

Protective effect of cannabidiol on hydrogen peroxide-induced apoptosis, inflammation and oxidative stress in nucleus pulposus cells

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Abstract. Cannabidiol, a major component of marijuana, protects nerves, and exerts antispasmodic, anti-inflammatory and anti-anxiety effects. In the current study, the protective effect of cannabidiol was observed to prevent hydrogen peroxide (H₂O₂)-induced apoptosis, inflammation and oxidative stress in nucleus pulposus cells. Nucleus pulposus cells were isolated from rats and cultured *in vitro*, and H₂O₂ was used to construct the nucleus pulposus cell model. Cell viability of the nucleus pulposus cells was assessed using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The ratio of apoptotic cells, and caspase-3 or cyclooxygenase-2 (COX-2) mRNA expression was analyzed by annexin V-fluorescein isothiocyanate/propidium-iodide staining and reverse transcription-quantitative polymerase chain reaction, respectively. The quantities of interleukin (IL)-1 β and interleukin-6 were measured using a series of assay kits. B-cell lymphoma 2 (Bcl-2) and inducible nitric oxide synthase (iNOS) protein expression levels were analyzed using western blotting. The present study identified that cannabidiol enhanced cell viability and reduced apoptosis in H₂O₂-treated nucleus pulposus cells *in vitro* using a lumbar disc herniation (LDH) model. In addition, cannabidiol reduced caspase-3 gene expression and augmented the Bcl-2 protein expression levels in the nucleus pulposus cells following H₂O₂ exposure. Pre-treatment with cannabidiol suppressed

the promotion of COX-2, iNOS, IL-1 β and IL-6 expression in the nucleus pulposus cells following H₂O₂ exposure. Taken together, these results suggest that cannabidiol potentially exerts its protective effect on LDH via the suppression of anti-apoptosis, anti-inflammation and anti-oxidative activities in nucleus pulposus cells.

Introduction

Lumbar disc herniation (LDH) that causes back and leg pain, numbness, muscle loss and sphincter disturbances, amongst other symptoms, is a common orthopedic disease (1). Waist LDH is divided into three types: i) Nuclear protrusion does not damage the fiber ring; ii) subligamentous extrusion of nucleus pulposus does not damage posterior longitudinal ligaments; iii) sequestration is a posterior longitudinal ligament rupture, in which nucleus pulposus is dislocated to the spinal canal (2). It is currently accepted that intervertebral disc degeneration or damage and inflammation of the surrounding soft tissue are the source of lower back pain (3). Oppression of nerves in locations away from the intervertebral disc causes nerve damage and degeneration of outstanding intervertebral discs. After the intervertebral disc ruptures, dislocation of nucleus pulposus stimulates nerve to produce chemical nerve root inflammation, which leads to sciatica (4). Therefore, the aim of conservative treatment should be regeneration of degenerated intervertebral discs and neural protection, with a focus on disc nucleus pulposus tissue reuptake (5).

Intervertebral disc degeneration, caused by a series of spinal degenerative diseases and their secondary pathological effects, including vascular injury and oxidative stress is common; however, the cause and specific mechanism remains unclear. The excessive apoptosis of intervertebral disc cells directly decreases the number of intervertebral disc cells, which results in intervertebral disc degeneration. Nucleus pulposus cells (NPCs) are important, as they maintain the normal intervertebral disc environment and repair degenerated intervertebral discs. Therefore, excessive apoptosis of NPCs is the direct cause of intervertebral disc degeneration (6). The

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apoptosis process is triggered through mitochondrial signaling pathways, which are dependent on hydrogen peroxide (H_2O_2)-mediated oxidative stress (7).

