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Ligustilide alleviated IL-1β induced apoptosis and extracellular matrix degradation of nucleus pulposus cells and attenuates intervertebral disc degeneration *in vivo*

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ABSTRACT

Intervertebral disc degeneration is a multifactorial and complicated degenerative disease that imposes a huge economic burden on society. However, there is no effective treatment that can delay and reverse the progression of disc degeneration. The inflammatory response causes the death of nucleus pulposus cells and the degradation of extracellular matrix are main factors of intervertebral disc degeneration. Ligustilide is a bioactive phthalide that is said to have an anti-inflammatory effect and anti-apoptosis effect on various disorders. Therefore, we further explored the protective effect of ligustilide on intervertebral disc degeneration and its potential mechanism. In this study, we found that ligustilide inhibited apoptosis, suppressed the expression of related inflammatory mediators (iNOS and COX-2) and decreased the expression of inflammatory cytokines (TNF-a and IL-6) in nucleus pulposus cells under IL-1β stimulation. At the same time, the degradation of extracellular matrix of nucleus pulposus cells induced by IL-1β was inhibited. In addition, we also found that ligustilide inhibits the inflammation response by inhibiting the NF-κB signaling pathway. Moreover, TUNEL assay and histological analysis showed that ligustilide could inhibit the apoptosis of nucleus pulposus cells and ameliorate the progression of intervertebral disc degeneration in punctured Rat IDD model. In summary, ligustilide may become a new potential treatment for intervertebral disc degeneration.

1. Introduction

Intervertebral disc degeneration (IDD) is a complicated and multifactorial disease [1]. IDD has been considered as one of the primary cause of Low back pain (LBP), which is puzzling 80% individuals around the world, associating with disabilities and contributing to heavy social costs worldwide [2,3]. However, there are major limitations in the clinical treatment of intervertebral disc degeneration, which can only partially relieve the symptoms induced by degeneration of the intervertebral disc, but cannot fundamentally reverse the degeneration of the intervertebral disc. Therefore, corresponding effective medicine that really ameliorates the progression of IDD should be developed.

Intervertebral disc is composed by nucleus pulposus (NP), annulus fibrosus (AF) and cartilaginous endplate (CEPs) [4]. The NP is the most important structure in the intervertebral disc, maintaining the stability of the intervertebral disc and buffering the impact of external force on the spine. At present, the molecular mechanism of IDD has attracted the attention of scholars. IDD is associated with cellular changes in the intervertebral disc, including increased cell death and degradation of the extracellular matrix [5]. Therefore, inhibition of cell death and degradation of extracellular matrix of NP cells may be therapeutic targets for delaying degeneration of the intervertebral disc.

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Inflammation and apoptosis have been considered as leading causes blamed for IDD [6,7]. As the disc degeneration progressed, the expression of IL-1β in the NP tissue increased significantly. Admittedly, IL-1β induces several inflammatory mediators such as such as cyclooxygenase-2 (COX-2), Prostaglandin E2 (PGE2), nitric oxide (NO), NO synthase (NOS) in NP cells [8]. In addition, disintegrin-like ADAM metallopeptidases with thrombospondin type 1 motif (ADAMTS) and matrix metalloproteinases (MMPs) that impairing the extracellular matrix (ECM) were also increased when IL-1β upregulated. Moreover, increased IL-1β promotes apoptosis of NP cells, which resulted in leakage of NP cells and reduction in synthesis of the ECM.

In recent studies, activation of NF-κB pathway has been shown to regulate inflammatory responses, including inflammatory mediator release [9]. Numerous studies showed that inflammation response was inhibited when NF-κB suppressed in IDD and OA disease [10–12]. Ligustilide is a bioactive phthalide derivative isolated from Cnidii Rhizoma and Angelicae Gigantis Radix which are both medicinal herbs used to treat inflammation disorders [13,14]. Previous studies showed that ligustilide could delay inflammation response in endothelial cells, nerve cells and macrophages [15–17], however, whether ligustilide can delay the inflammatory reaction of NP cells is still unclear.

