Cordycepin inhibits LPS-induced inflammatory and matrix degradation in the intervertebral disc

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Cordycepin is a component of the extract obtained from *Cordyceps militaris* and has many biological activities, including anti-cancer, anti-metastatic and anti-inflammatory effects. Intervertebral disc degeneration (IDD) is a degenerative disease that is closely related to the inflammation of nucleus pulposus (NP) cells. The effect of cordycepin on NP cells in relation to inflammation and degeneration has not yet been studied. In our study, we used a rat NP cell culture and an intervertebral disc (IVD) organ culture model to examine the inhibitory effects of cordycepin on lipopolysaccharide (LPS)-induced gene expression and the production of matrix degradation enzymes (MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5) and oxidative stress-associated factors (nitric oxide and PGE2). We found a protective effect of cordycepin on NP cells and IVDs against LPS-induced matrix degradation and macrophage infiltration. In addition, western blot and luciferase assay results demonstrated that pretreatment with cordycepin significantly suppressed the LPS-induced activation of the NF-κB pathway. Taken together, the results of our research suggest that cordycepin could exert anti-inflammatory and anti-degenerative effects on NP cells and IVDs by inhibiting the activation of the NF-κB pathway. Therefore, cordycepin may be a potential treatment for IDD in the future.

1 **Cordycepin inhibits LPS-induced inflammation and matrix degradation in**

2 **intervertebral discs**

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Abstract

Cordycepin is a component of the extract obtained from *Cordyceps militaris* and has many

biological activities, including anti-cancer, anti-metastatic and anti-inflammatory effects.

Intervertebral disc degeneration (IDD) is a degenerative disease that is closely related to the

inflammation of nucleus pulposus (NP) cells. The effect of cordycepin on NP cells in relation to

inflammation and degeneration has not yet been studied. In our study, we used a rat NP cell

culture and an intervertebral disc (IVD) organ culture model to examine the inhibitory effects of

cordycepin on lipopolysaccharide (LPS)-induced gene expression and the production of matrix

degradation enzymes (MMP-3, MMP-13, ADAMTS-4, and ADAMTS-5) and oxidative stress-

associated factors (nitric oxide and PGE2). We found a protective effect of cordycepin on NP

cells and IVDs against LPS-induced matrix degradation and macrophage infiltration. In addition,

western blot and luciferase assay results demonstrated that pretreatment with cordycepin

significantly suppressed the LPS-induced activation of the NF-κB pathway. Taken together, the

results of our research suggest that cordycepin could exert anti-inflammatory and anti-

degenerative effects on NP cells and IVDs by inhibiting the activation of the NF-κB pathway.

Therefore, cordycepin may be a potential treatment for IDD in the future.

Introduction

42 Intervertebral disc degeneration (IDD) is thought to be a significant contributor to the

43 development of low back pain (LBP) [1,2]. In addition, LBP is one of the most common

44 musculoskeletal complaints, estimated to reach up to 2.8% of healthcare visits in the United

45 States [3]. The mechanism of IDD contains a complex biochemical cascade. One of the most

46 important features of IDD is the loss of proteoglycan (PG) content of intervertebral discs (IVDs).

47 This process is closely related to the gene expression and activity of disintegrins and

48 metalloproteinases with thrombospondin motifs (ADAMTSs), matrix metalloproteinases (MMPs)

49 and tissue inhibitors of metalloproteinases (TIMPs) [4,5]. As a strong promoter of inflammation,

50 previous studies have shown that lipopolysaccharide (LPS) can induce gene upregulation and the

51 production of various proinflammatory cytokines and matrix-degrading enzymes, including

52 MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5 in NP cells, thus causing a reduction in PG

53 content and IDD [6,7]. Moreover, proinflammatory cytokine such as interleukin-1 (IL-1β) and

54 tissue necrosis factor-α (TNF-α) also play important roles in IDD [8]. Cytokines do not directly

55 degrade the IVD like MMPs or ADAMTSs do, instead they accelerate IDD by promoting the

56 production of inflammatory substances by the disc cells [9]. As a TLR ligand, LPS can also

57 initiate TLR signaling in NP cells, leading to the increased expression of proinflammatory

58 cytokines and MMPs [10,11]. In addition to MMPs and ADAMTSs, cytokines can also induce

59 chemokine ligand (CCL) expression in NP cells [12]. And according to previous research, CCLs

60 can promote macrophage migration into the IVD, exacerbating the inflammatory state and

61   causing pain [12,13].

62   *Cordyceps militaris* is a traditional Chinese medicine that has been widely used for decades.

63   Recent studies have demonstrated that bioactive components isolated from Cordyceps species

64   have various pharmacological functions [14,15]. Cordycepin (3'-deoxyadenosine) is one of the

65   most widely studied components of *C. militaris* and has diverse bioactivities, such as anti-cancer,

66   anti-metastatic and anti-inflammatory effects [16-18]. Many studies have shown that anti-

67   inflammatory treatments is an effective therapy for treating IDD in vitro [19,20]. However, the

68   results may be quite different between in vitro and in vivo, and the precise delivery of

69   therapeutic agents to IVDs without causing tissue damage and while still maintaining an

70   appropriate concentration for a long time is very difficult. To address some aspects of this

71   problem, the present study used both in vitro and ex vivo models to investigate the inhibitory

72   effect of cordycepin on LPS-induced inflammation and matrix decrease in intervertebral discs.

73   Based on our findings, we suggest that cordycepin may be a potential treatment for IDD in the

74   future.

75

Materials and Methods

Reagents and animal ethics

78   Cordycepin, LPS and dimethylmethylene blue (DMMB) were purchased from Sigma (St. Louis,

79   USA). Cordycepin was dissolved in DMSO; the final concentration of DMSO in the medium

80   was less than 0.05%. The same volume of DMSO was added to the control and LPS groups in all

81   experiments. The NF-κB luciferase reporter and the pRL-TK plasmids were purchased from

82   Promega (Madison, USA). Sprague Dawley rats were euthanized via the abdominal injection a

83   lethal dose of pentobarbital sodium. All of the animal work was conducted according to relevant

84   national and international guidelines and was approved by the Animal Experimental Ethical

85   Committee of Shanghai Ninth People's Hospital (Approval number: 2013-47).

86

87   **NP cell isolation and culture**

88   Nucleus pulposus (NP) cells were isolated from the lumbar spines of Sprague Dawley rats (6-8

89   weeks old, mixed male and female). The spines was separated between each of the lumbar discs,

90   and then, a sterile scalpel blade was used to completely remove the nucleus pulposus. Before

91   digestion with trypsin and collagenase, the nucleus pulposus was washed with PBS to remove

92   other cells that may have been attached to the surface of nucleus pulposus. NP cells were

93   cultured in complete medium (high-glucose DMEM with 10% FBS, 100 U/ml penicillin and 100

94   μg/ml streptomycin) up to passage 2-3.

95

96   **Cell viability assay**

97   A commercial kit (Cell Counting Kit-8, CCK-8; Dojindo) was used to evaluate the potential

98   cytotoxic effect of cordycepin. NP cells were plated in 96-well plates at a density of 5×10^3 per

99   well, incubated with various concentrations of cordycepin for 24 h, administered 10 µl of CCK-8

100 solution and incubated for an additional 2 h. The optical density (OD) of each well was measured

101 at 450 nm. The culture medium was used as a blank. Cell viability was calculated as follows: cell

102 viability = [OD (with cordycepin) - OD (blank)] / [OD (without cordycepin) - OD (blank)].

103

104 **ELISA assessments**

105 NP cells were incubated with various concentrations of cordycepin for 2 h and then stimulated

106 with 10 µg/ml LPS for 24 h. The PGE2, MMP-3 and MMP-13 levels in the culture medium were

107 measured using commercially available enzyme-linked immunosorbent assay kits according to

108 the manufacturer's instructions (R&D Systems).

109

110 **Measurement of nitric oxide**

111 Nitric oxide (NO) was measured by Griess reagent [21]. Briefly, NP cells were incubated with

112 100 µM cordycepin for 2 h and then stimulated with 10 µg/ml LPS (Sigma) for 24 h. Then, 50 µl

113 of each culture supernatant was incubated at room temperature for 15 min with 50 µl of Griess

114 reagent (Sigma) in a 96-well plate. The absorbance at 540 nm was measured. A standard curve

115 was made using $NaNO_2$ to calculate the NO concentration of each sample.