Cannabidiol was isolated from marijuana during the 1940s, and *in vivo* experiments demonstrated that cannabidiol stimulated cannabinoid receptor type I, which affected mental activity, whilst also exerting anticonvulsant (8), sedative hypnotic (9), anti-anxiety (10), antipsychotic (11), anti-inflammatory (12) and nerve protective (13) effects. Preclinical and clinical studies have indicated that the pharmacokinetic properties of cannabidiol are good; following injection it quickly passes through the blood-brain barrier, exerting a marked protective effect on the cranial nerve (14). Furthermore, cannabidiol exerts its neuroprotective effects through multiple channels (15). The aim of the current study was to evaluate the protective effect of cannabidiol by investigating whether its administration prevented H_2O_2 -induced apoptosis, inflammation and oxidative stress in NPCs.

Materials and methods

Reagents. Gibco Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) was obtained from Thermo Fisher Scientific, Inc. (Waltham, MA, USA) and HyClone fetal bovine serum (FBS) was obtained from GE Healthcare Life Sciences (Logan, UT, USA). Cannabidiol (>98% purity) was supplied by Sigma-Aldrich (St. Louis, MO, USA) and its chemical structure is demonstrated in Fig. 1. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was obtained from Beyotime Institute of Biotechnology (Haimen, China) and the Invitrogen TRIzol reagent was obtained from Thermo Fisher Scientific, Inc. A reverse transcription-polymerase chain reaction (RT-PCR) kit was obtained from Takara Biotechnology Co., Ltd. (Dalian, China) and the Applied Biosystems ABI Prism 7900HT Real-Time PCR system was obtained from Thermo Fisher Scientific, Inc. Invitrogen interleukin (IL)-1 β and IL-6 assay kits were obtained from Thermo Fisher Scientific, Inc.

Animals. A total of 40 healthy male Sprague-Dawley rats (weight, 362 ± 35 g, 10 rats per treatment group) were obtained from the Center of Experimental Animals of Xi'an Jiaotong University (Xi'an, China) and selected as NPC donors. All rats were housed with their respective groups at a temperature of $22 \pm 1^\circ\text{C}$ under a 12-h light/dark cycle with food and water available *ad libitum*. The study protocol was approved by the Animal Use and Care Committee for Research and Education of Xi'an Jiaotong University.

Cell isolation and culture. Rat NPCs were isolated according to a previously described explant culture method (16). Briefly, rats were anesthetized with 10% chloral hydrate (i.p.; 4 ml/kg body weight). The lumbar intervertebral discs were resected from the spinal column. The gel-like nucleus pulposus tissue was separated from the annulus fibrosus under aseptic conditions. The gelatinous nucleus pulposus tissue samples were obtained from the rats and sliced into small sections. Under aseptic conditions, skin and tissue were separated at the thigh, and then the annulus fibrosus was incised to separate the gel-like nucleus pulposus tissue. The tissues were

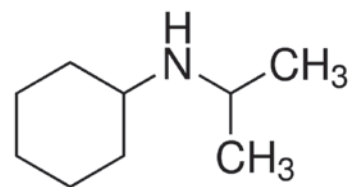


Figure 1. Chemical structure of cannabidiol.

digested with 0.1% type-2 collagenase (Sigma-Aldrich) in DMEM/F-12 at 2,000 \times g. The cells were cultivated with 0.1% type-2 collagenase in DMEM/F-12 in an incubator at 37°C under an atmosphere of 5% carbon dioxide for 4 h. Following cultivation, the suspension was filtered through mesh (pore size, a $70\text{-}\mu\text{m}$; Thermo Fisher Scientific, Inc.). The filtered cells were washed with DMEM/F-12 3 times and then placed in 25-cm^2 culture flasks (Thermo Fisher Scientific, Inc.). Finally, the NPCs were incubated with DMEM/F-12, 10% FBS, 100 U/ml streptomycin, 100 U/ml penicillin at 37°C under an atmosphere of 5% carbon dioxide. The NPCs were chondrocyte-like cells, identified by immunohistostaining of type II collagen and aggrecan.

Establishment of NPC apoptosis models. Briefly, NPCs were plated onto a 6-well plate at a density of 1×10^6 cells/well. NPCs were exposed to $200\text{ }\mu\text{M}$ H_2O_2 for 24 h to induce damage.