Thus, in this study, we investigated the anti-inflammatory effect and anti-apoptosis effect of ligustilide on IL-1β-stimulated NP cells and explored the potential mechanism. Furthermore, the protective role of ligustilide against IDD was also confirmed in a puncture-induced rat IDD model *in vivo*.

2. Material and method

2.1. Ethics statement

All surgical interventions, treatments and postoperative animal care procedures were performed in strict accordance with the Animal Care and Use Committee of Wenzhou Medical University.

2.2. Reagents and antibodies

Ligustilide ($C_{12}H_{14}O_2$) (purity > 98%) was purchased from Chengdu Herbpurify CO., LTD (Chengdu, China). Dimethylsulfoxide (DMSO) and Collagenase II were purchased from Sigma-Aldrich (St Louis, MO, USA). The primary antibody of β-actin, MMP13, Collagen II, Aggrecan, Lamin B were purchased from Abcam (Cambridge, UK). The Cleaved-caspase3, Bax and Bcl-2, iNOS, COX-2, IκBα and NF-κB(p65) antibodies were obtained from CST (MA, USA). Second antibody, such as Alexa Fluor®488 labeled and Alexa Fluor®594 labeled Goat Anti-Rabbit IgG (H + L) were obtained from Abcam (Cambridge, UK). The 4′,6-diamidino-2-phenylindole (DAPI) was obtained from Beyotime (Shanghai, China).

2.3. Surgical procedure

Sprague–Dawley rats (200–250 g) were purchased from Shanghai Laboratory Animal Center and randomly classified into three groups, including control group (n = 12), IDD group (saline injected after surgery, n = 12) and ligustilide group (ligustilide injected after surgery, n = 12). As description in the previous study [18], IDD group and ligustilide group rats were anesthetized by 2% (w/v) pentobarbital (40 mg/kg) and puncturing the whole layer of annulus fibrosus though the tail skin by needles (26G). All the needles were kept in the discs for 1 min. After surgery, the ligustilide was immediately injected intraperitoneally to deliver a dose of 10 mg/kg/day in ligustilide group and same amount of saline was injected in IDD group every day until the rats were sacrificed.

2.4. Isolation and cultivation of NP cells

SD rats were sacrificed by an overdose of sodium pentobarbital. As described in the previous study [18], NP tissues were carefully collected. Then, the tissues were digested by collagenase II (2 mg/ml 0.1%) for 4 h at 37 °C. Next, tissues were transferred to DMEM/F12 Culture medium (Gibco, Invitrogen, Grand Island, NY) with 15% fetal bovine serum (FBS; Gibco, Invitrogen, Grand Island, NY) and antibiotics (1% penicillin/streptomycin) in the incubator at 5% CO_2 at 37 °C. When NP cells are incubated about 48 h, medium was changed for first time. Harvesting the NP cells by 0.25% Trypsin-EDTA (Gibco, Invitrogen) when confluent. Next, NP cells were passage into 10-cm culture plates at the appropriate density. The complete medium was changed every other day and the first two and three passage NP cells were used in our experiments.

2.5. Cell viability analysis

The cytotoxicity of ligustilide on NP cells were determined by cell counting kit-8 (CCK-8; Dojindo Co, Kumamoto, Japan) referring to the protocol. As described in the previous study [18], NP cells were treated with ligustilide for 24 h and washed by phosphate-buffered saline (PBS) for one time, and then 100 μl of DMEM/F12 containing 10 μl of CCK-8 solution was added to each well of the plate for 2 h at 37 °C. Then measured the OD at 450 nm using a micro-plate reader. All experiments were performed more than three times.

