity. So, doses ranging from 5 to 100 μM were used in subsequent experiments.

Effect of cordycepin on IL-1β-stimulated GAG release

As shown in **Figure 2**, IL-1β significantly induced the release of GAG ($P < 0.05$). Compared to IL-1β treated samples, higher concentration of cordycepin (25, 100 μM) could reduce IL-1β-stimulated GAG release. No significant effect

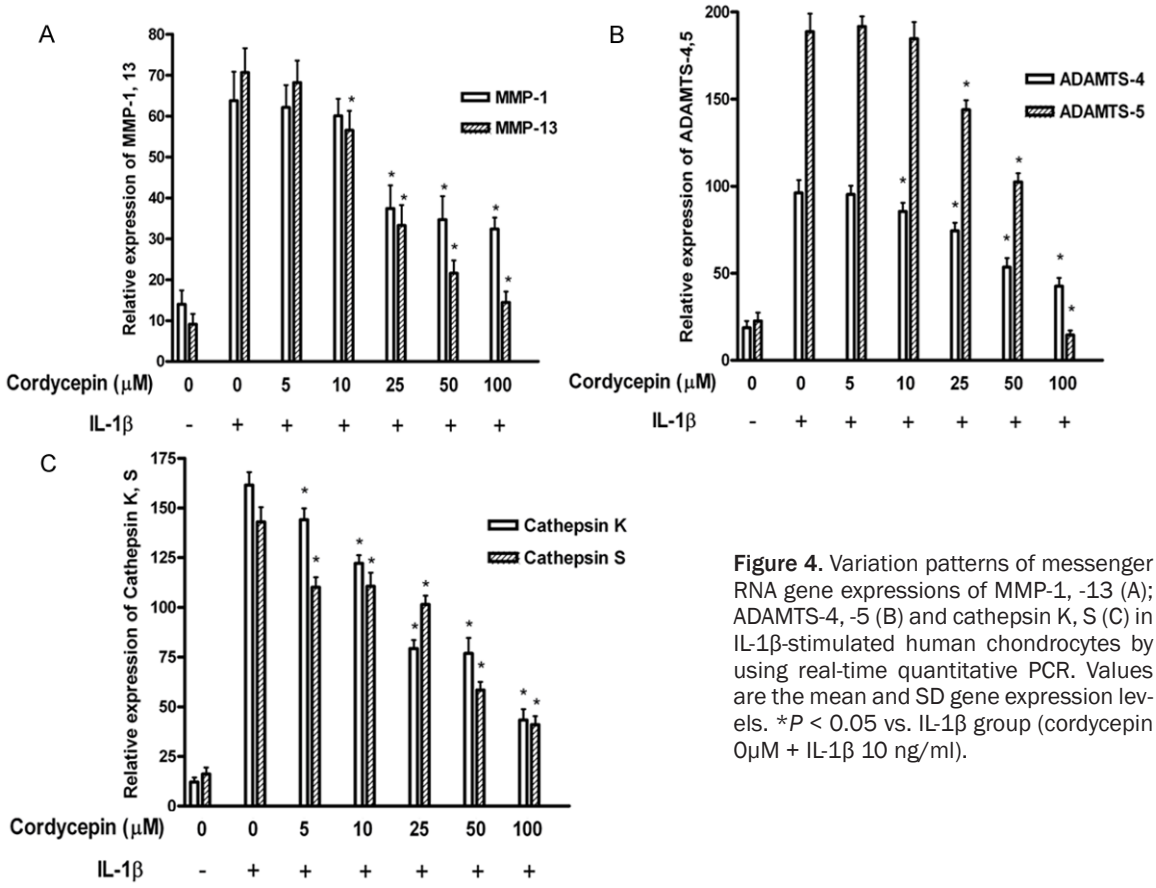


Figure 4. Variation patterns of messenger RNA gene expressions of MMP-1, -13 (A); ADAMTS-4, -5 (B) and cathepsin K, S (C) in IL-1β-stimulated human chondrocytes by using real-time quantitative PCR. Values are the mean and SD gene expression levels. **P* < 0.05 vs. IL-1β group (cordycepin 0μM + IL-1β 10 ng/ml).

was observed at the lower concentrations (5, 10 μM). According to our gross histological results by safranin O staining, cordycepin could remarkably decrease the IL-1β-stimulated GAG lost (**Figure 3**).

