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Original Article

Piperine mediates LPS induced inflammatory and catabolic effects in rat intervertebral disc

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Abstract: Piperine is an exact of the active phenolic component from Black pepper. It has been reported to have many biological activities including anti-oxidant, anti-inflammatory and anti-tumor effects. Intervertebral disc degeneration (IDD) is a degenerative disease closely relate to inflammation of nucleus pulposus (NP) cells. This study aimed to assess the anti-inflammatory and anti-catabolic effects of piperine in rat intervertebral disc using in vitro and ex vivo analyzes. We demonstrated that piperine could inhibit LPS induced expression and production of inflammatory factors and catabolic proteases in NP cells culture model. It significantly inhibited multiple inflammatory factors and oxidative stress-associated genes (IL-1β, TNF-α, IL-6, iNOS), MMPs (MMP-3, MMP-13), ADAMTS (ADAMTS-4, ADAMTS-5) mRNA expression and NO production in a concentration-dependent manner. Moreover, piperine could reverse the LPS-induced inhibition of gene expression of aggrecan and collagen-II. Histologic and dimethylmethylene blue analysis indicated piperine could also against LPS induced proteoglycan (PG) depletion in a rat intervertebral disc culture model. Western blot results showed that piperine inhibited the LPS-mediated phosphorylation of JNK and activation of NF-κB. Finally, our results demonstrated the ability of piperine to antagonize LPS-mediated inflammation of NP cells and suppression of PG in rat intervertebral disc, suggesting a potential agent for treatment of IDD in future.

Keywords: Piperine, inflammation, nucleus pulposus cells, intervertebral disc degeneration

Introduction

Intervertebral disc degeneration (IDD) is considered to be one of the most important reasons to the development of low back pain (LBP). In fact, intervertebral disc degeneration related conditions are among the most common causes of disability among all workers aged 18 to 64 years [1]. IDD is associated with disc herniation, spinal stenosis, facet hypertrophy, spondylolysis and other pathology that may engender symptoms, and spine clinicians are tying various surgical and non-surgical therapies to combat these symptoms.

The mechanism of IDD contains a complex biochemical cascade. One of the most important features of IDD is the breakdown of the Intervertebral disc (IVD) extracellular matrix (ECM). This process is closely related to the gene expression and activity of matrix metalloproteinases (MMPs), disintegrins and metalloproteinases with thrombospondin motifs (ADAMTSs) and tissue inhibitors of metalloproteinases (TIMPs) [2, 3]. Moreover, proinflammatory cytokine such as Interleukin-1β (IL-1β) and tissue necrosis factor-α (TNF-α) also play an important role in IDD. Cytokines are a byproduct of and stimulant of the inflammatory cascade. They do not directly degrade the IVD like MMPs or ADAMTSs; instead they accelerate IDD by promoting the production of inflammatory substance by the disc cells.

Piperine is an exact of the active phenolic component from Black pepper (*Piper nigrum*). It has been reported to have many biological activities including anti-oxidant, anti-inflammatory and anti-tumor effects [5]. It was reported that piperine decreased inflammation by inhibiting the activation of MAPKs and reduced cytokine production in isolated cerulein-treated pancre-

Table 1. Sequences of primers used in real-time polymerase chain reaction (Real-time 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
IL-1β	Forward	GCACAGTTCCCCAACTGGTA
	Reverse	GGAGACTGCCCATTCTCGAC
IL-6	Forward	CATTCTGTCTCGAGCCCACC
	Reverse	AGTCTCCTCTCCGGACTTGT
TNF-α	Forward	TCGTAGCAAACCACCAAGCA
	Reverse	TCGTAGCAAACCACCAAGCA
iNOS	Forward	ACACAGTGTCGCTGGTTTGA
	Reverse	AGAAACTTCCAGGGGCAAGC
Aggrecan	Forward	CAGATGGCACCCTCCGATAC
	Reverse	GACACACCTCGGAAGCAGAA
Collagen II	Forward	GGCCAGGATGCCCGAAAATT
	Reverse	ACCCCTCTCTCCCTTGTCAC
β-actin	Forward	AACCTTCTTGCAGCTCCTCCG
	Reverse	CCATACCCACCATCACACCCT

atic acinar cells [4]. In addition, Ying et al. reported piperine-dose-dependently inhibited LPS and IL-1β induced inflammation via NF-κB signal path in RAW 264.7 and chondrocyte cells [6, 7]. In spite of these beneficial results, no study has yet assessed the anti-inflammation effects of piperine in IVD. In the present study, we investigate whether piperine could inhibit LPS induced inflammation and degeneration of IVD in rat Nucleus Pulposus (NP) cell culture and IVD organ culture model.

