Neuroprotective effects of curcumin alleviate lumbar intervertebral disc degeneration through regulating the expression of iNOS, COX-2, TGF- β 1/2, MMP-9 and BDNF in a rat model

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Abstract. Curcumin is a natural product with antimutagenic, antitumor, antioxidant and neuroprotective properties. However, to the best of our knowledge, curcumin has yet to be investigated for the treatment of lumbar intervertebral disc degeneration (LIDD). The aim of the present study was to investigate whether curcumin can alleviate LIDD through regulating the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, transforming growth factor (TGF)- β 1/2, matrix metalloproteinase (MMP)-9 and brain-derived neurotrophic factor (BDNF) in a rat model of LIDD. The results of the present study suggest that pretreatment with curcumin can prevent the development of LIDD in rats. It was revealed that treatment with curcumin significantly reduced interleukin (IL)-1 β and IL-6, iNOS, COX-2 and MMP-9 levels in rats with LIDD. In addition, treatment with curcumin reduced the mRNA expression levels of TGF- β 1 and TGF- β 2, whereas it increased the mRNA expression levels of BDNF in rats with LIDD. In conclusion, the present findings indicate that curcumin may exert protective effects on LIDD development, exerting its action through the regulation of iNOS, COX-2, TGF- β 1/2, MMP-9 and BDNF.

Introduction

Lumbar intervertebral disc degeneration (LIDD) in humans is a complex chronic disease mainly characterized by low back pain (1). LIDD and cervical spondylosis exhibit the highest morbidity among intervertebral disc-related pathologies. Previous studies have reported that 70-80% of adults

suffer from low back pain-related diseases, thus making LIDD an important health issue (2,3). In the United States, 1-2% of the population has been reported to suffer from obvious intervertebral disc protrusion, whereas ~200,000 new cases are reported each year (3,4). Annular disruption, herniation of the nucleus pulposus, and oppression of the nerve root and cauda equina syndrome are among the main manifestations of LIDD (3). Although an early age of onset for low back pain is not uncommon, with symptoms sometimes appearing in patients as young as 20 years old, usually symptoms appear later in life, in patients between 30 and 40 years of age (4).

Low back pain can often be misdiagnosed and believed to be a result of previous trauma; however, in most cases careful examination reveals that patients suffer chronic pain in the waist and lower extremities without traumatic etiology (5). Often, low back pain is transient, and symptoms can be relieved after rest (6). A previous study reported that low back pain may be induced by external forces, including excessive standing, stooping or physical exertion; these causes frequently interact with each other, accelerating the onset and progression of back and leg pain (7). In most cases, pain starts in the lower waist region and gradually extends to regions innervated by the sciatic nerve, as well as the buttocks. Lesions in the spinal column can also result in lower back pain that can spread to the rear side of the thigh (8).

Turmeric (*Curcuma longa*) is a well-known plant, commonly used as a food additive and natural dye. It is also used in Chinese traditional medicine (9). Curcumin (Fig. 1) is the main active compound found in turmeric, which has previously been reported to possess antioxidant properties that can prevent the oxidative damage caused by free radicals to proteins, lipids, saccharides and nucleic acids. Free radicals and oxidative stress have been implicated in inflammation and several diseases, including cardiovascular and cerebrovascular pathologies, and dermatological conditions (10,11). The present study investigated the putative protective effect of curcumin on a rat model of LIDD, which was revealed to be exerted through regulation of the expression of inducible nitric oxide synthase (iNOS), cyclooxygenase (COX)-2, transforming growth factor (TGF)- β 1/2, matrix metalloproteinase (MMP)-9 and brain-derived neurotrophic factor (BDNF).

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Materials and methods

Animals and LIDD model. Sprague-Dawley rats (250-280 g; age, 8-10 weeks; male) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) and housed under temperature-controlled ($22\pm 2^{\circ}\text{C}$) conditions with a 12/12 h light/dark cycle and free access to food/water. Rats were randomized into 3 groups: Control group ($n=10$) containing healthy rats, LIDD model group ($n=10$) containing rats to which LIDD was induced surgically, and curcumin-treated group ($n=10$), which consisted of curcumin-treated LIDD rats. Rats in the control and LIDD model groups were treated with saline. To induce LIDD, rats were anesthetized via inhalation of 1.5-3% isoflurane (Shanghai Jingke Scientific Instrument Co., Ltd., Shanghai, China) with an oxygen carrier. Subsequently, they were placed in a supine position on a heated pad, offering anterior access to the lumbar spine. A gas tight microsyringe (Hamilton Company, Reno, NV, USA) attached to a custom 33-gauge needle was inserted through the anterior of the appropriate discs to a controlled depth of 2.5 mm. In the control group, rats were anesthetized without the induction of LIDD. All experiments were approved by the Animal Ethics Committee of The First Affiliated Hospital of Chinese PLA General Hospital (Beijing, China). Subsequently, rats in the curcumin-treated group were treated with curcumin (200 mg/kg/day; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 5 weeks.