116

Immunofluorescence

117

118   NP cells were stimulated with 10 μg/ml LPS in the presence or absence of cordycepin (100μM)

119   in a 24-well plate for 5 days. Then, the cells were fixed with 4% paraformaldehyde for 30 min,

120   treated with 0.1% Triton X-100 for 10 min, and blocked with 2% bovine serum albumin (Sigma)

121   for 1 h. After being washed by PBS, the NP cells were incubated with an anti-collagen II

122   antibody (1:50, Cell Signaling Technology) overnight at 4 °C and then exposed to Alexa Fluor®

123   594-conjugated secondary antibodies (1:100 dilution; Life Technologies) for 60 min at room

124   temperature. Then, after being washed by PBS, the NP cells were counterstained with DAPI and

125   phalloidin. Laser confocal microscopy (OLYMPUS) was used for observation and imaging. The

126   integral optical density (IOD) of each picture was measured using the Image-Pro Plus 6.0

127   software (Media Cybernetics). The immunofluorescence results are expressed as IOD /cell

128   number per view.

129

Cell migration assay

130

131   A total of 1×10^5 RAW 264.7 cells were seeded on a Matrigel-coated polycarbonate membrane

132   insert (8.0 μm pores) in a transwell apparatus (Costar) and maintained in 100 μl of complete

133   medium (high-glucose DMEM with 10% FBS and 1% antibiotic). NP cells were also cultured in

134   complete medium with or without 10 μg/ml LPS or 100 μM cordycepin in the lower chamber for

135   24 h. Then, the inserts were washed with PBS, and the cells on the top surface of the insert were

136   carefully removed by using a cotton swab. The cells on the bottom surface of the insert were

137   fixed with 4% paraformaldehyde for 10 min, followed by staining with 0.1% crystal violet for 20

138   min, and then subjected to an inspection and cell count via microscopy. The cells were counted

139   under 200× magnification, and the counts of 5 randomly chosen fields were averaged for each

140   sample.

141

142   **RNA isolation and PCR**

143   NP cells were incubated with various concentrations of cordycepin for 2 h and then stimulated

144   with 10 μg/ml LPS for 24 h. Then, the total RNA of the NP cells was isolated using TRIzol

145   reagent (Invitrogen) following the manufacturer's instructions. Reverse transcription was carried

146   out from 1 μg of RNA using the 1st Strand cDNA Synthesis Kit (TAKARA) for first-strand

147   complementary DNA (cDNA) synthesis. The relative gene expression was determined by real-

148   time PCR. Real-time PCR was performed using the SYBR Premix Ex Taq kit (TAKARA) with

149   the ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems) according to the

150   manufacturer's instructions. The primers were designed and selected using BLAST. Gene

151   expression was measured using the $2^{-\Delta\Delta Ct}$ method [22]. The primer sequences are summarized in

152   Table 1 and we used β-actin as the internal control.

153   **Table 1. Sequences of the primers used in the polymerase chain reaction (PCR)**

Gene	Primer sequences (5′-3′)	
MMP-3	Forward	TTTGGCCGTCTCTTCCATCC
	Reverse	GCATCGATCTTCTGGACGGT
MMP-13	Forward	ACCATCCTGTGACTCTTGCG

	Reverse	TTCACCCACATCAGGCACTC
ADAMTS-4	Forward	ACCGATTACCAGCCTTTGGG
	Reverse	CCGACTCCGGATCTCCATTG
ADAMTS-5	Forward	CCGAACGAGTTTACGGGGAT
	Reverse	TGTGCGTCGCCTAGAACTAC
iNOS	Forward	ACACAGTGTCGCTGGTTTGA
	Reverse	AGAAACTTCCAGGGGCAAGC
Cox-2	Forward	ATCAGAACCGCATTGCCTCT
	Reverse	GCCAGCAATCTGTCTGGTGA
CCL3	Forward	TGCCAAGTAGCCACATCCAG
	Reverse	CACAGTGTGAGCAACTGGGA
CCL2	Forward	TAGCATCCACGTGCTGTCTC
	Reverse	CAGCCGACTCATTGGGATCA
Aggrecan	Forward	CAGATGGCACCCTCCGATAC
	Reverse	GACACACCTCGGAAGCAGAA
Collagen II	Forward	GGCCAGGATGCCCGAAAATTA
	Reverse	ACCCCTCTCTCCCTTGTCAC
β-actin	Forward	AACCTTCTTGCAGCTCCTCCG
	Reverse	CCATACCCACCATCACACCCT

154

Western blotting

156 For pathway related protein assays, NP cells were pretreated with various concentrations of

157 cordycepin for 2 h and then stimulated with 10 µg/ml LPS for 30 min. For collagen II and

158 aggrecan protein assays, NP cells were cultured with various concentrations of cordycepin and

159 10 µg/ml LPS for 5 days. Then, all cells were washed twice with cold PBS, and the total protein

160 was extracted using an RIPA lysis buffer. The protein concentration was quantified using a BCA

161 Protein Assay Kit (Thermo Scientific), Samples (20 µg of protein) were loaded into gel,

162 separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes

163 (Millipore). The transfer membranes were blocked with 5% fat-free milk at room temperature for

164 1 h and then incubated with primary antibodies against ERK1/2, p-ERK1/2, JNK, p-JNK, p38, p-

165 p38, IκBα, p-IκBα, p65, p-p65 (1:1000, Cell Signaling Technology) aggrecan and collagen-II

166 (1:1000, Abcam) at 4 °C overnight. After three washes with TBST (TBS with Tween20), the

167 membranes were incubated with appropriate secondary antibodies that were conjugated with

168 IRDye 800CW at room temperature for 1 h. Immunoreactive bands were detected using the

169 Odyssey infrared imaging system (LI-COR). The β-actin antibody (1:2000, Cell Signaling

170 Technology) was used as a control.

171

Luciferase assay

173 In addition to western blotting, we also used a luciferase assay to investigate the effect of

174 cordycepin on NF-κB activity. One day before transfection, NP cells were transferred to a 96-

175 well plate at a density of 5×10^3 cells/well. NP cells were cotransfected with 20 ng of NF-κB

176 luciferase reporter and 5 ng of pRL-TK plasmids for each well. Lipofectamine 2000 (Invitrogen)

177 was used as a transfection reagent. After transfection, the cells were cultured for 24 h and then

178 pretreated with cordycepin (10, 50 or 100 μM) and/or stimulated with 10 μg/ml LPS for 24 h.

179 Then, the cells were harvested for the luciferase assay. A Dual-Luciferase Reporter Assay

180 System (Promega) was used for firefly and renilla luciferase activity measurements.

181

182 **Organ culture**

183 Sprague Dawley rats (6-8 weeks old, mixed male and female) were euthanized. Then, the motion

184 segment was isolated from each lumbar vertebra, including the upper and lower end plate and the

185 whole disc. Intervertebral discs were cultured in a 24-well plate with 1 ml of complete DMEM

186 medium (high-glucose DMEM with 10% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin).

187 Additional NaCl was added to the medium to increase the osmolarity to 410 mOsm/kg. Then,

188 100 µmol (10 µl) cordycepin (cordycepin group) or the same volume of saline (control and LPS

189 groups) was injected into each disc through the annulus fibrosus using a microsyringe (5 µl,

190 Hamilton). Then, the IVD was maintained for 7 days with or without 10 µg/ml LPS and 100 µM

191 cordycepin. The culture medium was replaced daily. A total of 30 Sprague Dawley rats,

192 including 90 lumbar segments, were used in the organ culture (30 lumbar segments for each

193 group).

194

195 **Dimethylmethylene blue assay**

196 A DMMB assay was used to measure the proteoglycan (PG) content during 7 days of IVD organ

197 culture. At specific time points, NP tissue was isolated from each cultured IVD and then digested

198 with 5 mg/ml papain (sigma) prior to the DMMB assay. The amount of PG was normalized by

199 the Total DNA of the NP tissue. The total DNA content was determined via an assay of total

200 DNA using the PicoGreen kit (Molecular Probes). The DMMB assay was performed as

201   previously described using chondroitin sulfate as a standard [23]. The PG level of each sample

202   was expressed as μg PG /60 ng DNA (equal to 10^4 cells).

203

204   **Histological analysis**

205   Discs were removed from the culture medium after 7 days and fixed in 4% paraformaldehyde.

206   After fixation, the discs were decalcified in EDTA for 14 days. Serial sagittal sections of discs

207   (5-μm thick) were obtained to prepare slides. NBT/DAPI staining was used to evaluate the cell

208   viability as previously described [24]. Live cells were defined as cells with both DAPI and NBT

209   staining, and cells with DAPI staining alone were registered as dead. The live/dead cell ratio was

210   calculated using fluorescence microscopy (OLYMPUS) at 400× magnification. We analyzed 3

211   sections from each IVD tissue, and for each section, we calculated 3 fields and took the average.