Cell viability assay. NPCs were plated in a 96-well plate at a density of 2×10^4 cells/well. After 24 h the medium was replaced with phosphate-buffered saline (PBS; Sangon Biotech Co., Ltd., Shanghai, China; control group), DMEM/F-12 containing H_2O_2 (model group) or cannabidiol (2.5 or $5\text{ }\mu\text{M}$ group) (17). For quantitative analysis of cell viability, 0.5% MTT solution ($20\text{ }\mu\text{l}$; Beyotime Institute of Biotechnology) with PBS was added to each well and incubated for 4 h at 37°C under an atmosphere of 5% carbon dioxide. The culture medium was replaced, and $200\text{ }\mu\text{l}$ dimethyl sulfoxide (Sangon Biotech Co., Ltd.) was added to each well and agitated for 20 min at room temperature. The optical density as measured at 450 nm using a microplate reader (ELx800; BioTek Instruments, Winooski, VT, USA).

Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) staining. NPCs (1×10^6 cells/well) were plated onto a 6-well plate. The medium was replaced after 24 h with PBS (control group), DMEM/F-12 containing H_2O_2 (model group) or cannabidiol (2.5 or $5\text{ }\mu\text{M}$ group) (17). Annexin V-FITC ($5\text{ }\mu\text{l}$) and PI ($5\text{ }\mu\text{l}$) were added and incubated for 10 min in the dark at room temperature, both were obtained from BD Biosciences (Franklin Lakes, NJ, USA). Cell apoptosis was examined using a BD FACSCanto II flow cytometer (BD Biosciences). Cell Quest Pro software was used for this data (version 4.01; BD Biosciences).

RT-quantitative PCR of caspase-3 and COX-2 mRNA expression levels. NPCs were plated onto 6-well plates at a density of 1×10^6 cells/well. After 24 h, the medium was replaced with PBS (control group), DMEM/F-12 containing H_2O_2 (model group) or cannabidiol (2.5 or $5\text{ }\mu\text{M}$ group) (17). Total RNA was extracted from NPCs using TRIzol reagent according to

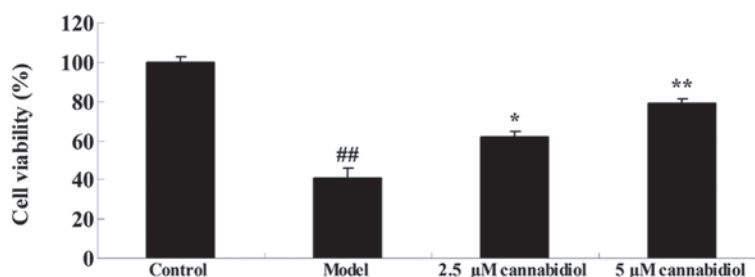


Figure 2. Effect of cannabidiol on cell viability in H_2O_2 -treated nucleus pulposus cells (model). ## $P<0.01$ vs. control group, * $P<0.05$ vs. model group and ** $P<0.01$ vs. model group.

Table I. Primers used for reverse transcription-quantitative polymerase chain reaction analysis of gene expression.

Gene	Primer sequence
Caspase-3	Forward 5'-GGCCTGCTTTTACCTCAGA3' Reverse 5'-CGTTTCCGCACAGGCTGCTT-3'
COX-2	Forward 5'-GTGTATCCCCCAGTCAAA-3' Reverse 5'-ACACTCTGTTGTGCTCCCGAA-3'
GAPDH	Forward 5'-TGTCTCCTGCGACTTCAACAG3' Reverse 5'-GAGGCCATGTAGGCCATGAG-3'

COX-2, cyclooxygenase 2.

the manufacturer's protocol and cDNA was transcribed from RNA using the RT-PCR kit according to the manufacturer's instructions. The qPCR system was performed using an ABI Prism 7900HT Real-Time PCR system according to the manufacturer's instructions. The SYBR Green I fluorescent dye (Thermo Fisher Scientific, Inc.) method was conducted to quantify the cDNA. Table I demonstrates the primer sequences (Sangon Biotech Co., Ltd.).