Histological evaluation. Rats were anesthetized using 35 mg/kg pentobarbital and then rats were sacrificed using decollation. The intervertebral discs and adjacent vertebral endplates between lumbar vertebrae L4 and L5 were acquired. The tissues were fixed with 4% paraformaldehyde for 24-48 h and decalcified with 20% EDTA for 21 days at room temperature. Subsequently, tissue samples were dehydrated through serial ethanol dilutions (95-100%) at room temperature and embedded in olefin. Embedded samples were sliced into 7- μm sections, and tissue morphology was examined using an image auto-analysis system (CMIAS-99B; Okolab s.r.l., Pozzuoli, Italy).

Collagen content and interleukin (IL) levels. Tissue samples from intervertebral discs were acquired and processed to measure type II collagen content (cat. no. E-EL-R0234c), and IL-1 β (cat. no. E-EL-R0012c) and IL-6 (cat. no. E-EL-R0015c) levels, using a microplate reader, according to the manufacturer's protocol (Wuhan Elabscience Biotechnology Co., Ltd., Wuhan, China).

Western blot analysis. Intervertebral discs were acquired, weighed and processed into a fine powder. Tissue samples were homogenized using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and total protein concentration was determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology, Haimen, China) according to the manufacturer's protocol. Equal amounts (80 μg) of extracted protein samples were separated by 10-12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes

Figure 1. Chemical structure of curcumin.

(EMD Millipore, Billerica, MA, USA). The membranes were blocked for 1 h with TBS containing Tween (0.01%) with 5% fat-free milk at room temperature, followed by an overnight incubation at 4°C with the following primary antibodies: Rabbit anti-rat iNOS antibody (cat. no. sc-649; 1:3,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), rabbit anti-rat MMP-9 antibody (cat. no. sc-10737; 1:2,000; Santa Cruz Biotechnology, Inc.) and rabbit anti-rat β -actin antibody (cat. no. sc-7210; 1:5,000; Santa Cruz Biotechnology, Inc.) which was used as a loading control. Subsequently, membranes were probed with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (cat. no. 14708; 1:5,000; Cell Signaling Technology, Inc., Danvers, MA, USA) for 1.5 h at room temperature. The bands were visualized with an Enhanced Chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and quantified using Quantity One software (version 3.0; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from intervertebral disc samples using TRIzol (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA (1 μg) was reverse transcribed into cDNA using Advantage RT-for-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). qPCR analyses were performed on cDNA (1 μl) with the Rotor-Gene Q real-time DNA amplification system (Qiagen China Co., Ltd., Shanghai, China) using SYBR-Green (Bio-Rad Laboratories, Inc.) according to the manufacturer's protocol. PCR reactions were performed as follows: An initial predenaturation step for 1 min at 95°C , followed by 40 cycles of amplification at 95°C for 15 sec, at 60°C for 30 sec and 72°C for 30 sec, and 4°C for 10 min. Sequences of the primers used for RT-qPCR are presented in Table I. The experimental results were expressed using $2^{-\Delta\Delta\text{Ct}}$ (12).

Statistical analysis. Statistical analysis was performed using SPSS software version 16.0 (SPSS, Inc., Chicago, IL, USA). Data were expressed as the mean $\pm$ standard deviation and all experiments were repeated three times. Statistical significance was assessed using one-way analysis of variance, followed by Dunnett's test for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Curcumin improves the histological profile of rats with LIDD. In order to evaluate the putative protective effects of curcumin on LIDD, intervertebral disc samples were acquired from sham-operated rats and from rats with surgically-induced LIDD. Histological evaluation of the samples

Table I. Sequences of primers used in reverse transcription-quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer
COX-2	5'-GGAGCATCCTGAGTGGGATGA-3'	5'-AAGCAGGTCTGGGTCTGAACCTTG-3'
TGF- β 1	5'-ATTCCTGGCGTTACCTTGG-3'	5'-AGCCCTGTATTCCGTCTCCT-3'
TGF- β 2	5'-GCAGAGTTCAGGGTCTTTTCG-3'	5'-GCTGGGTGGAGATGTTAGG-3'
BDNF	5'-TCTCCCTGCCTCATCCCT-3'	5'-CAGAGTCTTCCTTTGCCTAC-3'
GAPDH	5'-GAGTCAACGGATTTGGTCGT-3'	5'-TTGATTTTGGAGGGATCTCG-3'

COX-2, cyclooxygenase-2; TGF, tumor growth factor; BDNF, brain-derived neurotrophic factor.