212   The cell viability was calculated as follows: (cell viability = live cells in field / total cells in field)

213   × 100%.

214   Sagittal sections were also stained with hematoxylin and eosin (HE) and Safranin O-fast green to

215   assess the degeneration of the IVD. Type II collagen and Aggrecan expression was detected

216   using mouse monoclonal antibodies (1:200; Abcam) and a horseradish peroxidase-conjugated

217   anti-mouse antibody (1:100; Dako), followed by color development with diaminobenzidine

218   tetrahydrochloride (DAB, Dako). The results of the type II collagen and aggrecan staining were

219   quantified as the IOD using the Image-Pro Plus 6.0 software. Cell and immunohistochemical

220   staining was performed following standard histochemical protocols.

221

Statistical analysis

223 All of the experiments were repeated 3 times. The data are expressed as the mean ± SD. A

224 statistical analysis was performed with a one-way analysis of variance (ANOVA), followed by

225 Duncan's post hoc test using SPSS 19.0 (IBM, Inc.). A P-value of less than 0.05 was considered

226 statistically significant.

227

Results

229 **Cell Viability assay in the cell culture model**

230 To study the potential cytotoxicity of cordycepin, we cultured NP cells with different

231 concentrations of cordycepin for 24 h. As shown in Fig. 1, cordycepin did not show any

232 cytotoxicity at concentrations of 10-100 μM (P>0.05).

233

234 **Cordycepin regulates LPS-induced matrix-degrading enzymes and extracellular matrix-**

235 **related gene expression in NP cells**

236 Monolayer cultures of NP cells were stimulated with 10 μg/ml LPS and 0, 10, 50 or 100 μM

237 cordycepin, followed by a PCR assay and ELISA to measure the mRNA and protein levels

238   respectively of various matrix-degrading enzymes. Cordycepin markedly inhibited the mRNA

239   expression of multiple MMPs (MMP-3 and MMP-13) and ADAMTSs (ADAMTS-4 and

240   ADAMTS-5) in a concentration-dependent manner (Fig. 2A-D). Cordycepin also counteracted

241   the LPS-induced gene downregulation of collagen-2 and aggrecan especially at a concentration

242   of 50 or 100 μM. (Fig. 2E, 2F). The ELISA results showed that 50 and 100 μM cordycepin

243   significantly inhibited MMP-3 and MMP-13 protein production (Fig. 2G, H). Moreover,

244   cordycepin also reversed the LPS-induced increased in the gene expression of Cox-2 and iNOS

245   at a concentration of 100 μM (Fig. 3A, B). Thus, cordycepin significantly inhibited PGE2 and

246   NO production as induced by LPS in NP cells at a concentration of 100 μM (Fig. 3C, D).

247

248   **Cordycepin protects NP cells from LPS-induced matrix degradation**

249   Given that cordycepin effectively suppressed multiple matrix-degrading enzymes that were

250   upregulated by LPS, we further analyzed the effect of cordycepin on antagonizing the LPS-

251   induced matrix degradation in NP cells. NP cells were stimulated with 10 μg/ml LPS in the

252   presence or absence of cordycepin (100 μM) for 5 days, followed by collagen-II fluorescence

253   staining and a western blot assay. These results indicate that the stimulation of NP cells with LPS

254   strikingly reduced the collagen-II and aggrecan content, while cordycepin significantly inhibited

255   the decrease of collagen-II and aggrecan compared to that observed in LPS group (Fig. 4 A-D).

256

Cordycepin inhibits the NP-mediated migration of macrophages after treatment with LPS

We examined the effect of NP cells that were treated with LPS on the migration of RAW 264.7 macrophages. Fig. 5A shows that after treatment with 10 μg/ml LPS, NP cells promoted the chemotactic migration of macrophages compared to untreated NP cells. However, cordycepin inhibited the migration of macrophages promoted by NP cells after treating with LPS (Fig. 5A, B). Moreover, as shown in Fig. 5C-E, 100 μM cordycepin reversed the LPS-induced gene upregulation of chemokine ligand 2 (CCL2, or monocyte chemotactic protein-1 (MCP-1)), a well-characterized macrophage chemotactic factor that is expressed in NP cells [25].

Cordycepin inhibits the LPS-induced activation of the NF-κB pathway in NP cells

To evaluate the potential involvement of signal transduction pathways and the mechanisms of the effects of cordycepin on LPS-stimulated NP cells, we measured the activation of the MAPK and NF-κB pathways via western blotting. The results show that 10 μg/ml LPS significantly activated the MAPK and NF-κB pathways in NP cells, while cordycepin inhibited the phosphorylation of IκBα and p65 in a dose-dependent manner (Fig. 6A, D, E). However, cordycepin did not influence the activation of the MAPK pathway (Fig. 6B, F-H). A luciferase assay also showed that cordycepin could inhibit the LPS-induced activation of the NF-κB pathway in NP cells (Fig. 6C).

276 **Cordycepin reverses the LPS-induced degeneration of IVDs in an organ culture model**

277 The NBT/DAPI staining showed that the cell viability of all groups at 7 days was still greater

278 than 80%, confirming the reliability of the organ culture model (Fig. 7A,B).

279 Fig. 8A shows cultured IVD sections that were stained with HE and Safranin-O fast green. On

280 the Safranin-O fast green sections, proteoglycans (PGs) stained red, and on the HE sections, they

281 stained purple. After 7 days of IVD organ culture, the presence of LPS resulted in severe PG loss

282 compared to the effects of co-incubation with cordycepin (Fig. 8A).

283 The immunohistochemical staining of collagen-II and aggrecan were markedly enhanced in the

284 NP area. Immunohistochemical staining showed that cordycepin effectively reversed the

285 decreased collagen-II and aggrecan expression induced by LPS (Fig. 8B). The quantification of

286 the Integral Optical Density (IOD) also indicates that the cordycepin group gained more

287 collagen-II and aggrecan staining at day 7 (Fig. 8C, P<0.01).

288 A DMMB assay was used to quantify the PG content of NP in an IVD culture. Similar to the

289 histological results, cordycepin significantly attenuated the PG loss induced by LPS (Fig. 8D,

290 P<0.01).

291

292 **Discussion**

293 The present study provides, for the first time, evidence that cordycepin exhibits pharmacological

294 anti-inflammation and anti-degeneration effects in LPS-induced NP cells and IVDs. Our results

295   also show that cordycepin blocks the LPS-induced activation of the NF-κB pathway, but not the

296   MAPK pathway in NP cells.

297   The expression of MMPs and ADAMTSs has been extensively studied in the process of IVD

298   degeneration. According to many studies [26-28], MMP-3 and MMP-13 are not expressed in

299   nondegenerated human discs but have increased expression in degenerated human discs. In

300   addition, MMP-3 protein expression has a positive correlation with IVD histomorphological

301   degenerative findings [27]. The upregulation of ADAMTSs has also been observed in

302   degenerative discs [29]. ADAMTS-4 and ADAMTS-5 have been reported as the most important

303   aggrecanases due to their strong capabilities in cleaving aggrecan among the 20 different

304   ADAMTSs [30,31]. Inhibitors of MMPs and ADAMTSs have a therapeutic effect on OA [32]

305   and IDD [33] in vitro and in vivo. Collagen-II and aggrecan are the main components of nucleus

306   pulposus, and the reduction of collagen-II content is highly correlated with disc degeneration

307   [34,35]. Our study shows that cordycepin can both prevent LPS-induced collagen-II and

308   aggrecan loss and promote their synthesis.

309   Many studies have revealed that NO and PGE2 play important roles in both the regulation of

310   cellular metabolism in discs under mechanical stress conditions and the pathology of

311   intervertebral disc degeneration [36-38]. According to previous reports, PGE2 increases the

312   excitability of rat sensory neurons and is involved in the development of sciatica in herniated

313   disc disease [39]. In addition, NO contributes to the development of radiculopathy by mediating

314   protein nitration, moreover, the symptoms can be relieved by its suppression [40]. Macrophages

315   also play a critical role in the inflammatory response that is associated with degenerative disc

316   disease and LBP [41,42]. Macrophages could upregulate many cytokines expression including

317   PGE2, and the interaction of macrophages and IVDs induce MMP-3 production which may

318   cause extracellular matrix resorption [43]. Previous studies have suggested that chemokine

319   ligand 2 (CCL2) and chemokine ligand 3 (CCL3) are important mediators of macrophage

320   infiltration into disc tissue [41,42]. In our research, LPS upregulated the gene expression of

321   CCL2 and CCL3 in NP cells, thereby promoting macrophage migration in NP cells and in a

322   RAW 264.7 cell co-culture model. However, cordycepin reversed LPS-induced CCL2 gene

323   upregulation of NP cells and inhibit macrophage migration, thus reducing the early-stage

324   inflammation of NP cells. We also demonstrated that cordycepin could inhibit LPS-induced

325   iNOS and Cox-2 gene overexpression and reduce the production of NO and PGE2 in NP cells.