2.6. Immunofluorescence

For immunofluorescence, as described in the previous study [18], The cells were rinsed by PBS for three-times before fixation using 4% paraformaldehyde and followed by permeation using the 0.5% Triton for 5 min. Nonspecific protein binding was blocked by 10% bovine serum albumin for 1 h at 37 °C, cells were rinsed by PBS and incubated with specific primary antibodies: Collagen II (1:200), MMP-13 (1:200) and p65 (1:200) in a humid chamber overnight at 4 °C. Next day, NP cells were incubated with Alexa Fluor®488/594 labeled conjugated second antibodies (1:400) for 1 h at 37 °C and the nuclear were labeled by DAPI for 5 min. Finally, five visual fields were randomly selected from each sample in a single blinded manner.

2.7. Western blot assay

RIPA lysis buffer containing with1 mM PMSF (Phenylmethanesulfonylfluoride) were applied to extract the total protein of NP cells and BCA protein assay kit (Beyotime) was used to measure the concentration of extracted protein. Then, the protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, USA). After non-specific proteins were blocked by 5% nonfat milk, the PVDF membranes were incubated with the specific primary antibody: cleaved caspase3 (1:1000), Bax (1:1000), Bcl-2 (1:1000), β-actin (1:1000), Aggrecan (1:1000), MMP13 (1:1000), iNOS (1:1000), COX-2 (1:1000), p65 (1:1000) and IκBα (1:1000) overnight at 4 °C. Then the membranes were rinsed and secondary antibodies to bond with respective primary antibody at room temperature. As described in the previous study [19], the bands were detected by electro chemiluminescence plus reagent (Invitrogen) and the intensity of these bands was quantified using Image Lab 3.0 software (Bio-Rad).

2.8. TUNEL method

After fixed by 4% paraformaldehyde for 15 mins, NP cells were rinsed by PBS for three times, then 0.1% Triton X-100the cells were used to increase membrane permeability for 10 min at 4 °C as previous study described [18]. According to the manufacturer's protocol, NP

cells were stained with *in situ* cell death detection kit (F. Hoffmann-La Roche Ltd., Basel, Switzerland) for 1 h. Nuclear of cells were stained with 40,6-diamidino-2-phenylindole (DAPI) after rinsed by PBS for three times in dark room. Apoptotic changes were observed, and image of apoptotic cells were counted by through a fluorescence microscope (Olympus).

2.9. Real-time PCR

TRIzol method (Invitrogen, USA) was used to extract the total RNA from NP cells. To reverse RNA to complementary DNA (cDNA) and amplification cDNA using Prime Script-RT reagent kit and SYBR Premix Ex Taq (Sangon, Shanghai, China). The expression of mRNA were measured using the △△Ct method as described previously [20]. The primers of COX-2 (F) 5′-GAGAGATGTATCCTCCCACAGTCA-3′ (R) 5′-GACCAGGCACCAGACCAAAG-3′; iNOS (F) 5′-CCTTACGAGGCGAAG AAGGACAG-3′, (R) 5′-CAGTTTGAGAGAGGAGGCTCCG-3′; IL-6 (F) 5′-GACAGCCACTCACCTCTTCA-3′, (R) 5′-TTCACCAGGCAAGTCTC CTC-3′; TNF-α (F) 5′-GTCAGATCATCTTCTCGA ACC-3′, (R) 5′-CAGAT AGATGGGCTCATACC-3′; IL-10 (F) 5′-CTCTGATACCTCAGTTCCCATT CTA-3′, (R) 5′-AAATAACAAACTGGTCACAGCTTTC-3′; TGF-β (F) 5′-CCCGCATCCCAGGACCTCTCT-3′, (R) 5′-CGGGGGACTGGCGAGCCT TAG-3′ were refer to previous study [21].

2.10. Safranin O-fast green staining

The SD rats were sacrificed by over dosage injection of 10% pentobarbital and the intervertebral discs of tails were harvested on 8 weeks post-surgery according to the changes of rat's MRI. 4% paraformaldehyde were used to fix the specimen for 48 h and the sample was decalcified for more than 30 days, then dehydrated and embedded in paraffin. The sample were cut into sections (5 μm thick). Safranin O-fast green staining was used to assess the disc degeneration. The histology score was evaluated according to a grading scale [22,23].