Figure 2. Effect of curcumin on the histological profile of rats with surgically-induced LIDD (magnification, x10). Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. LIDD, lumbar intervertebral disc degeneration.

revealed extensive intervertebral tissue injury in rats with surgically-induced LIDD, which was absent in sham-operated healthy rats. Treatment with curcumin appeared to prevent tissue injury in rats with surgically-induced LIDD (Fig. 2).

Curcumin reduces type II collagen content in rats with LIDD. The effects of curcumin on the type II collagen content of intervertebral disc samples were evaluated in rats with LIDD. The results revealed that in rats with surgically-induced LIDD, type II collagen content was significantly increased compared with in sham-operated normal rats. Treatment with curcumin was demonstrated to significantly reduce type II collagen content in rats with LIDD, as compared with in untreated LIDD rats (Fig. 3).

Curcumin reduces IL-1 β and IL-6 levels in rats with LIDD. IL-1 β and IL-6 levels were assessed in intervertebral disc samples, and it was revealed that LIDD induced a significant increase in IL-1 β and IL-6 levels compared with in the sham group. Treatment with curcumin significantly suppressed IL-1 β and IL-6 levels in rats with LIDD compared with in the untreated group (Fig. 4).

Curcumin reduces the protein expression of iNOS and MMP-9 in rats with LIDD. The expression levels of iNOS and MMP-9 were assessed using western blot analysis, and it was revealed that LIDD induced a significant increase in protein levels compared with in the sham group. Treatment with curcumin significantly reduced iNOS and MMP-9 levels in intervertebral disc samples (Figs. 5 and 6).

Curcumin reduces mRNA levels of COX-2 and TGF- β 1/2 in rats with LIDD. In order to evaluate the mRNA expression

Figure 3. Curcumin reduced type II collagen content in rats with LIDD. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. **P<0.01 compared with the control group; ##P<0.01 compared with the LIDD group. LIDD, lumbar intervertebral disc degeneration.

levels of COX-2 and TGF- β 1/2, RT-qPCR was employed. Results demonstrated a significant upregulation in COX-2 and TGF- β 1/2 mRNA expression levels in rats with LIDD compared with in the control group. Treatment with curcumin appeared to significantly reduce the mRNA expression levels of COX-2 and TGF- β 1/2 in intervertebral disc samples (Figs. 7 and 8).

Curcumin increases the mRNA expression levels of BDNF in rats with LIDD. The present study also examined the effect of curcumin on the mRNA expression levels of BDNF in rats with surgically-induced LIDD. In rats with LIDD, BDNF mRNA appeared significantly downregulated compared with in the control group. Treatment with curcumin significantly increased BDNF mRNA expression levels in intervertebral disc samples compared with in untreated rats (Fig. 9).

Figure 4. Effect of curcumin on IL-1 β and IL-6 levels. Treatment with curcumin reduced (A) IL-1 β and (B) IL-6 levels in rats with LIDD when compared with the untreated group. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the LIDD group. IL, interleukin; LIDD, lumbar intervertebral disc degeneration.

Figure 5. Effect of curcumin on iNOS protein levels. (A) Western blot analysis of iNOS protein expression in intervertebral disc samples. (B) Statistical analysis revealed that treatment with curcumin significantly reduced iNOS expression in rats with LIDD when compared with the untreated group. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the LIDD group. iNOS, inducible nitric oxide synthase; LIDD, lumbar intervertebral disc degeneration.

Figure 6. Effect of curcumin on MMP-9 protein levels. (A) Western blot analysis of MMP-9 protein expression in intervertebral disc samples. (B) Statistical analysis revealed that treatment with curcumin significantly reduced MMP-9 expression in rats with LIDD when compared with the untreated group. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the LIDD group. MMP-9, matrix metalloproteinase-9; LIDD, lumbar intervertebral disc degeneration.

Figure 7. Effect of curcumin on COX-2 mRNA levels. COX-2 mRNA appeared significantly upregulated in intervertebral disc samples of rats with LIDD when compared with the control group. Treatment with curcumin significantly downregulated COX-2 mRNA levels. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. ** $P < 0.01$ compared with the control group; ## $P < 0.01$ compared with the LIDD group. COX-2, cyclooxygenase-2; LIDD, lumbar intervertebral disc degeneration.

Discussion

LIDD is characterized by degeneration, necrosis and apoptosis of the nucleus pulposus, dehydration and degradation of the extracellular matrix, as well as changes in collagen content. These characteristics result in the gradual disappearance of the nucleus pulposus and its boundaries with the fibrous rings, and in the progressive development of fibrosis (5). As a consequence, intervertebral disc tissues gradually lose their normal structure and functionality (13). In the present study, it was revealed that curcumin significantly improved tissue injury, reduced type II collagen content and downregulated IL-1 β and IL-6 levels in intervertebral tissue samples from rats with LIDD.