Materials and methods

NP cell isolate and culture

Nucleus Pulposus (NP) cells were isolated from Sprague-Dawley rats lumbar spines, using standard enzymatic digestion and culture in complete media (high glucose DMEM with 10% FBS and 1% antibiotic) up to passage 2-3. Cells were incubated with piperine (Sigma) at concentrations of 0, 10, 50 and 100 mg/ml for 2 h, then stimulate with LPS (Sigma) at 10 μg/ml for 24 h. Piperine was dissolved with DMSO, the final concentration of DMSO in medium was less than 0.05%.

Organ culture

Sprague Dawley rats were euthanized by injecting a lethal dose of chloral hydrate. Then isolate the motion segment from each lumbar vertebrae including upper and lower end plate and whole disc. The lumbar IVD tissue was cultured in 24-well plate with hypertonic DMEM medium. Additional NaCl was added to medium to raise the osmolarity to 410 mOsm/kg. The medium was replaced every day. The disc tissue was maintained for 14 days with or without 10 μg/ml LPS and various concentrations of piperine. We used nitro blue tetrazolium (NBT)/4, 6-diamino-2-phenyl indole (DAPI) staining to evaluate cell viability in organ culture. At the end of culture period, the IVD tissue was treated with 0.75 mg/ml of NBT (Sigma) in a fresh complete medium and incubate at 37°C for 24 h, then fixed with 4% paraformaldehyde for NBT/DAPI staining.

Cell viability assay in cells culture model

Cell viability was measured using Cell Counting Kit-8 (CCK-8; Dojindo, Japan). NP cells were plated in 96-well plates at a density of 5×10^3 per well, incubate with various concentrations of piperine for 24 h, then add 10 μl CCK-8 solution incubate for 2 h. Absorbance of each wells was measured at 450 nm. The culture medium was used as a blank. Cell viability was calculated as: cell viability = [absorbance (with piperine)-absorbance (blank)]/[absorbance (without piperine)-absorbance (blank)].

Dimethylmethylene blue assay for proteoglycan content

DMMB assay was used to measure proteoglycan (PG) content at 7 and 14 days of IVD organ culture. At specified time point, NP tissue was isolated from each cultured IVD and then digested with 5 mg/ml papain (Sigma) prior to DMMB assay. The DMMB assay was quantified by cell numbers of NP tissue. Cell numbers were determined by assay of total DNA using PicoGreen kit (Molecular Probes, Carlsbad, CA). The DMMB assay was performed as described using chondroitin sulfate as standards [8]. The PG level of each sample was expressed as μg PG/10^4 cells.

RNA isolate and real-time PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer's instruction. Reverse transcription was carried out from 1 μg RNA using 1st Strand cDNA Synthesis Kit (TAKARA, Dalian, China) for first-strand complementary DNA (cDNA) synthesis. Relative gene expression was determined by real-time PCR using SYBR Premix Ex Taq kit (TAKARA, Dalian, China) with ABI Prism 7500 Fast Real-Time PCR system (Applied Biosystems, Foster city, CA, USA) according to manufacturer's instruction. Primer was designed and selected using BLAST. Gene expression was measured using the $^{\Delta\Delta}$Ct method. β-actin was used as the internal control. The primer sequences are summarized in **Table 1**.

Western blot

In short, the cells were washed twice with cold PBS, then extracted total protein using RIPA lysis buffer. The protein was separated by 10% SDS-PAGE and transferred to PVDF membranes. Transfer membranes were blocked with 5% fat-free milk for 1 h, and then incubated with primary antibodies against ERK1/2, p-ERK-1/2, JNK, p-JNK, p38, p-p38, IκBα, p-IκBα (1:1000, Cell Signaling Technology, USA) over night. After three washes, the membranes were incubated with appropriate secondary antibodies conjugated with IRDye 800CW. Immunoreactive bands were detected using Odyssey infrared imaging system (LI-COR).

Measurements of MMP-3 and MMP-13 by ELISA

The level of MMP-3 and MMP-13 in culture medium was measured by ELISA kit according to manufacturer's instructions (R&D Systems, MN, USA).

Measurement of nitric oxide

NP cells were incubated with piperine (Sigma) at concentrations of 0, 10, 50 and 100 μg/ml for 2 h, then stimulate with LPS (Sigma) at 10 mg/ml for 24 h. 100 μl of each culture supernatant were incubated at room temperature for 15 min with 100 μl of Griess reagent (Sigma), then read the absorbance at 540 nm. A standard are curve using $NaNO_2$ was then used to calculate the NO concentration.

Histologic analysis and cell viability assay in organ culture model

Discs were removed from culture after 0, 7, 14 days and fixed in 4% paraformaldehyde. After fixation, discs were decalcified in EDTA for 14 days. Serial sagittal sections of discs (5-μm thick) were obtained to prepare slides. NBT/DAPI staining was used to evaluate cell viability as previously described [9]. Live cells were defined as cells with both DAPI and NBT stains, and cells with DAPI signals alone were registered dead. Live/dead cells radio was calculated using fluorescence microscopy (OLYMPUS, JAPAN) at 400× magnification. We analyzed 3 sections from each IVD tissue, and for each section, we calculated 3 fields and took the average. Cell viability was calculated as: cell viability = live cells in field/total cells in field × 100%.