2.11. Magnetic resonance imaging method

After 8 weeks of surgery, the disc degeneration of rats was evaluated by MRI examination. All rats were anesthetized by an intraperitoneal injection of 10% pentobarbit (40 mg/kg). As previous study described [18], Magnetic resonance imaging was performed on all rats to evaluate the signal and structural changes in sagittal T2-weighted images using a 3.0 T clinical magnet (Philips Intera Achieva 3.0MR). The degree of IDD was evaluated by Pfirrmann grading system [24].

2.12. Statistical analysis

All experiments were performed at least three times. The results were expressed as mean ± S.D. Statistical analyses were performed using SPSS statistical software program 20.0. Data were analyzed by one-way analysis of variance (ANOVA) followed by the Tukey's test or *t*-tests (and nonparametric tests) for comparison between control and treatment groups. Nonparametric data (Pfirrmann grading) were analyzed by the Kruskal–Wallis H test. Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effects of ligustilide on nucleus pulposus cells' viability

The chemical structure of ligustilide is shown in Fig. 1A above. In order to evaluate whether ligustilide has toxic effects on NP cells, NP cells were treated with ligustilide in a dose dependent manner for 24 h and 72 h. And then CCK8 was applied to determine the cellular viability of NP cells. As shown in Fig. 1B and C, the results shown that ligustilide was not toxic to NP cells when the concentration of ligustilide is less than 30 μM, and ligustilide could slightly increase the cellular activity of NP cells, especially at the concentration of 5 and 10 μM. Furthermore, to estimate whether ligustilide can alleviate the toxic effects of IL-1β on NP cell. We found out that IL-1β could significantly decreased the cellular viability of NP cells, but ligustilide could reversed this phenomenon in a dose dependent manner, especially at the concentration of 5 and 10 μM (Fig. 1D). Therefore, the concentration of ligustilide applied in our subsequent experiments was more than 5 μM.

3.2. Ligustilide inhibit the generation of iNOS, COX-2, TNF-α and IL-6 in IL-1β stimulated nucleus pulposus cells

To investigate the effects of ligustilide in NP cells under inflammation condition induced by IL-1β, the expression of iNOS, COX-2, TNF-α and IL-6 by western blots and RT-PCR. As shown in Fig. 2A, B, C, the protein expression and mRNA expression of iNOS and COX-2 was significantly increased in NP cells under IL-1β stimulation, however, the increment was inhibited by ligustilide in a dose dependent manner. What's more, TNF-α and IL-6 was upregulated in NP cells under IL-1β stimulation according to Real time PCR results. And ligustilide treatment could inhibit the increase of TNF-α and IL-6 in a dose dependent in NP cells after IL-1β treatment, especially at the concentration of 10 μM. In addition, in order to investigate the effects of ligustilide on anti-inflammatory cytokines like IL-10 and TGF-β, which might be beneficial for IDD recovery, we measured the mRNA expression of these cytokines. And the results suggested that IL-1β promoted the expression of IL-10 and inhibited the expression of TGF-β, but the results showed that ligustilide had no effect on the expression of IL-10 and TGF-β. These results indicate that ligustilide could prominently suppress IL-1ß induced inflammatory mediators and cytokines production at the gene and protein levels in a dose-dependent manner.