326   The MAPK and NF-κB pathways play important roles in the regulation of the inflammatory

327   response [44,45]. According to previous studies, cordycepin exerts anti-inflammatory effect via

328   suppressing the NF-κB pathways in many types of cells [18, 46,47]. And cordycepin has also

329   been reported to induce apoptosis in various cancer cells via MAPK pathway [48]. In our study,

330   cordycepin inhibited the NF-κB pathway by inhibiting the phosphorylation of IκBα and p65 in

331   LPS-stimulated NP cells. In contrast, the phosphorylation of Erk, JNK and p38 of the MAPK

332   pathway did not significantly change. This result was consistent to the data reported previously

333   [18,46].

334   Given that the in vitro experiment showed encouraging results, we further study the potential

335   therapeutic effects of cordycepin in an organ culture model. Although the permeability of the

336   annulus fibrous and endplate is very limited, our previous and other studies have confirmed that

337   LPS and bioactive molecules can penetrate into the nucleus pulposus and exert bioactive effects

338   [23,49]. Our results clearly indicate that high concentrations of LPS (10 μg/ml) can induce the

339   degeneration of cultured IVDs in a relatively short period (7 days), while cordycepin can

340   significantly reduce the LPS-induced PG loss in cultured IVD. A similar study reported that

341   cordycepin may suppress IL-1β-stimulated catabolic enzyme, COX-2 and iNOS gene expression

342   in chondrocytes, exerting chondroprotective effect and interfering with the inflammatory

343   response [50]. Considering the similarity between chondrocytes and NP cells, this previous study

344   support our results to some extent. However, we focus more on the early stage of inflammation

345   of NP cells in the present study, while the chondrocytes used in the previous study were from

346   subjects with advanced-stage osteoarthritis.

347   Previous study have reported fibrin-genipin annulus fibrosus sealant as a drug delivery system

348   for IDD, and it can maintain the bioactivity of the drug for over 20 days [51]. Based on our study,

349   cordycepin needs to be directly deliver to IVDs before exerting its anti-degenerative effects.

350   Future investigations are required to determine if such a system can effectively deliver

351   cordycepin and allow it to exert its effects in IVDs.

352   In conclusion, our results show that cordycepin exhibits a strong anti-inflammatory and anti-

353   catabolic effect by inhibiting LPS-induced NF-κB activation in NP cells. Our organ culture

354   model also demonstrates an anti-degeneration effect of cordycepin in IVDs. Cordycepin may be

355   a potential new agent for treating IDD in the future.

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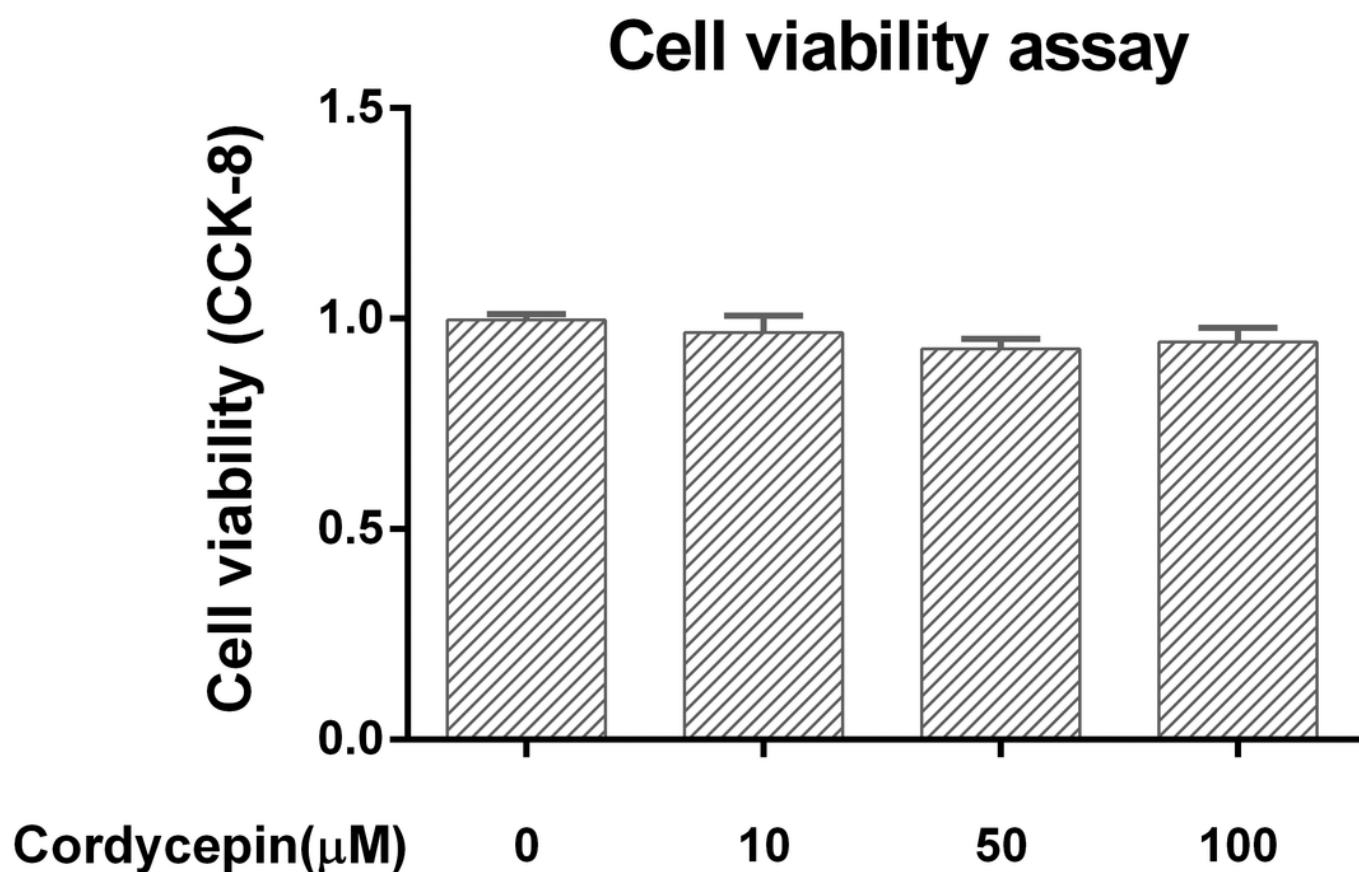
506

1

Cell viability assay in the NP cell culture model.

We used CCK-8 to measure the NP cell viability in a monolayer culture model. Cordycepin did not show any cytotoxicity at concentrations of 10-100 μM.

2

Cordycepin regulates the LPS-induced matrix-degrading enzymes and extracellular matrix related gene expression in NP cells.

We used PCR (A-F) and an ELISA assay (G, H) to investigate the effect of cordycepin on the LPS-induced gene expression and matrix-degrading enzyme secretion of NP cells. (A-F) Real-time PCR indicated that cordycepin downregulated the LPS-induced gene over-expression of MMP-3, MMP-13, ADAMTS-4 and ADAMTS-5. Moreover, cordycepin also counteracted the LPS-induced gene downregulation of collagen-2 and aggrecan especially at concentration of 50 mM or 100 µM. (G, H) An ELISA assay of MMP-3 and MMP-13 demonstrated that cordycepin inhibited LPS-induced MMPs secretion. The values are presented as the mean ± standard deviation. *P<0.05 compared to the LPS group; **P < 0.01 compared to the LPS group; ##P < 0.01 compared to the control group.