Sagittal sections from all time points were also stained with hematoxylin and eosin (HE) and Safranin O-fast green to assess the degeneration of IVD. Type II collagen expression was detected using a mouse monoclonal antibody (1:200; Abcam, Cambridge, MA) and a horseradish peroxidase-conjugated anti-mouse antibody (1:50; Dako, Denmark), followed by colour development with diaminobenzidine tetrahydrochloride (DAB, Dako). The results of type II collagen staining were quantified in integrated optical density (IOD) with the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MD, USA). All staining and immunohistochemical staining were performed following standard histochemical protocols.

Statistical analysis

All experiments were repeated 3 times. Data were expressed as mean ± SD. The statistical analysis was performed with a one-way analysis of variance (ANOVA) for multiple comparisons using SPSS 19.0 (IBM, Inc., NY, USA). *P*-value less than 0.05 was considered to be statistically significant.

Results

Cell viability assay in cells culture model

To study potential cytotoxicity of piperine, we cultured NP cells with different concentrations of piperine for 24 h. As shown in **Figure 1A**, piperine did not show any cytotoxicity at concentrations of 10-200 μg/ml ($P > 0.05$).

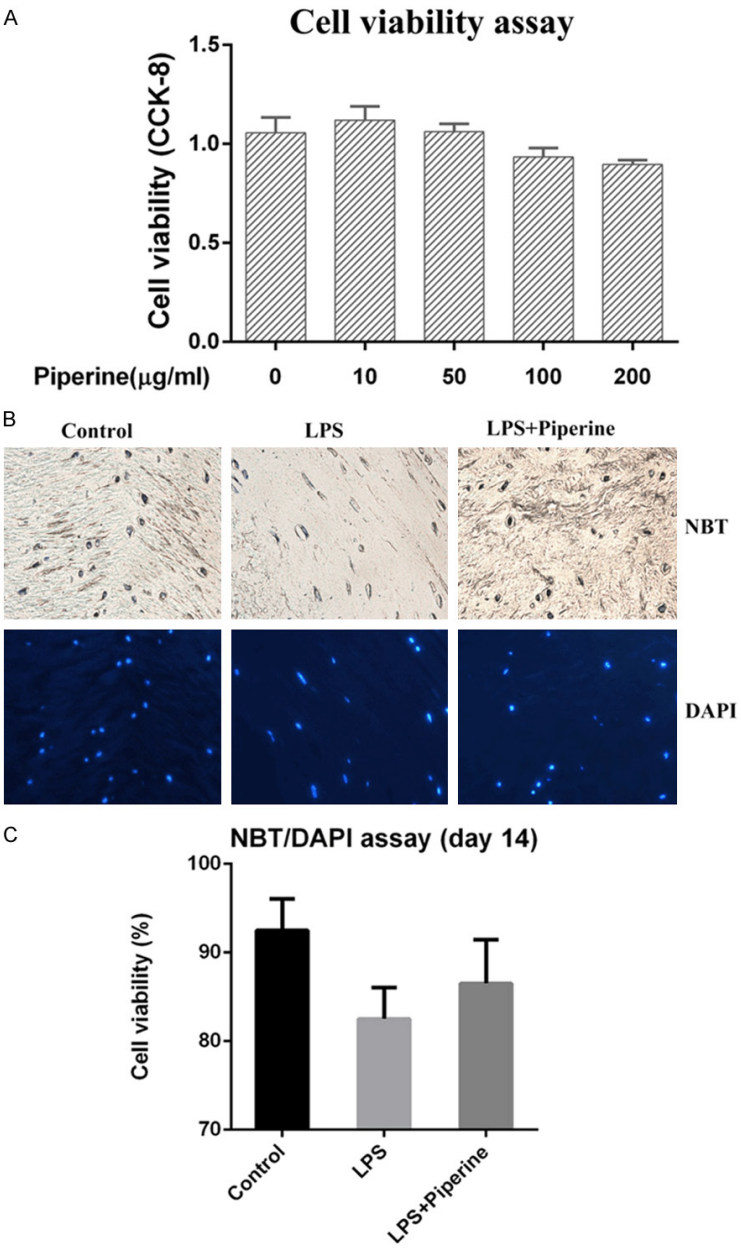

Figure 1. Cell viability assay in NP cells culture and IVD organ culture model. A. Effect of piperine on cell viability assay in cell culture model using CCK-8. Piperine did not show any cytotoxicity at concentrations of 10-200 mg/ml. B, C. Cell viability assay in organ culture model using NBT/DAPI staining. Cell viability of cultured IVD decreased gradually over time, especially in LPS group. The cell viability of all groups at 14 days was still greater than 80%. Values presented as mean ± standard deviation.