Measurement of IL-1 β and IL-6. NPCs were plated onto 96-well plates at a density of 1×10^4 cells/well. The medium was replaced 24 h later with PBS (control group), DMEM/F-12 containing H_2O_2 (model group) or cannabidiol (2.5 or 5 μ M group) (17). An Invitrogen ELISA kit (Thermo Fisher Scientific, Inc.) was used to measure the quantities of IL-1 β and IL-6 according to the manufacturer's instructions.

Western blot analysis of Bcl-2 and inducible nitric oxide synthase (iNOS). NPCs were plated onto 6-well plates at a density of 1×10^6 cells/well. After 24 h, the medium was replaced PBS (control group), DMEM/F-12 containing H_2O_2 (model group) or cannabidiol (2.5 or 5 μ M group) (17). The NPCs were prepared in RIPA lysis buffer (Beyotime Institute of Biotechnology) for 30 min on ice. The cytochylema was subsequently centrifuged at $12,000 \times g$ for 10 min at $4^\circ C$ and the supernate was collected. The cytochylema protein concentration was determined using a commercial bicinchoninic acid protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Equal protein (50 ng) was resolved on 12% SDS-PAGE gel (Thermo Fisher Scientific, Inc.) and transferred to

nitrocellulose membranes (EMD Millipore, Billerica, MA, USA). After the membranes were washed 3 times for 5 min, the membranes were incubated with polyclonal rabbit anti-Bcl-2 (cat. no. sc-492; 1:1,500; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) polyclonal rabbit anti-iNOS (cat. no. sc-650; 1:1,500; Santa Cruz Biotechnology, Inc.) and polyclonal rabbit anti- β -actin (cat. no. D110007; 1:500; Sangon Biotech Co., Ltd.) overnight at $4^\circ C$. The membranes were subsequently incubated with corresponding monoclonal mouse anti-rabbit horseradish peroxidase-conjugated secondary antibody (cat. no. D110059-0100; 1:5,000; Sangon Biotech Co., Ltd.) for 2 h at room temperature.

Statistical analysis. All statistical analysis was conducted using SPSS 18.0 software (SPSS, Inc., Chicago, IL, USA) and data are presented as means \pm standard deviation. Values were evaluated by one way analysis of variance, followed by Duncan's multiple range tests and $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of cannabidiol on cell viability in H_2O_2 -treated NPCs. NPC viability was analyzed by MTT assay. The results indicated reduced cell viability in NPCs treated with 200 μ M H_2O_2 when compared with the control group ($P<0.01$; Fig. 2). A significant increase was observed in the 2.5 and 5 μ M cannabidiol-treated groups, compared with cell viability of NPC following 200 μ M H_2O_2 treatment (Fig. 2). These results indicate that cannabidiol may exert protective effects on NPCs.

Effect of cannabidiol on apoptosis in H_2O_2 -treated NPCs. To analyze the effect of cannabidiol on apoptosis in H_2O_2 -treated NPCs, the rate of apoptosis was quantified by flow cytometry. As presented in Fig. 3, a significant increase in the rate of apoptosis was observed after 24 h of exposure to 200 μ M H_2O_2 . The increased apoptotic rate was suppressed by 2.5 or 5 μ M cannabidiol treatment in the NPCs exposed to 200 μ M H_2O_2 .

Effect of cannabidiol on caspase-3 gene expression in H_2O_2 -treated NPCs. To further investigate the effect of cannabidiol on apoptosis in H_2O_2 -treated NPCs, caspase-3 gene expression was quantified using qPCR. As presented in Fig. 4, H_2O_2 exposure (200 μ M) increased caspase-3 gene expression of the NPCs when compared with that of the control group.