A MMP-3

B MMP-13

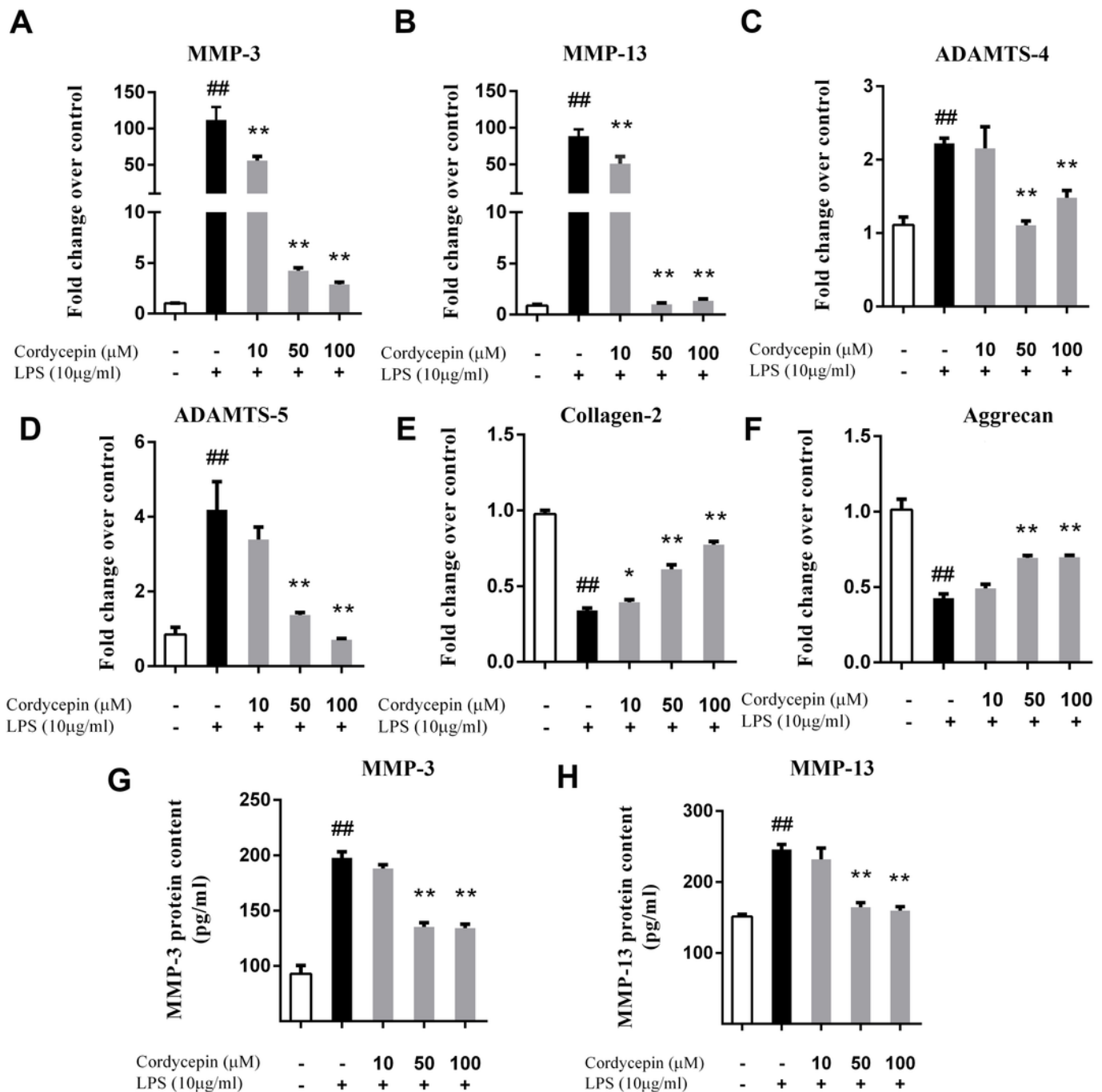
C ADAMTS-4

D ADAMTS-5

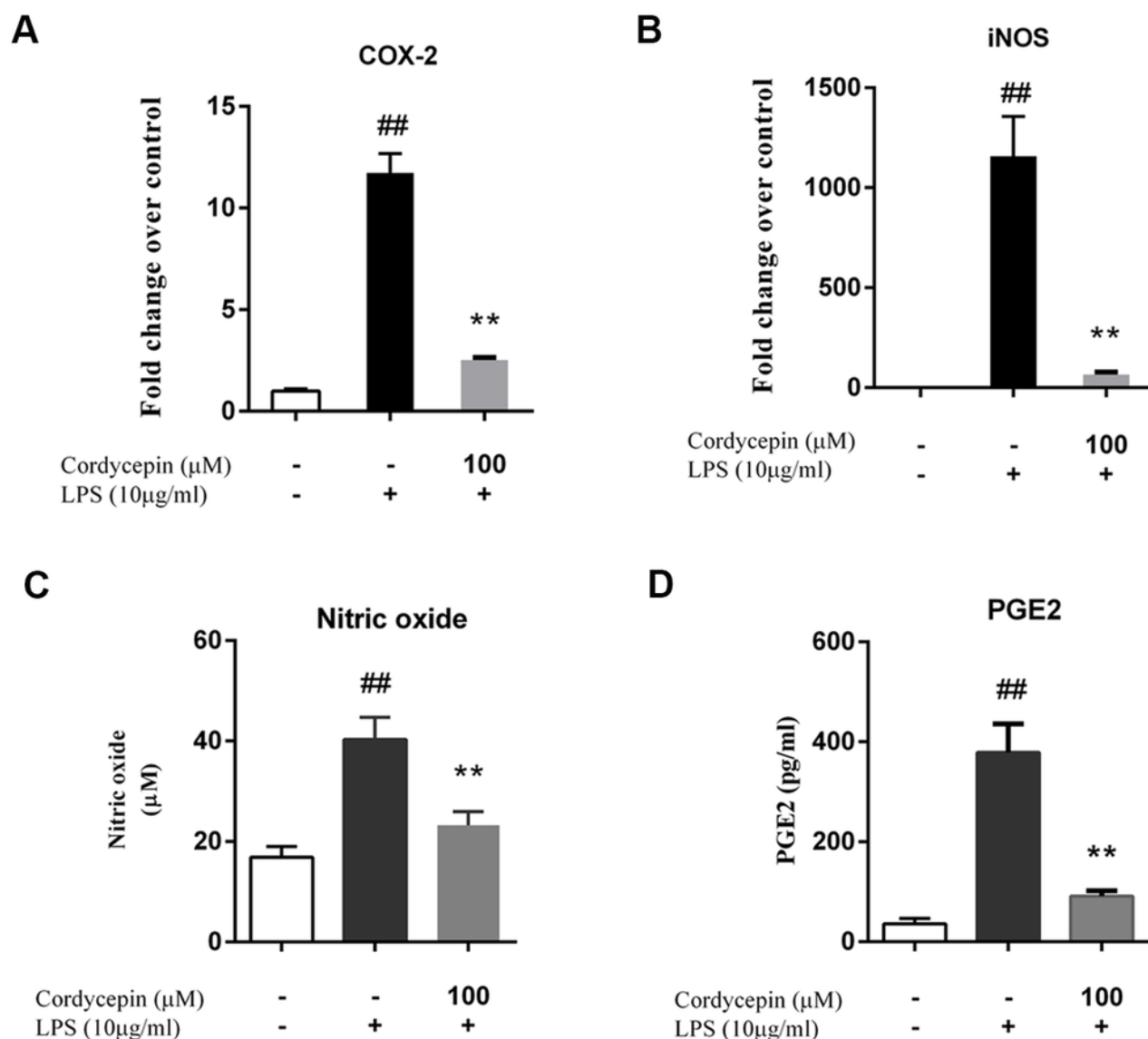
E Collagen-2

F Aggrecan

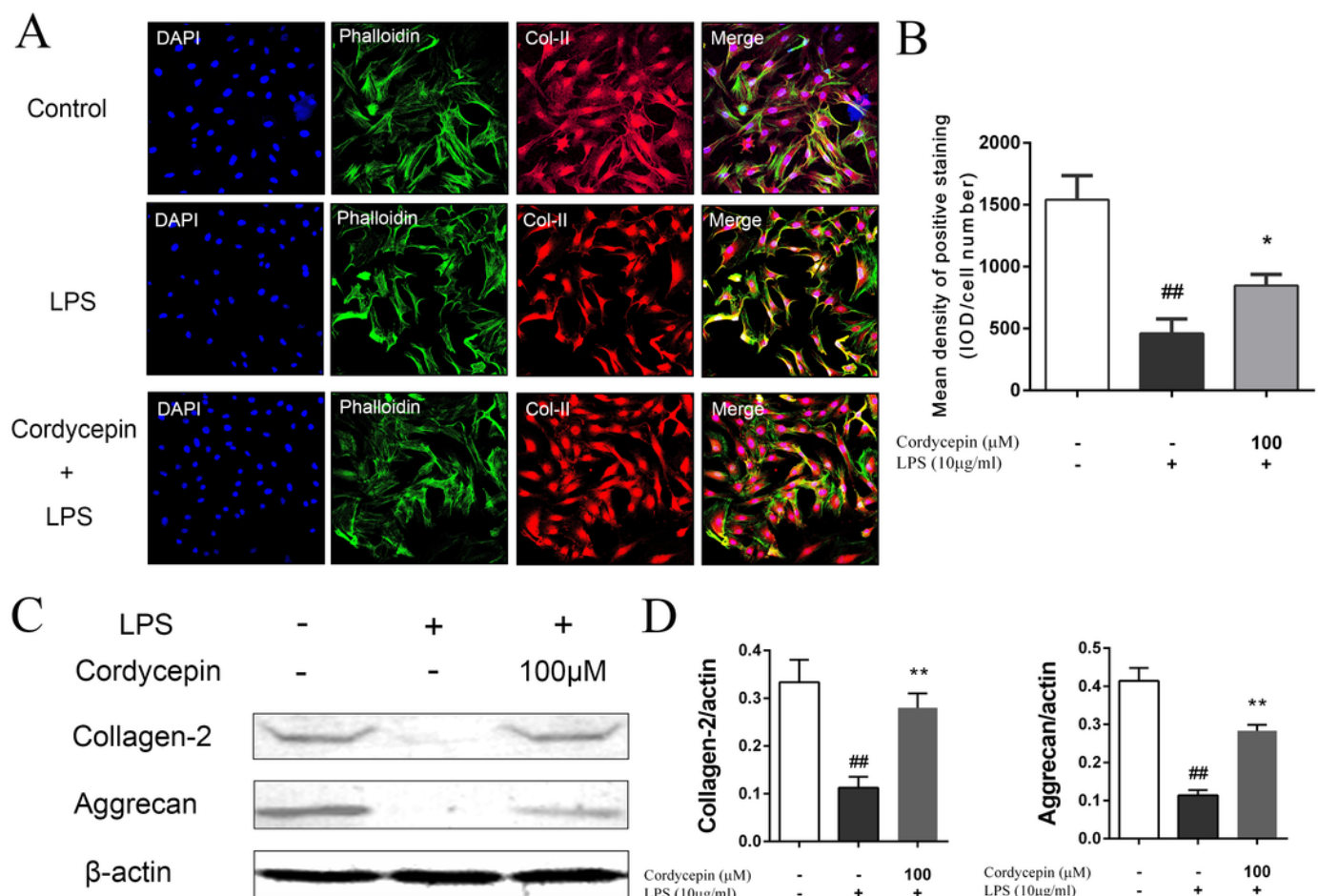
G MMP-3

H MMP-13

3

Cordycepin decreases the LPS-induced production of PGE2 and NO in NP cells.

(A, B) Real-time PCR indicated that cordycepin reversed the LPS-induced increased gene expression of Cox-2 and iNOS. (C) The NO content was measured using the Griess reaction; the results showed that cordycepin inhibited LPS-induced NO production in NP cells. (D) An ELISA assay demonstrated that cordycepin inhibited the LPS-induced PGE2 production in NP cells. The values are presented as the mean ± standard deviation. **$P < 0.01$ compared to the LPS group; ##$P < 0.01$ compared to the control group.

Cordycepin reverses LPS-induced matrix degradation in NP cells.

NP cells were treated with or without 100 µM cordycepin and 10 µg/ml LPS for 5 days and then fixed with 4% paraformaldehyde and used to perform fluorescence staining. (A, B) Collagen-II (Col-II) immunofluorescent staining showed that cordycepin reduced the LPS-induced collagen-II decrease in NP cells. (C, D) Western blotting results showed that cordycepin significantly reversed LPS-induced