Piperine inhibits LPS-induced expression and production of inflammatory factors and catabolic proteases in NP cells culture model

Monolayers culture of NP cells was stimulated with 10 μg/ml LPS and 0, 10, 50 or 100 μg/ml piperine respectively. Followed by real-time PCR and ELISA assay for mRNA and protein level of various matrix-degrading enzymes. ELISA assay showed that 50 and 100 μg/ml piperine significantly inhibited MMP-3 and MMP-13 secretion of NP cells induced by LPS (**Figure 2B**). Piperine also markedly inhibited multiple MMPs (MMP-3, MMP-13) and ADAMTS (ADAMTS-4, ADAMTS-5) mRNA expression in a concentration-dependent manner (**Figure 2A**). As shown in **Figure 3A**, we observed a dose-dependent inhibition of expression of inflammatory factors genes (IL-1β, TNF-α, IL-6). Moreover, piperine could reverse the LPS-induced inhibition of gene expression of aggrecan and collagen-II at 50 and 100 μg/ml concentration (**Figure 3B**). Piperine also significantly inhibited iNOS gene expression and NO production induced by LPS in NP cells (**Figure 4**).

To evaluate the potential involvement of the signal transduction pathways and the mechanisms of effects of piperine on LPS induced NP cells, we measured the activation of the MAPKs and NF-κB by Western blot. The result showed that 10 mg/ml LPS could significantly activate the MAPKs and NF-κB pathways on NP cells, while piperine could inhibit the phosphorylation of IκBα and JNK in a dose dependent manner (**Figure 5**).

Cell viability assay in organ culture model

The NBT/DAPI staining showed cell viability of cultured IVD decreased gradually over time (**Figure 1B**, **1C**). However, the cell viability of all groups at 14 days was still greater than 80%, confirming the reliability of organ culture model.

Piperine inhibits LPS induced degeneration of IVD in organ culture model

At day 0, the NP showed a homogenous basophilic staining with many vacuoles of various sizes. The basophilic staining progressively lost