Effects of cordycepin on gene expression of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K and cathepsin S

The gene levels of MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K and cathepsin S were examined by real-time quantitative PCR after 24 h. As to MMPs (**Figure 4A**), IL-1β at 10 ng/ml increased the transcript levels of MMP-1, -13 (*P* < 0.01, date not shown). Treatment with cordycepin (25, 50, 100 μM) partly blocked the up-regulation of MMP-1, -13 induced by IL-1β (*P* < 0.05). For ADAMTS system (**Figure 4B**), gene expressions of ADAMTS-4 and -5 in the absence of IL-1β increased remarkably and cordycepin ranged from 25-50 μM could abolish this effect (*P* < 0.05). In addition, a notable increase in the cathepsin K and cathepsin S was observed after treatment of IL-1β. A significant inhibition of IL-1β-stimulated cathepsin K and cathepsin S expressions was observed at 5, 10, 25, 50, 100 μM of cordycepin (**Figure 4C**).

Effects of cordycepin on IL-1β-stimulated NO synthesis and gene expression of iNOS and COX-2

Similarly, IL-1β alone highly stimulated NO production nearly 3.3 fold. Its synthesis was inhibited by cordycepin in a dose-dependent manner down to near normal level at concentration of 100 μM. A concentration of cordycepin ranging from 10-100 μM was viewed effectible (*P* < 0.05). And cordycepin ranged from 10-100 μM could prevent IL-1β-stimulated gene expression of iNOS and COX-2 (*P* < 0.05) (**Figure 5**).

Discussion

In this study, we have for the first time showed the cartilage-protective role of cordycepin *in vitro* by decreasing GAG release and NO production, as well as inhibiting the gene expressions of catabolic enzymes, including MMP-1,

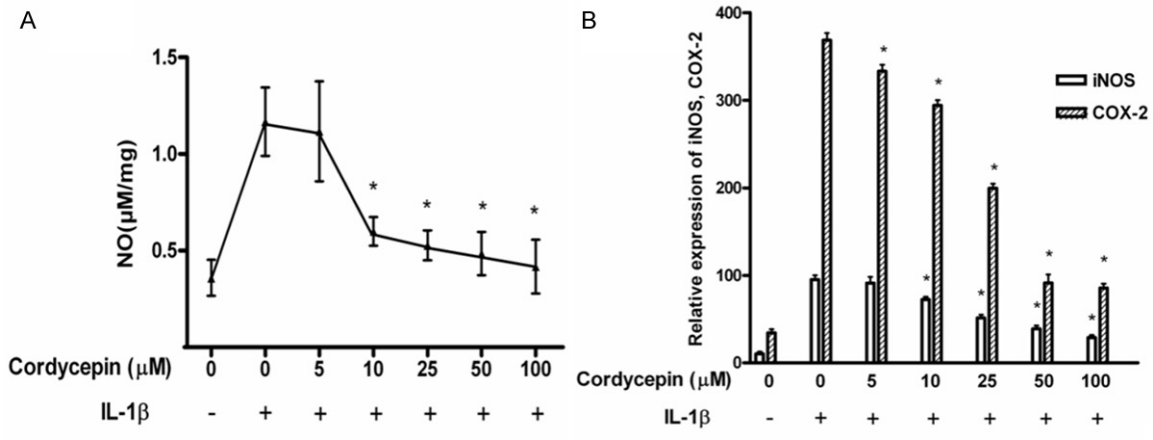


Figure 5. Effects of cordycepin on NO synthesis (A) in IL-1β-induced cartilage explants and gene expressions of iNOS and COX-2 (B) in IL-1β-induced human chondrocytes. Supernatants were collected and NO concentration was determined by Griess reaction. Results were normalized to the weight of the explants. Data represent mean ± SD. **P* < 0.05 vs. IL-1β group (cordycepin 0 μM + IL-1β 10 ng/ml).

MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K and cathepsin S, and inflammatory mediators, such as iNOS and COX-2 in a dose-dependent manner, in IL-1β-induced cartilage explants and human OA chondrocytes.

We have demonstrated that cordycepin could inhibit the release of GAG, a major component of aggrecan. And aggrecan degradation is a fundamental manifestation of OA. As a most prominent catabolic factor in OA cartilage, IL-1β was used to stimulate in GAG release in cartilage explants culture. Mathy-Hartert *et al*. have reported that IL-1β (10 ng/ml) highly stimulated GAG release (1.51 ± 0.23 fold) [24]. In the present study, we found IL-1β (10 ng/ml) alone significantly induced the release of GAG, which was consistent with their study. And this effect was effectively inhabited by cordycepin in a dose-dependent way. Moreover, we also examined relative proteoglycan loss from the cartilage by Safranin O stain, a qualitative method widely used for detecting the content of GAG. The gross histological results also indicated the suppressive action of cordycepin on IL-1β-induced GAG release. Therefore, cordycepin might act as a chondroprotective role based on the suppression of IL-1β-induced aggrecan loss, and the optimal concentration was about 100 μM. Since the aggrecan depletion is mainly contributed to various degradation enzyme systems, including MMPs, ADAMTS, and cathepsin, we desired to know whether cordycepin could exert its effects through these enzyme systems on protecting articular cartilage.

For MMPs system, cordycepin has been reported to inhibit IL-1β-induced MMP-1 and MMP-3 expressions in cultured RA synovial fibroblasts [8]. Another study by Lee *et al*. demonstrated cordycepin attenuated UVB-induced MMP-1 and -3 expressions in a dose-dependent manner by blocking UVB-induced NF-κB activation [5]. Further study by Noh *et al*. showed cordycepin could decrease TPA-induced MMP-9 expression through inhibition of AP-1 activation in MCF-7 cells [25]. These results revealed the close relationship between cordycepin and MMPs. IL-1β is central to the inflammatory process in OA via increasing production of MMP and inducing a reduction of TIMPs [13, 26]. In this *in vitro* model of cartilage inflammation induced by IL-1β, the mRNA levels of MMP-1 and MMP-13 increased significantly after treatment of IL-1β. We also showed novel evidence that cordycepin interfered with IL-1β-induced expression of MMP-1 and -13. So cordycepin exerted as an inhibitor of MMP activity and might inhibit cartilage and bone destruction. However, little is known about the exact mechanism of cordycepin on IL-1β-induced chondrocytes. According to the previous reports, MAPK families, especially P38 and JNK, were required for IL-1β-induced of MMPs [27, 28]. As cordycepin could block IL-1β-mediated phosphorylation of P38 and JNK in synovial fibroblasts, we speculated the effect of cordycepin on IL-1β-induced chondrocytes was mediated via P38/JNK pathway. Also notably, cordycepin also blocked IL-1β-induced up-regulation of MMP-1 and -13

in a dose-dependent manner and the highest concentration (100 μM) was exhibited the most protective effect.