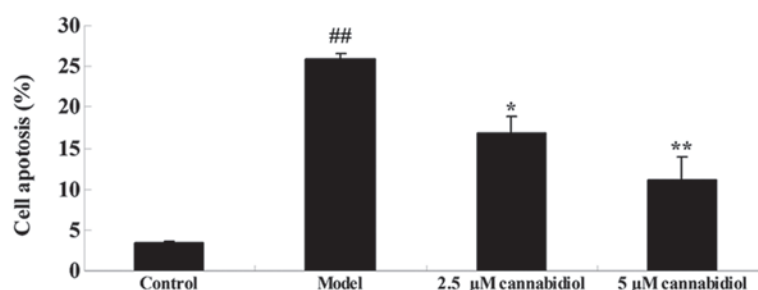


Figure 3. Effect of cannabidiol on apoptosis in H_2O_2 -treated nucleus pulposus cells (model). ## P <0.01 vs. control group, * P <0.05 vs. model group and ** P <0.01 vs. model group.

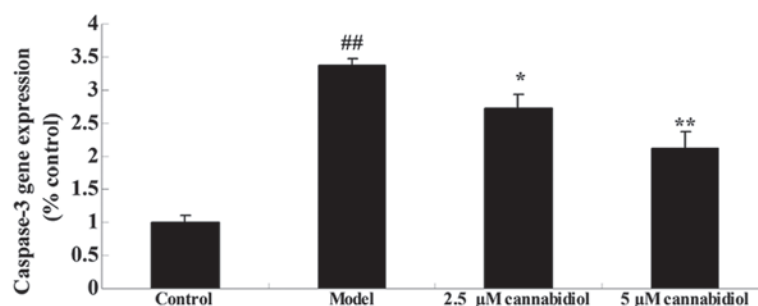


Figure 4. Effect of cannabidiol on caspase-3 gene expression in H_2O_2 -treated nucleus pulposus cells (model). ## P <0.01 vs. control group, * P <0.05 vs. model group and ** P <0.01 vs. control group.

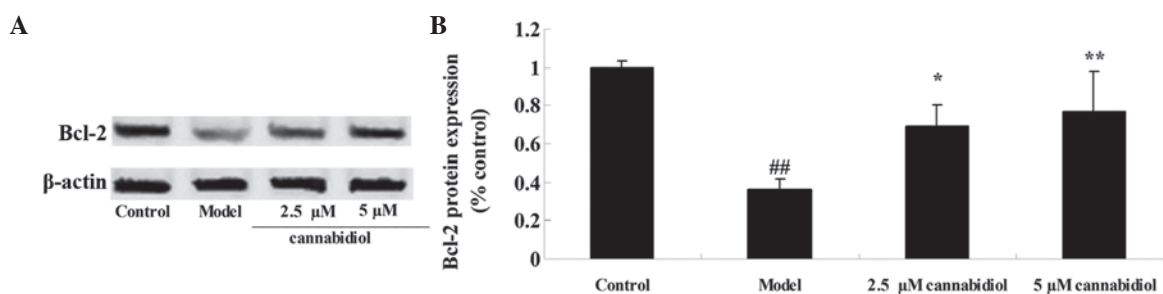


Figure 5. Effect of cannabidiol on the expression levels of Bcl-2 in H_2O_2 -treated NP cells (model). (A) Western blot analysis demonstrating the effect of cannabidiol on the Bcl-2 protein expression levels. (B) Statistical analysis of Bcl-2 protein expression level in H_2O_2 -treated NP cells. ## P <0.01 vs. control group, * P <0.05 vs. model group and ** P <0.01 vs. model group. Bcl-2, B-cell lymphoma 2; NP, nucleus pulposus.