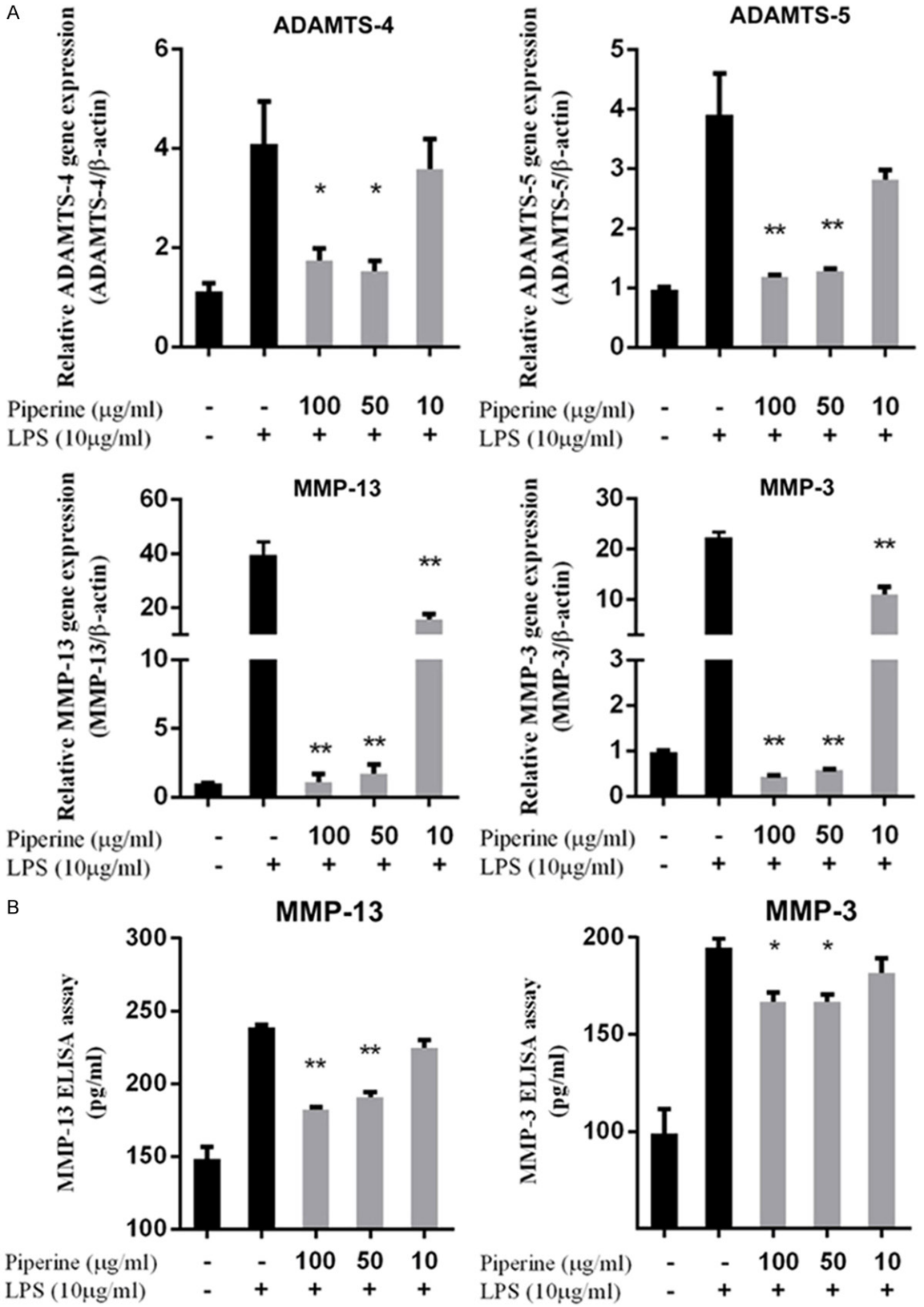

Figure 2. Real time PCR and ELISA assay of MMPs and ADAMTSs. We use real time PCR (A) and ELISA assay (B) to investigate the effect of piperine on LPS-induced gene expression and proteases secretion of NP cells. A. Piperine downregulated LPS induced production of catabolic proteases in NP cells. LPS induced gene overexpression of ADAMTS-4, ADAMTS-5, MMP-13, and MMP-3 was significantly reversed by piperine. B. ELISA assay showed inhibition

of piperine on LPS-activated MMPs secretion. Piperine significantly inhibited MMP-3 and MMP-13 secretion of NP cells induced by LPS. Values presented as mean ± standard deviation. $^{*}P < 0.05$ compared with LPS group; $^{**}P < 0.01$ compared with LPS group.

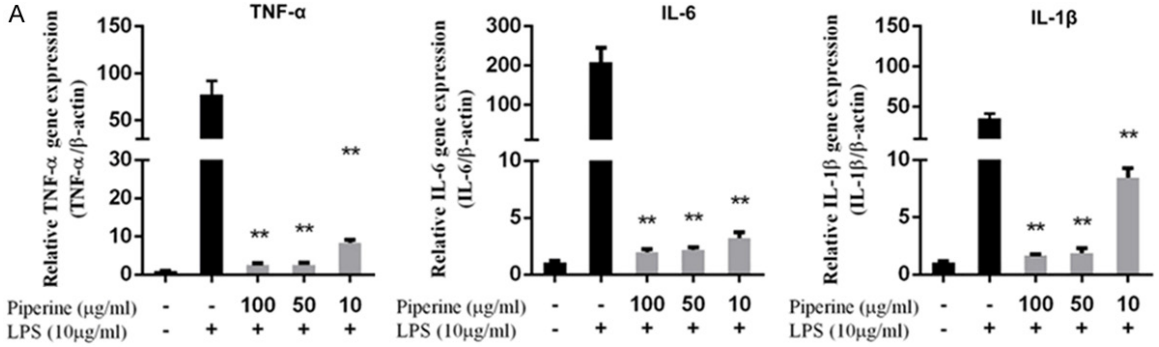

Figure 3. Piperine downregulated LPS induced inflammation and extracelluar matrix production related gene expression in NP cells. A. Piperine inhibits LPS induced IL-6, IL-1b, TNF-α mRNA expression. B. Piperine also could reverse the LPS-induced inhibition of gene expression of aggrecan and collagen-II. Values presented as mean ± standard deviation. $^{*}P < 0.05$ compared with LPS group; $^{**}P < 0.01$ compared with LPS group.