collagen-II and aggrecan loss. These results indicated that cordycepin could protect NP cells from LPS-induced matrix degradation. *P < 0.05 compared to the LPS group; ##P < 0.01 compared to the control group.

5

Cordycepin inhibits LPS-induced CCL2 expression and macrophage migration in NP cells.

Macrophage migration was measured using a 24-well cell culture insert system. The results of crystal violet staining (A) and a positive cell count (B) showed that LPS promoted NP-mediated macrophage migration, which was inhibited by cordycepin. (C, D) Real-time PCR indicated that cordycepin inhibited the LPS-induced increased gene expression of CCL2 but not CCL3. The values are presented as the mean ± standard deviation. *P < 0.05 compared to the LPS group; **P < 0.01 compared to the LPS group; ##P < 0.01 compared to the control group.

A

LPS(without NP)	Control	LPS(with NP)	Cordycepin+LPS

B

Positive Cell number per field

Cordycepin (μM)	-	-	-	100
LPS (10μg/ml)	+	-	+	+
NP cells	-	+	+	+

**

C CCL-2

Fold change over control

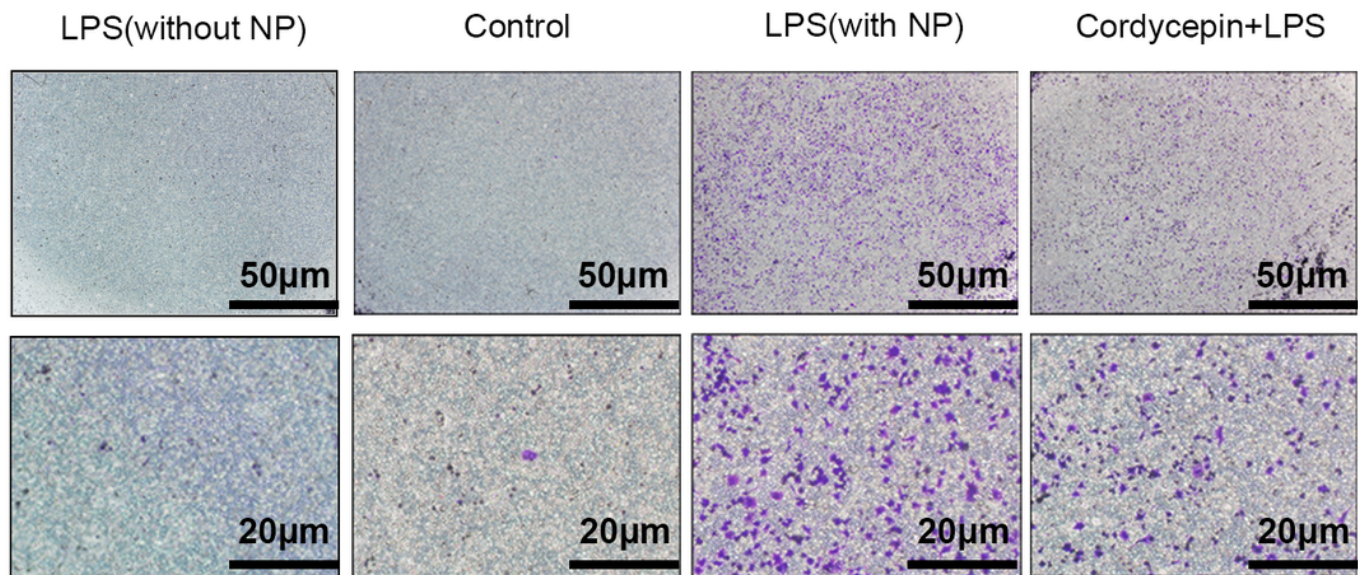
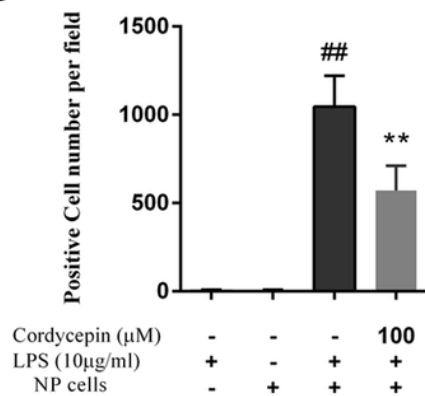
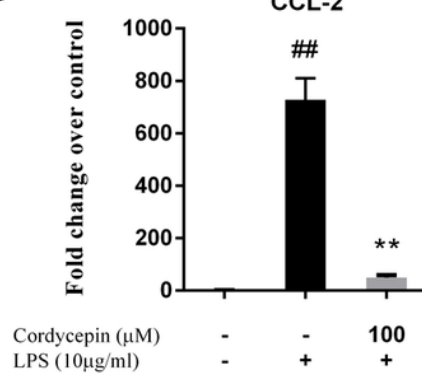
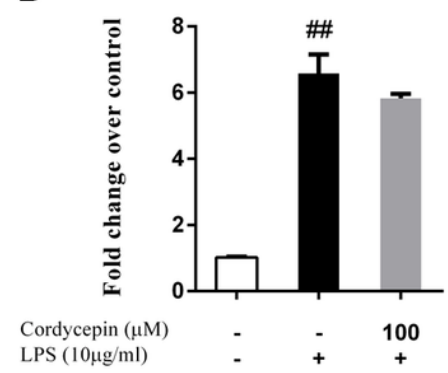
Cordycepin (μM)	-	-	100
LPS (10μg/ml)	-	+	+