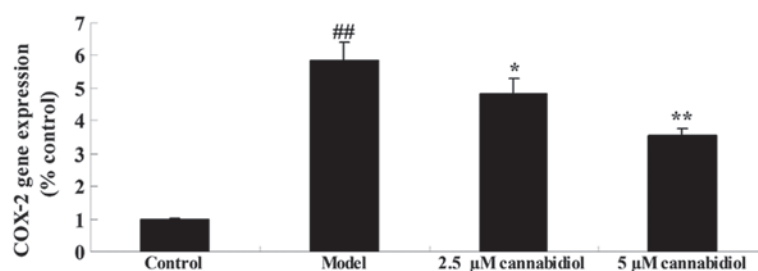


Figure 6. Effect of cannabidiol on COX-2 gene expression in H_2O_2 -treated nucleus pulposus cells (model). ## P <0.01 vs. control group, * P <0.05 vs. model group and ** P <0.01 vs. model group. COX-2, cyclooxygenase 2.

Treatment with 2.5 or 5 μ M cannabidiol significantly inhibited H_2O_2 -induced caspase-3 gene expression (Fig. 4).

Effect of cannabidiol on the expression levels of Bcl-2 in H_2O_2 -treated NPCs. To elucidate the effect of cannabidiol on

the expression levels of Bcl-2 in H_2O_2 -treated NPCs, the protein expression levels of Bcl-2 were measured by western blot analysis. The results indicated that H_2O_2 exposure (200 μ M) induced a significant reduction in the Bcl-2 protein expression level in NPCs following treatment for 24 h compared with the control

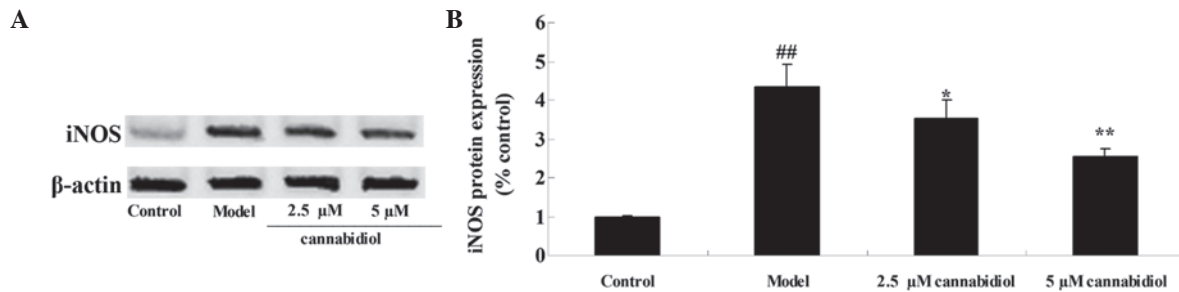


Figure 7. Effect of cannabidiol on the expression level of iNOS in H_2O_2 -treated NP cells (model). (A) Western blot analysis demonstrating the effect of cannabidiol on the iNOS protein expression levels. (B) Statistical analysis of iNOS protein expression level in H_2O_2 -treated NP cells. ^{##} $P < 0.01$ vs. control group, ^{*} $P < 0.05$ vs. model group and ^{**} $P < 0.01$ vs. control group. iNOS, inducible nitric oxide synthase; NP, nucleus pulposus.

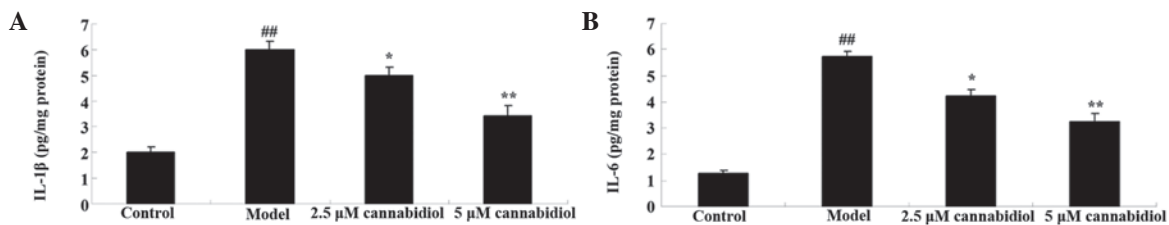


Figure 8. Effect of cannabidiol on IL-1 β and IL-6 in H_2O_2 -treated NP cells (model). Effect of cannabidiol on (A) IL-1 β and (B) IL-6 in H_2O_2 -treated NP cells. ^{##} $P < 0.01$ vs. control group, ^{*} $P < 0.05$ vs. model group and ^{**} $P < 0.01$ vs. control group. IL, interleukin; NP, nucleus pulposus.