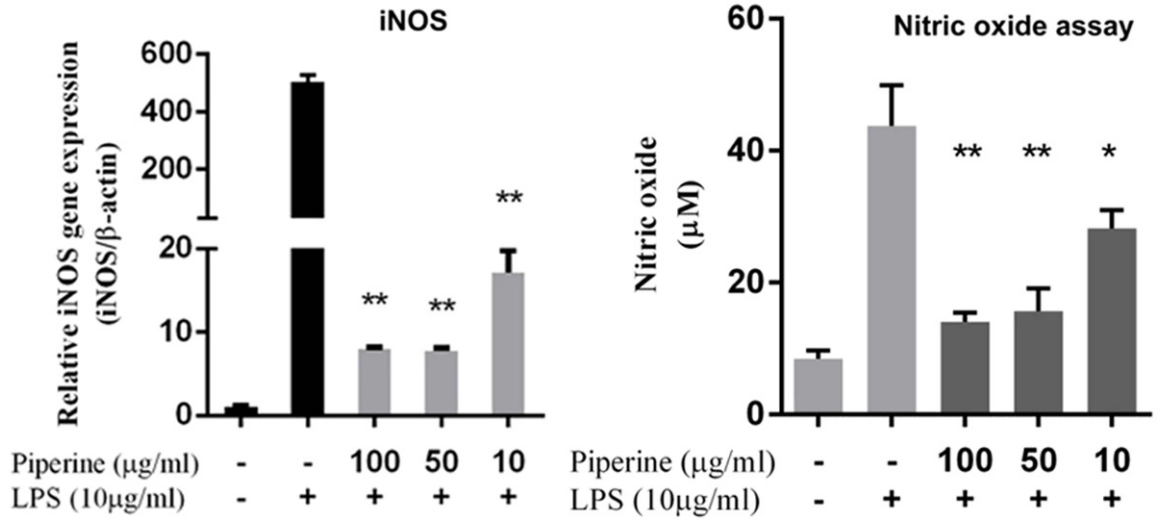

Figure 4. Effect of piperine on LPS-induced NO production and iNOS expression in NP cell. Piperine could reverse the LPS-induced NO production and iNOS expression in NP cells. Values presented as mean ± standard deviation. $^{*}P < 0.05$ compared with LPS group; $^{**}P < 0.01$ compared with LPS group.

and the vacuoles grew bigger as time in culture increased (**Figure 6**). These changes were more significant when cultured with LPS (**Figure 6**). However, when cultured with 100 μg/ml piperine and 10 μg/ml LPS, the piperine significantly attenuates LPS-induced IVD degeneration. The

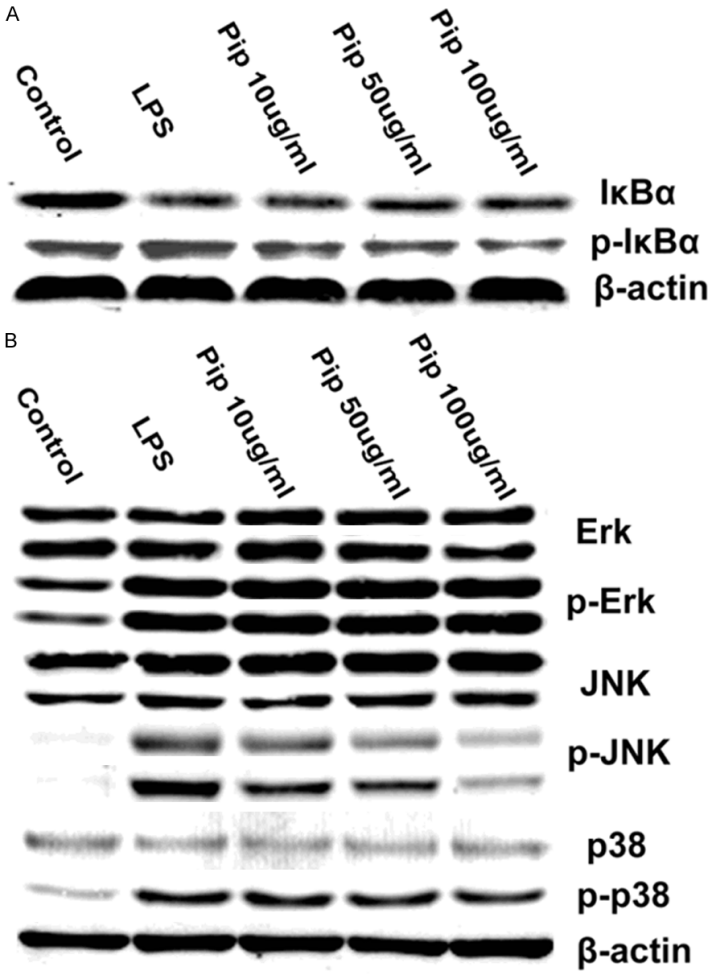

Figure 5. Effects of piperine on LPS-induced MAPK signal pathways and NF-kB activation in NP cells. To evaluate the mechanism of piperine on LPS treated NP cells, to MAPK signaling pathways (ERK, p38, and JNK) and IκBα were evaluated by western blot. A. Piperine could significantly inhibit the phosphorylation of IκBα induced by LPS. B. LPS enhanced the phosphorylation of ERK, p38 and JNK, while piperine inhibits the LPS-induced activation of JNK.

Safranin O-fast green staining also showed similar trend of cultured IVD. Proteoglycan (PG) loss accumulated with time, and LPS induced more PG loss compared to control. By contrast, piperine significantly reduced the PG loss of IVD caused by LPS (**Figure 6A**, **6B**).

The immunohistochemical staining of collagen-II was markedly enhanced in NP and annulus fibrosus (AF) area. Immunohistochemical staining showed piperine effectively reversed the decreased of collagen-II induced by LPS (**Figure 6C**). Quantification of IOD also indicated piperine group had more collagen-II staining at day 7 (**Figure 6E**, $P < 0.01$).