As to ADAMTS system, the regulation of IL-1β on mRNA levels of ADAMTS-4 and -5 is still somewhat controversial. Catabolic agents, such as IL-1α, IL-1β and oncostatin M, could enhance the expression of ADAMTS-4 [29, 30], whereas ADAMTS-5 mRNA was little up-regulated by IL-1β. IL-1 and OSM synergized to increase gene expression of the ADAMTS-4, but not ADAMTS-5 [31]. On the contrary, IL-1β (20 ng/ml) significantly elevated both ADAMTS-4 and -5 expressions in primary cultured rabbit articular chondrocytes [32]. Recently, a study by Imada *et al*. indicated that the expression of both aggrecanases was conspicuously increased by IL-1β (10 ng/ml) in human articular chondrocytes [33]. Also, they have found the up-regulation of ADAMTS-4 and -5 exhibited differently from individual to individual. Therefore, IL-1β-mediated adjustment of their expression may concern with individual differences. In this study, we showed both ADAMTS-4 and -5 mRNA levels were enhanced by IL-1β (10 ng/ml). And similar to MMPs, cordycepin suppressed IL-1β-enhanced expression of ADAMTS-4 and -5 mRNA in human articular chondrocytes. Actually, mRNA levels of ADAMTS-4 and -5 were well associated with enzyme activity, thus cordycepin seemed to play a protective role in article metabolism by interfering the aggrecanases-mediated degradation of articular cartilage [33, 34].

Another significant finding of this study was that cordycepin could regulate the expression of cathepsin K and cathepsin S on IL-1β-induced human chondrocytes. Both cathepsin K and cathepsin S belong to cysteine proteases, which are regard as ly-sosomal enzymes of papain family. Cathepsin K was found to cleave native type I and II collagens, suggesting a possible link to the development of OA [35]. Moreover, cathepsin K also had the aggrecan-degradation activity and cleaved aggrecan complexes at specific cleavage sites [36]. Down-regulation of gene expression and protein synthesis of cathepsin K could partly reduce the degradation of OA cartilage in experimental OA [37]. And cathepsin S is also viewed as a most relevant factor to the degenerative process by degrading various extracellular matrix [38]. Using immunohistochemistry, expression of cathepsin S was found in the synovium in OA-affected joint [39]. However, the effects of cordycepin on the expression of cathepsin K and S have yet to be elucidated. Hou et al. found that IL-1β could stimulate the expression of cathepsin K at both transcript and protein levels [39]. We demonstrated IL-1β (10 ng/ml) stimulation significantly increased gene expressions of cathepsin K and S in human OA chon-drocytes and pretreatment with cordycepin could abolish this effect. Taken together, these novel results indicate that cordycepin may inhibit cartilage degradation in OA by modulating gene expressions of various degradation enzyme systems, including MMPs, ADAMTS, and cathepsin.

In addition, as an anti-inflammatory traditional medicinal agent, cordycepin suppressed the synthesis of NO in lipopolysaccharide (LPS)-stimulated RAW 264.7 macrophage cells by decreasing gene expression of iNOS and COX-2 via blocking NF-κB activation and the NO production-inhibitory activity of cordycepin was also verified by Won *et al*. [2, 3]. In our experiment, we demonstrated cordycepin inhibited IL-1β-induced COX-2 and iNOS expression at the transcriptional level as well as blocked NO production. NO was considered to be a risk factor as for it mediated a lot of pathological processes, such as suppression of proteoglycan synthesis, apoptosis and dedifferentiation [40, 41]. And both COX-2 and iNOS genes were involved in OA pathogenesis in cartilage and could be potential as therapeutic tar [42, 43]. So, we speculated that cordycepin could modulate the inflammatory process in arthritis.

In summary, we showed that pretreatment of human OA cartilage explants and chondrocytes with cordycepin could inhibit IL-1β-induced GAG release and decrease gene expressions of catabolic enzymes, including MMP-1, MMP-13, ADAMTS-4, ADAMTS-5, cathepsin K and cathepsin S. Furthermore, cordycepin blocked NO production and suppressed IL-1β-enhanced iNOS and COX-2 mRNA levels in a dose-dependent manner. Thus, our results suggest that cordycepin may possess chondroprotective effect by preventing cartilage denegation and interfering inflammatory response in the pathogenesis of OA. Therefore, we presume cordycepin is a novel therapeutic candidate with both anti-catabolic and anti-inflammatory actions against arthritic diseases.

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Disclosure of conflict of interest

None.

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