group (Fig. 5A and B). Treatment with 2.5 or 5 μ M cannabidiol was demonstrated to prevent this reduction (Fig. 5A and B).

Effect of cannabidiol on COX-2 gene expression in H_2O_2 -treated NPCs. To investigate the effect of cannabidiol on COX-2 in H_2O_2 -treated NPCs, COX-2 gene expression was quantified by qPCR. As presented in Fig. 6, 200 μ M H_2O_2 significantly increased the COX-2 gene expression compared with the control group. Treatment with 2.5 or 5 μ M cannabidiol inhibited this increase (Fig. 6).

Effect of cannabidiol on the expression level of iNOS in H_2O_2 -treated NPCs. To clarify the effect of cannabidiol on the expression level of iNOS in H_2O_2 -treated NPCs, the protein expression levels of iNOS were measured by western blot analysis. As presented in Fig. 7A and B, 200 μ M H_2O_2 significantly increased the level of iNOS protein compared with the control group. Notably, treatment with 2.5 or 5 μ M cannabidiol inhibited the increased iNOS protein expression in H_2O_2 -treated NPCs (Fig. 7A and B).

Effect of cannabidiol on IL-1 β and IL-6 in H_2O_2 -treated NPCs. The effect of cannabidiol on inflammation in H_2O_2 -treated NPCs was also investigated in the present study by measurement of IL-1 β and IL-6 levels. As presented in Fig. 8A and B, 200 μ M H_2O_2 significantly increased the levels of IL-1 β and IL-6 in the NPCs. Notably, administration of 2.5 or 5 μ M cannabidiol to the H_2O_2 -treated NPCs inhibited the increase in IL-1 β and IL-6 levels (Fig. 8A and B).

Discussion

LDH is a common type of clinical disease. Due to changes in the way individuals work and live, the incidence of LDH

is increasing and is affecting individuals at a younger age (2). According to statistics, ~85-90% of the patients exhibiting LDH demonstrate a satisfactory recovery following appropriate non-surgical treatment (2). The present study demonstrated that cannabidiol treatment exerted a protective effect on NPCs *in vitro* by increasing cell viability and decreasing apoptosis following H_2O_2 exposure. Kwiatkoski *et al* (18) confirmed that cannabidiol administered to treat cryogenic spinal cord injury resulted in higher motor scores in rats.

Previous studies found that apoptosis may be involved in intervertebral disc tissue degeneration of pathophysiological changes, and indicates that apoptosis was important in the process of intervertebral disc degeneration (19). Excessive cell apoptosis results in a reduction in the activity of intervertebral disc cells and a subsequent decrease in extracellular matrix change in synthesis and composition, contributing to the pathology of intervertebral disc degeneration (20). Numerous studies regarding signal transduction pathways have indicated that intervertebral disc cells may be associated with caspase-3 and Bcl-2 signal transduction and apoptosis, regulation of apoptosis may be a method of preventing intervertebral disc degeneration (21). In the present study, it was observed that treatment with cannabidiol markedly reduced the level of caspase-3 gene expression and increased the Bcl-2 protein expression level in the NPCs that had undergone H_2O_2 exposure. Mecha *et al* (17) demonstrated that cannabidiol treatment protects oligodendrocyte progenitor cells by decreasing caspase-3 gene expression and increasing the expression of anti-apoptotic Bcl-2.