DMMB assay was using for quantifying PG content of NP in culture IVD. PG content was gradually decreased over time. And just like the histological results, the PG content of NP was less in LPS group compared to control and piperine group (**Figure 6D**, $P < 0.01$).

Discussion

Piperine has been widely used in various food recipes. It also has been studied for its anti-inflammatory and anti-oxidant effect [10-12]. However, there is no study focus on the potential therapeutic effect of piperine in Intervertebral disc degeneration (IDD). Our research is the first to shed light on anti-inflammatory and anti-oxidate effects of piperine in NP cells and IVD. Our findings reveal that piperine has anti-inflammatory and anti-catabolic effects on IVD both in vitro and ex vivo. It's well known that LPS markedly induce inflammatory such as IL-1β, TNF-α, IL-6. And LPS-induced overexpression of iNOS could enhance NO production which is cytotoxic to cells and is involved in the pathogenesis of various inflammatory. Many researches also have shown LPS could upregulate various cartilagede-grading enzymes, including MMP-3, MMP-13, ADAMTS-4 and AD AMTS-5 in NP cells [13, 14]. In our research, we demonstrate that piperine could reverse the inflammatory mediators, cartilage-degrading enzymes expression and NO production induced by LPS in NP cells. Further, we use organ culture model to prove that piperine could inhibit LPS induced PG loss in NP tissue. These results suggest piperine has potential therapeutic effect for IDD.

MAPKs and NF-κB pathways play an important role in the regulation of inflammatory response [15, 16]. In our research, we used western blot to study the potential mechanism of piperine in anti-inflammation. We found piperine mainly inhibit phosphorylation of JNK of MAPKs and could also inhibit NF-κB pathway by inhibit