**

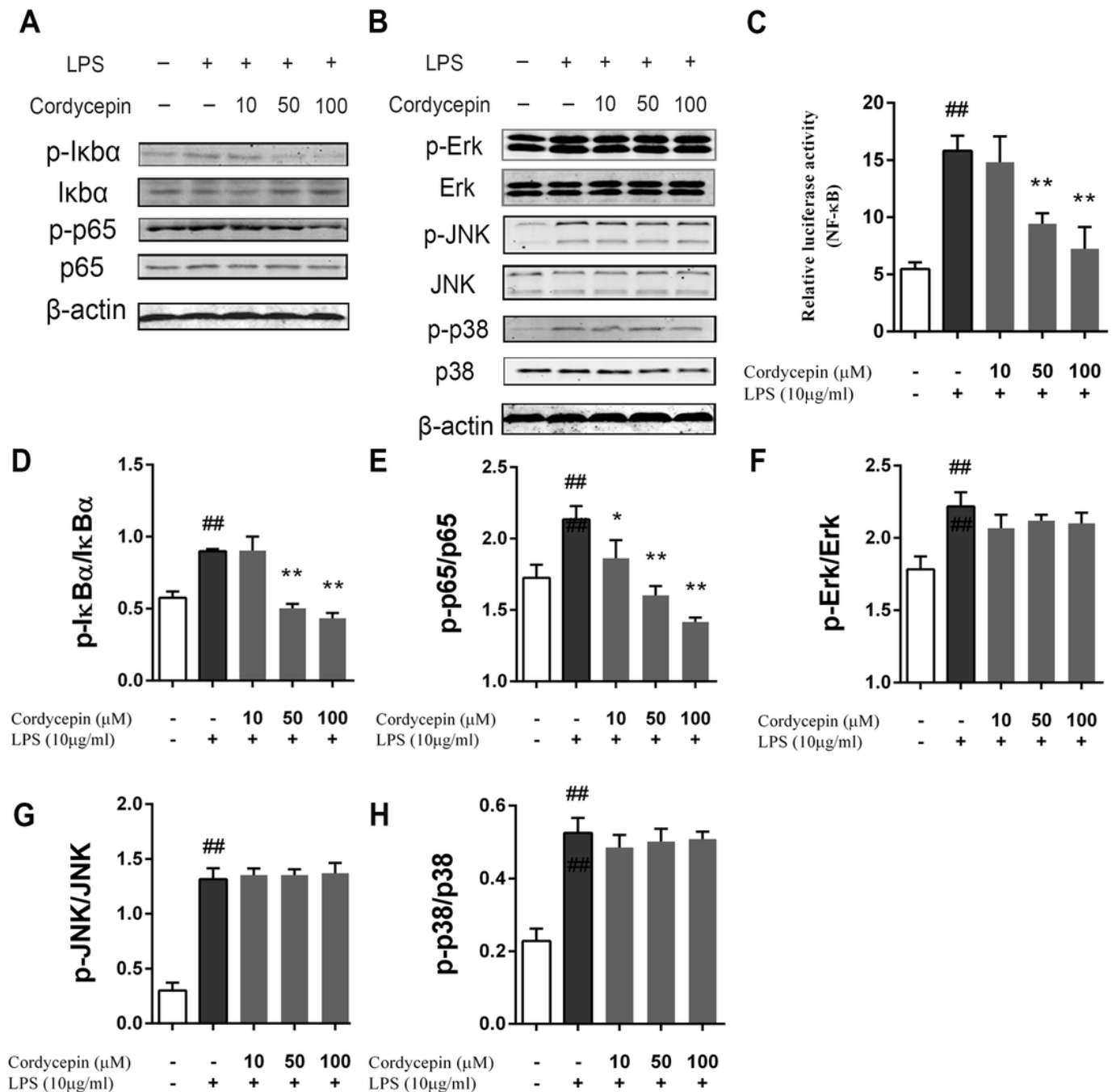
D CCL-3

Fold change over control

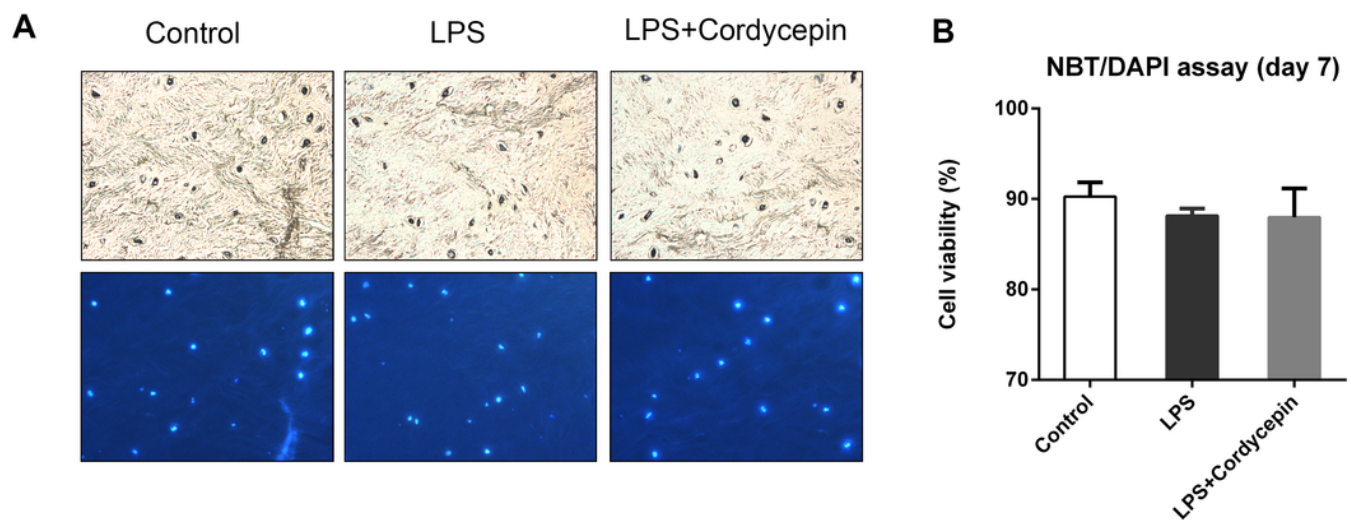
Cordycepin (μM)	-	-	100
LPS (10μg/ml)	-	+	+