In normal circumstances within the body, the generation of reactive oxygen species (ROS) and the active oxygen removal systems are in a state of dynamic balance. Various circumstances can result in increased ROS production and/or a reduced ability of the body to remove ROS, causing a state of

oxidative stress. When the body is in a state of oxidative stress, tissue cells contain elevated levels of molecular oxygen, which the body is unable to remove. This leads to increased tissue lipid peroxidation levels, causes abnormal oxidative DNA damage and protein expression, and ultimately damages the body (22). In the present study, H₂O₂-treated NPCs exhibited an increased COX-2 gene expression level. Administration of cannabidiol significantly inhibited the H₂O₂-stimulated increase in COX-2 gene expression. Castillo *et al* (23) reported that cannabidiol inhibited hypoxic-ischemic brain damage by reducing COX-2 and iNOS expression levels. Wheal *et al* (24) demonstrated that cannabidiol improves vasorelaxation through COX-1/2 activation in Zucker diabetic fatty rats.

During LDH, persistent compression of the spinal cord or nerve root by an inflamed outstanding intervertebral disc, activates iNOS, producing an increased quantity of nitric oxide (NO), thus increasing the NOS expression levels in the damaged area (25). As compression of the spinal cord or nerve root by the outstanding intervertebral disc cannot be removed, spinal cord ischemia, hypoxia and blocked energy generation are observed. This results in increased enzyme activity and insufficient substrate for NO synthesis. Thus, the NOS (resulting from insufficient substrate) promotes superoxide anion and H₂O₂ production, tissue damage, kills neurons surrounding the spinal cord and leads to nerve cell degeneration and necrosis. Finally, iNOS induces motor neuron death, causing the loss of cells that synthesize NO, resulting in reduced NOS activity and NO content in the serum (26,27). The present study provides evidence that treatment with cannabidiol significantly suppressed the enhanced level of iNOS protein resulting from H₂O₂ exposure. Furthermore, De Filippis *et al* (28) indicated that cannabidiol reduced intestinal inflammation via suppression of iNOS expression. Esposito *et al* (29) confirmed that cannabidiol suppressed IL-1 β and iNOS expression *in vivo*, which inhibits β -amyloid induced neuroinflammation.

The major clinical manifestation of intervertebral disc degeneration is lower back pain, and clinical treatments include conservative treatment, intervertebral disc resection and spinal fusion; however, these treatments cannot fundamentally prevent degeneration (30). At present, the study of lumbar disc degeneration is concentrated within the field of molecular biology (31). During investigation of the mechanisms underlying lumbar disc degeneration, the focus is predominantly on genetic factors, high loading of intervertebral discs and nutritional disorders; analyzing cytokines and inflammatory transmitters is also considered to be important (32,33). IL-1, IL-4, and tumor necrosis factor- α expression levels, within the intervertebral discs of the lumbar region, contribute to synthesis and decomposition of the matrix, inflammation and neurotransmission, and are important in disc cell apoptosis (34). The findings of the present study indicate that cannabidiol decreased the increased levels of IL-1 β and IL-6 in H₂O₂-treated rats. Barichello *et al* (35) demonstrated that cannabidiol reduced the host immune response and exerted an anti-inflammatory effect in Wistar rats that were submitted to pneumococcal meningitis. Hao *et al* (36) suggested that cannabidiol protects against doxorubicin-induced cardiomyopathy by modulating antioxidant and anti-inflammatory activity.

In conclusion, the current study demonstrated that cannabidiol treatment exerts a protective effect on H₂O₂-treated NPCs. However, the underlying mechanism of the protective effect of cannabidiol remains unknown, but appears to be partly mediated by anti-apoptosis, anti-inflammation and anti-oxidative activities, as well as by protecting mitochondrial function. These data provide a potential mechanism of cannabidiol reversing the effects upon H₂O₂-exposed NPCs, and support the therapeutic rationale for the use of cannabidiol to treat human LDH.

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