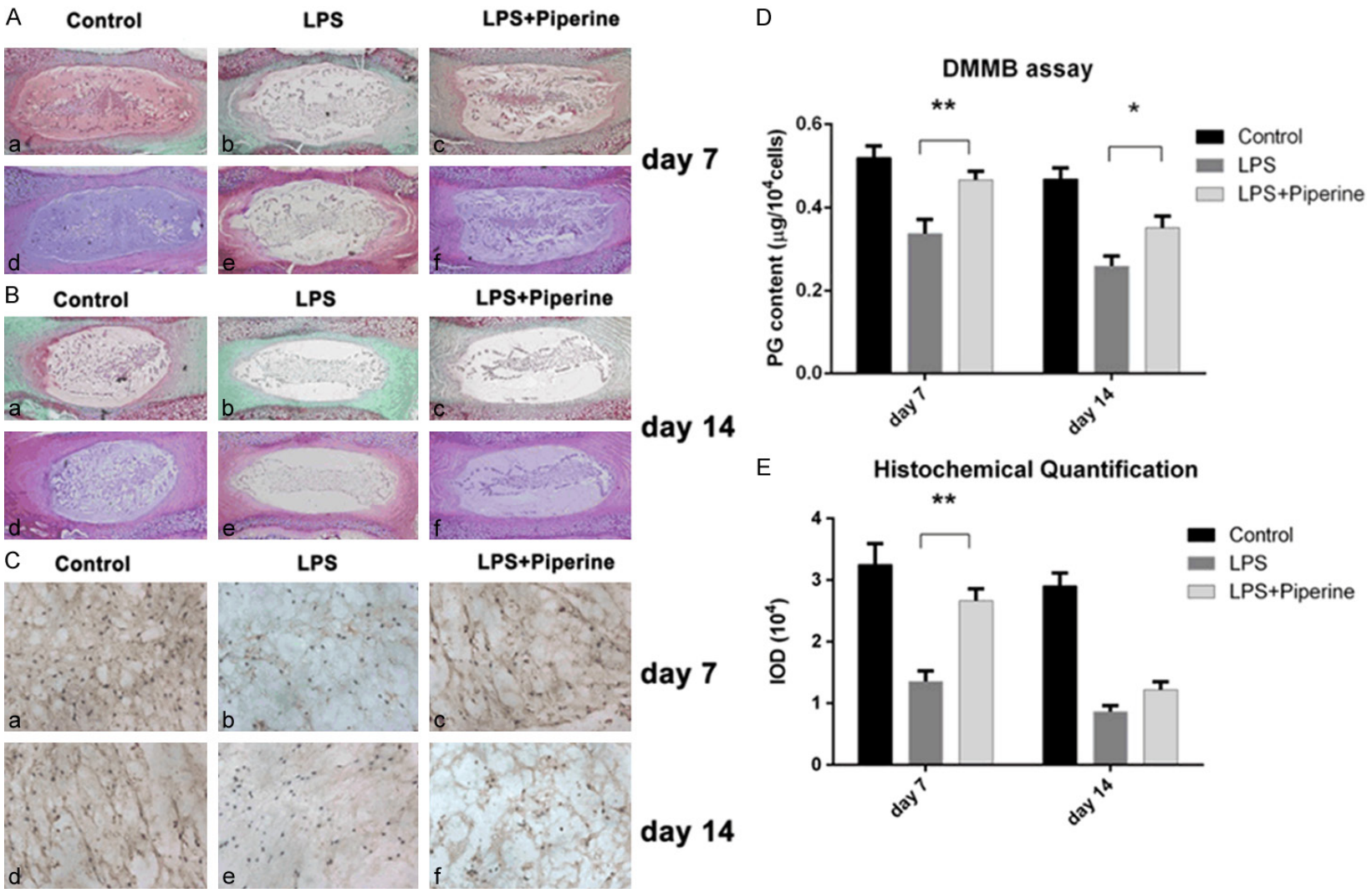

Figure 6. HE, Safranin O-fast green and type II collagen immunohistochemical staining of IVD organ (magnification: 40× for HE and Safranin O-fast green staining; 400× for collagen II immunohistochemistry). According to HE and Safranin O-fast green staining at day 7 (Aa-f) and day 14 (Ba-f), the cultured IVD organ gradually degenerative over time, especially in LPS group (Ab, Ae, Bb, Be). Collagen II immunohistochemical staining at day 7 (Ca-c) and day 14 (Cd-f) also show more staining of collagen-II in piperine group (Cc, Cf) compared with LPS group (Cb, Ce). D. DMMB assay was using for quantifying PG content of NP in culture IVD, the PG content of NP was less in LPS group compared with piperine group. E. The results of type II collagen staining were quantified in integrated optical density (IOD). Quantification of IOD also indicated piperine group had more collagen-II staining at day 7 compared with LPS group. Values presented as mean ± standard deviation. $^{*}P < 0.05$ compared with LPS group; $^{**}P < 0.01$ compared with LPS group.

phosphorylation of IκBα in LPS pretreated NP cells. In contrast, the phosphorylation of Erk and p38 of MAPKs were not significantly changed. These findings are partly different from previous researches. Two recent studies by the same authors reported piperine could inhibit NF-κB pathway in different cells after pretreated with LPS or IL-1β, but they did not report whether MAPKs pathway involved [6, 7]. Another research showed piperine could inhibit IL-1β induced p38 MAPK activation in human gastric cancer TMK-1 cells [17]. And Bang et al. [18] found that piperine only slightly inhibit phosphorylation of Erk in fibroblast-like synoviocytes stimulated by IL-1β. The MAPKs pathway does not only control inflammation, but also several other cellular functions, such as cell growth and differentiation, and it may have different functions in different cells [19, 20]. So we speculated these differences may attribute to the different cell types.

It is still unknown that whether disc degeneration can be halted or reversed. Current treatment of IDD is mainly concerned with symptom management rather than treatment of underlying causes. There is no treatment existed to completely regenerate the degenerated disc, the clinical results of spinal fusion and disc arthroplasty are still imperfect [21, 22]. Researches of the molecular mechanism of IDD update our understanding the biology underlying this process and show some potential therapeutic target.

The degeneration of IVD has been attributed to many different factors. The expression of MMPs and ADAMTSs has been extensively studied in the process of IVD degeneration. MMPs not only directly decompose extracellular matrix (ECM), but also indirectly contribute to disc degeneration via activation of latent enzymes [23]. According to many researches [24-27], MMP-1, MMP-3 and MMP-13 do not express in nondegenerated human discs, but increase expression in degenerated human discs. And MMP-1, MMP-3 protein expression correlated highly with IVD histomorphological degenerative findings [24]. Upreguation of ADAMTSs is also seen in degenerative discs [28]. Among the 20 different ADAMTSs, ADAMTS-4 and ADAMTS-5 are classified as the major aggrecanases for their high efficiency in cleaving aggrecan [29, 30]. Inhibition of MMPs and ADAMTSs has demonstrated efficacy and therapeutic potential in slowing the progression of OA [31] and IDD [32] in vitro and in vivo. Inflammation mediators also play an important role in IDD. They increase production of degradative enzymes [33], induce cell apoptosis [34], inhibit production of ECM [35] and establish a positive feedback loop to induce more serious inflammation [36]. There were some other drugs with anti-inflammatory and anti-catabolic effect in NP cells reported [37, 38], but most of them did not show ex vivo or in vivo results. Our results show piperine has anti-inflammatory and anti-catabolic effect by down-regulation of NF-κB and JNK activation in NP cells and IVD organ. Piperine may be a potential agent for treatment of IDD.

However, delivering therapeutic agents precisely to the avascular IVD without unintended damage and remain appropriate concentration for a long time is difficult. Therapeutic agents also have to overcome low acidity, limited nutrition, hypoxia and altered mechanics in degenerative IVD environment. More researches should be done in the future to solve these problems.

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Disclosure of conflict of interest

None.

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