As intervertebral disc degeneration progresses, mechanical pressure can be exerted on the nerve roots of the spinal cord.

Figure 8. Effect of curcumin on (A) TGF-β1 and (B) TGF-β2 mRNA levels. TGF-β1/2 mRNA appeared significantly upregulated in intervertebral disc samples of rats with LIDD compared with the control group. Treatment with curcumin significantly downregulated TGF-β1/2 mRNA levels. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. **P<0.01 compared with the control group; ##P<0.01 compared with the LIDD group. TGF, transforming growth factor; LIDD, lumbar intervertebral disc degeneration.

Figure 9. Effect of curcumin on BDNF mRNA expression levels. BDNF mRNA appeared significantly downregulated in intervertebral disc samples of rats with LIDD compared with the control group. Treatment with curcumin significantly increased BDNF mRNA expression levels. Sham, control group; LIDD, LIDD model group; curcumin, LIDD model + 200 mg/kg/day curcumin group. **P<0.01 compared with the control group; ##P<0.01 compared with the LIDD group. BDNF, brain-derived neurotrophic factor; LIDD, lumbar intervertebral disc degeneration.

This can result in spinal cord anoxia and ischemia, which block energy production. Under ischemic conditions, the activity of the iNOS enzyme is potentiated, whereas the substrate of the enzyme, required for NO production, is depleted (14). Under these circumstances, iNOS promotes the production of reactive oxygen species, such as superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), which cause tissue damage and cell necrosis (15). These processes can have deleterious effects in spinal cord neurons. When motor neurons become necrotic, NOS activity is reduced and NO levels drop (15,16). The results of the present study revealed that curcumin can significantly reduce iNOS levels in intervertebral disc samples from rats with LIDD. The present results are in accordance with a previous study, which reported the anti-inflammatory capabilities of curcumin, exerted through the downregulation of iNOS (9).

COX-2 is a multi-functional enzyme responsible for catalyzing the conversion of arachidonic acid into prostaglandins (PG), which is implicated in several inflammatory processes (17). It exerts diverse functions associated with cellular proliferation and apoptosis, and its expression has been revealed to be upregulated in certain tumors (17). A previous study reported that the PG content in degenerating intervertebral discs is markedly increased (18). PG can inhibit the synthesis of proteins and polysaccharides in intervertebral disc cells, whereas this depletion in the nucleus pulposus is one of the main causes of LIDD (18). The results of the present

study demonstrated that curcumin significantly inhibited COX-2 mRNA expression in rats with LIDD.

As a key enzyme for metabolic processes of the extracellular matrix under physiological and pathological states, MMP-9 has been reported to directly degrade collagen and polysaccharides, resulting in depolymerization of proteoglycan, whereas it can also precipitate the activation of other MMPs (19). The expression of MMP-9 has been demonstrated to be upregulated in vertebrae of degenerative processes (20). The present study revealed that curcumin can significantly suppress MMP-9 expression in rats with LIDD. In addition, Li *et al* reported that curcumin inhibited atherosclerosis in coronary heart disease, through its inhibitory effects on MMP-9 and tumor necrosis factor-α (21).

TGF-β1 is a multi-functional regulatory factor implicated in various processes, including cellular growth and differentiation. It can have conflicting actions, inducing apoptosis in some circumstances and triggering anti-apoptotic mechanisms in others (22). The present study demonstrated that curcumin can significantly downregulate TGF-β1 and TGF-β2 mRNA expression levels in rats with LIDD. The present findings are in accordance with a previous study reporting that curcumin can improve neural functionality through suppressing the expression of tumor necrosis factor-α, IL-1β, TGF-β1 and TGF-β2 following spinal cord injury (10). BDNF can sustain and facilitate the growth, development and regeneration of sensory, cholinergic, dopaminergic and GABAergic neurons (23). It has also been reported to prevent motor neuron death after sciatic nerve transection, and to rescue nucleus neurons following spinal cord incision (24). The results of the present study revealed that curcumin can significantly upregulate BDNF mRNA expression levels in intervertebral disc tissue of rats with LIDD. Fanaei *et al* (25) reported that the effects of curcumin may be mediated through enhancing serum BDNF levels in women with premenstrual syndrome. The results of the present study demonstrated that curcumin upregulated BDNF expression in LIDD.

In conclusion, the present study demonstrated that curcumin can significantly improve tissue injury, reduce type II collagen content, and suppress IL-1β and IL-6 levels in a rat model of LIDD. Curcumin appeared to exert regulatory effects on iNOS, COX-2, TGF-β1/2, MMP-9 and BDNF in intervertebral disc tissue. These results suggest that curcumin may have a role as a novel therapeutic agent for the treatment of LIDD.

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