##

6

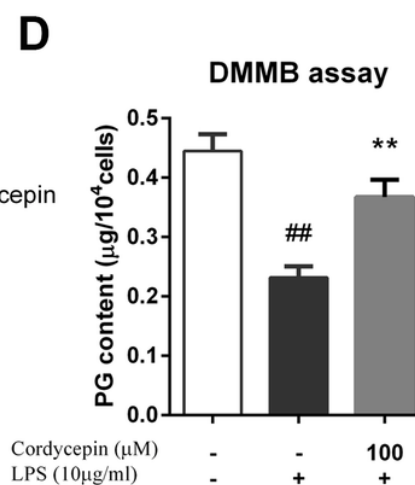
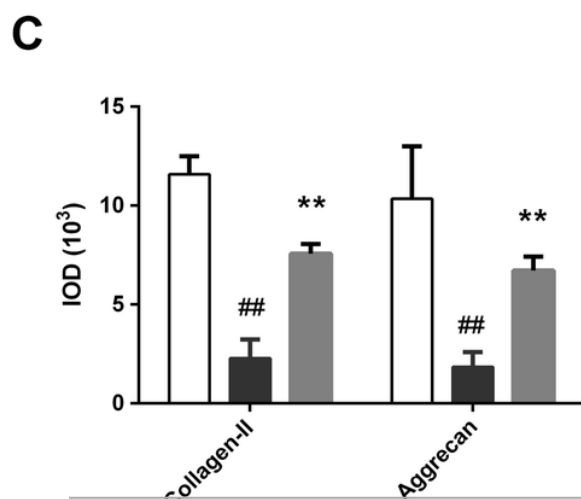
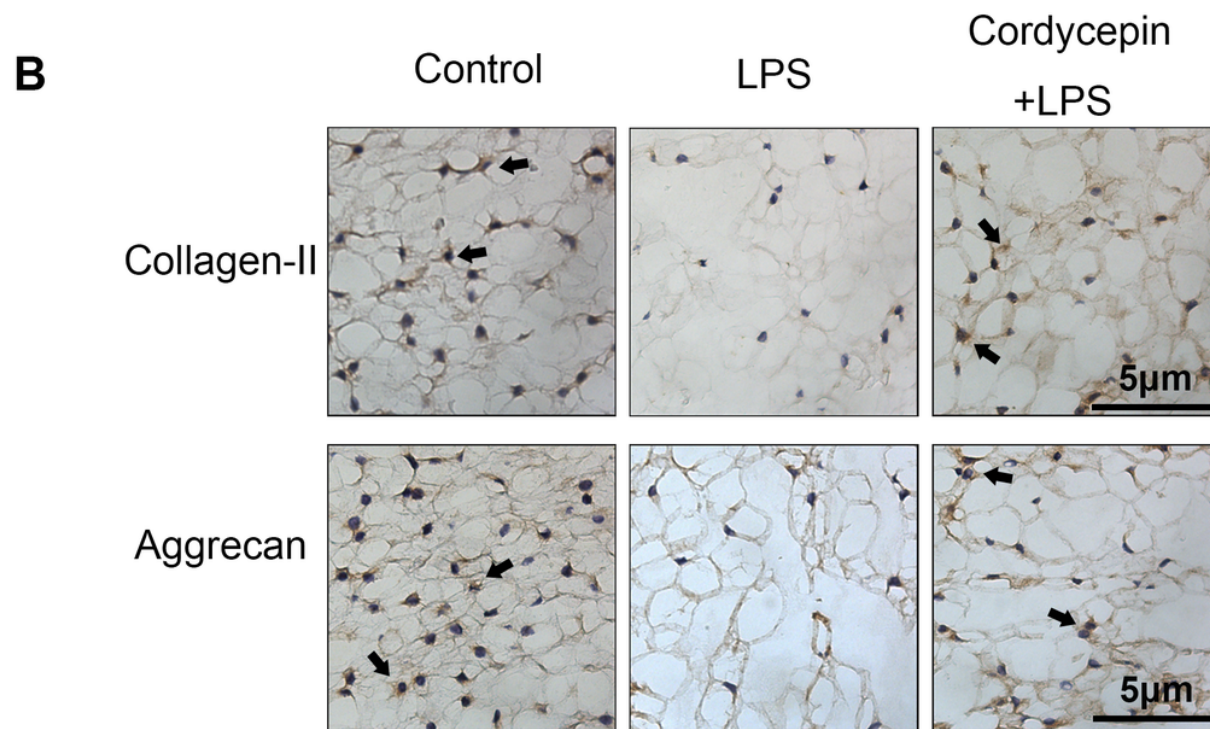
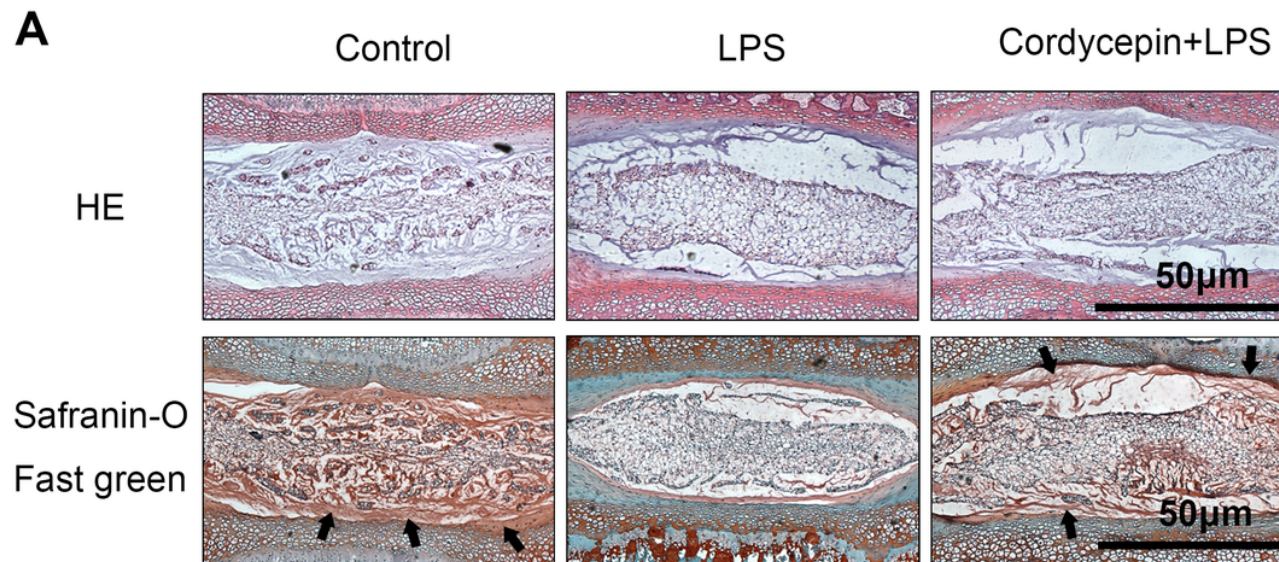
Effect of cordycepin on the LPS-induced activation of the NF-κB and MAPK pathways.

NP cells were pretreated with various concentrations of cordycepin for 2 h and then stimulated with 10 μg/ml LPS for 24 h. Then, western blotting was performed to evaluate the mechanism of cordycepin on LPS-treated NP cells. (A) Cordycepin significantly inhibied the phosphorylation of IκBα and p65 induced by LPS. (B) Cordycepin did not influence the phosphorylation of ERK, p38 or JNK enhanced by LPS. (C) NP cells were transfected with a NF-κB luciferase reporter, and the NF-κB pathway activity was determined by luciferase assay using a commercially available kit. (D-H) Quantitative analysis of western blotting data show cordycepin significantly inhibited the activation of the NF-κB pathway induced by LPS at concentrations of 50 and 100μM. The values are presented as the mean ± standard deviation. *P < 0.05 compared to the LPS group; **P < 0.01 compared to the LPS group; ##P < 0.01 compared to the control group.

7

Cell viability assay in the IVD organ culture model.

(A) NBT/DAPI staining on the 7th day of IVD organ culture. (B) Cell viability of the organ culture model. Cell viability = (NBT positive cell number / DAPI positive cell number) ×100%.

8

Histological assessments and DMMB assay.

The lumbar spine discs of rat were cultured with or without LPS and cordycepin for 7 days. Then, the discs were divided into two groups: one group was fixed and used to prepare serial section slides for staining. Fresh nucleus pulposus tissue was removed from another group of discs for DMMB assay. (A) HE and Safranin O-fast green staining. (B) Collagen II and Aggrecan immunohistochemistry staining. (C) The results of immunohistochemical staining were quantified in integral optical density (IOD). (D) A DMMB assay was used to quantify the PG content of NP in an IVD culture. Both chemical staining and immunohistochemical staining showed that cordycepin protected the intervertebral disc from LPS-induced PG loss. The black arrow shows the PG content in Safranin O-fast green staining and positive immunohistochemical staining. The values are presented as the mean ± standard deviation. **P < 0.01 compared to the LPS group; ##P < 0.01 compared to the control group.

Sequences of the primers used in the polymerase chain reaction (PCR)

1   **Table 1. Sequences of the primers used in the polymerase chain reaction (PCR)**

Gene		Primer sequences (5′-3′)
MMP-3	Forward	TTTGGCCGTCTCTTCCATCC
	Reverse	GCATCGATCTTCTGGACGGT
MMP-13	Forward	ACCATCCTGTGACTCTTGCG
	Reverse	TTCACCCACATCAGGCACTC
ADAMTS-4	Forward	ACCGATTACCAGCCTTTGGG
	Reverse	CCGACTCCGGATCTCCATTG
ADAMTS-5	Forward	CCGAACGAGTTTACGGGGAT
	Reverse	TGTGCGTCGCCTAGAACTAC
iNOS	Forward	ACACAGTGTCGCTGGTTTGA
	Reverse	AGAAACTTCCAGGGGCAAGC
Cox-2	Forward	ATCAGAACCGCATTGCCTCT
	Reverse	GCCAGCAATCTGTCTGGTGA
CCL3	Forward	TGCCAAGTAGCCACATCCAG
	Reverse	CACAGTGTGAGCAACTGGGA
CCL2	Forward	TAGCATCCACGTGCTGTCTC
	Reverse	CAGCCGACTCATTGGGATCA
Aggrecan	Forward	CAGATGGCACCCTCCGATAC
	Reverse	GACACACCTCGGAAGCAGAA
Collagen II	Forward	GGCCAGGATGCCCGAAAATTA
	Reverse	ACCCCTCTCTCCCTTGTCAC
β-actin	Forward	AACCTTCTTGCAGCTCCTCCG
	Reverse	CCATACCCACCATCACACCCT