3.3. Ligustilide protect nucleus pulposus cells against IL-1β induced apoptosis in vitro

It is fairly well-known that apoptosis contributes to the progression of IDD. Therefore, our study further examined the effect of ligustilide on apoptosis of NP cells. As shown in Fig. 3A, IL-1β promotes the expression of pro-apoptotic proteins such as Bax and Cleaved-caspase 3 and decreased the expression of anti-apoptotic proteins such as Bcl-2 in NP cells according to western blots results. Whereas, ligustilide could promotes the expression of Bcl-2 and decreased the expression of Bax and Cleaved-caspase 3 in NP cells under IL-1β stimulation in a dose dependent manner. In addition, TUNEL results showed that IL-1β increased the TUNEL positive NP cells, but ligustilide prevented the increment of NP cells under IL-1β stimulation in a dose dependent manner. In a conclusion, ligustilide protect NP cells against IL-1β induced apoptosis in NP cells *in vitro*.

3.4. Ligustilide alleviated IL-1β induced extracellular matrix degradation in nucleus pulposus cells in vitro

Extracellular matrix is the major component of the intervertebral disc, degradation of extracellular matrix was recognized as one of the culprits for accelerating intervertebral disc degeneration. We further tested the function of ligustilide on inhibition in the degradation of extracellular matrix. The expression of Collagen II, aggrecan (the major components of extracellular matrix) and MMPs (matrix metalloproteinase) such as MMP13 in NP cells were evaluated by western blots or Real-time PCR. As shown in Fig. 4A and B, the protein expression of aggrecan was decreased and but protein expression of MMP13 was increased in NP cells after IL-1β treatment for 24 h. However, ligustilide promoted the expression of aggrecan and inhibited the expression of MMP13 in NP cells under IL-1β simulation in a dose dependent manner. Besides, western blots results also showed that ADAMTS5, the contributor of ECM degradation, was increased with the stimulation of IL-

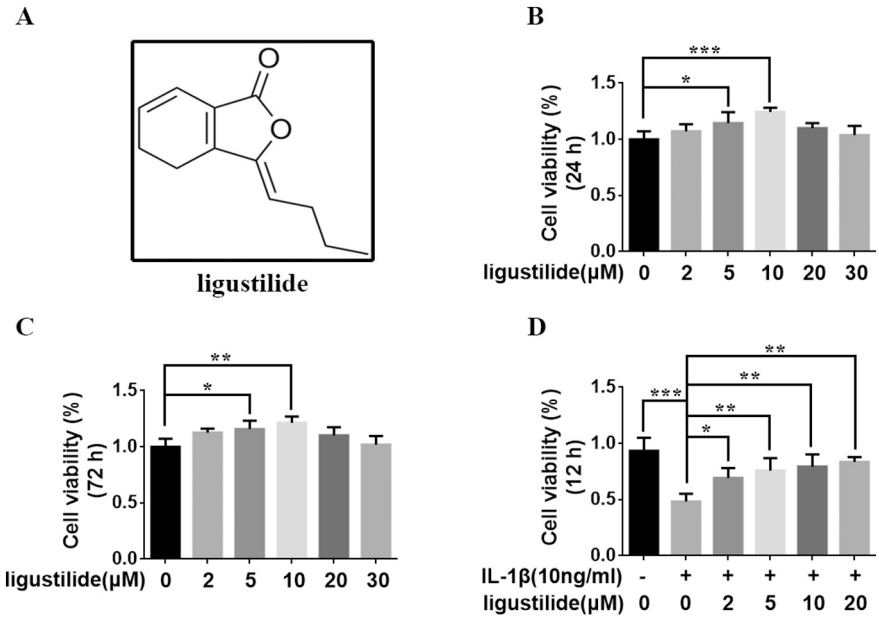

Fig. 1. Effects of ligustilide on the cell viability of NP cells.

(A) Chemical structure of ligustilide. (B) The cytotoxic effect of liguatilide on NP cells was determined at various concentrations for 24 h using a CCK8 assay. (C) The cytotoxic effect of liguatilide on NP cells was determined at various concentrations for 72 h using a CCK8 assay. (D) The effect of ligustilide on cell viability of NP cells in a dose dependent under IL-1β stimulation for 12 h. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as ***P < 0.001, **P < 0.01, *P < 0.05, *ns* P > 0.05.

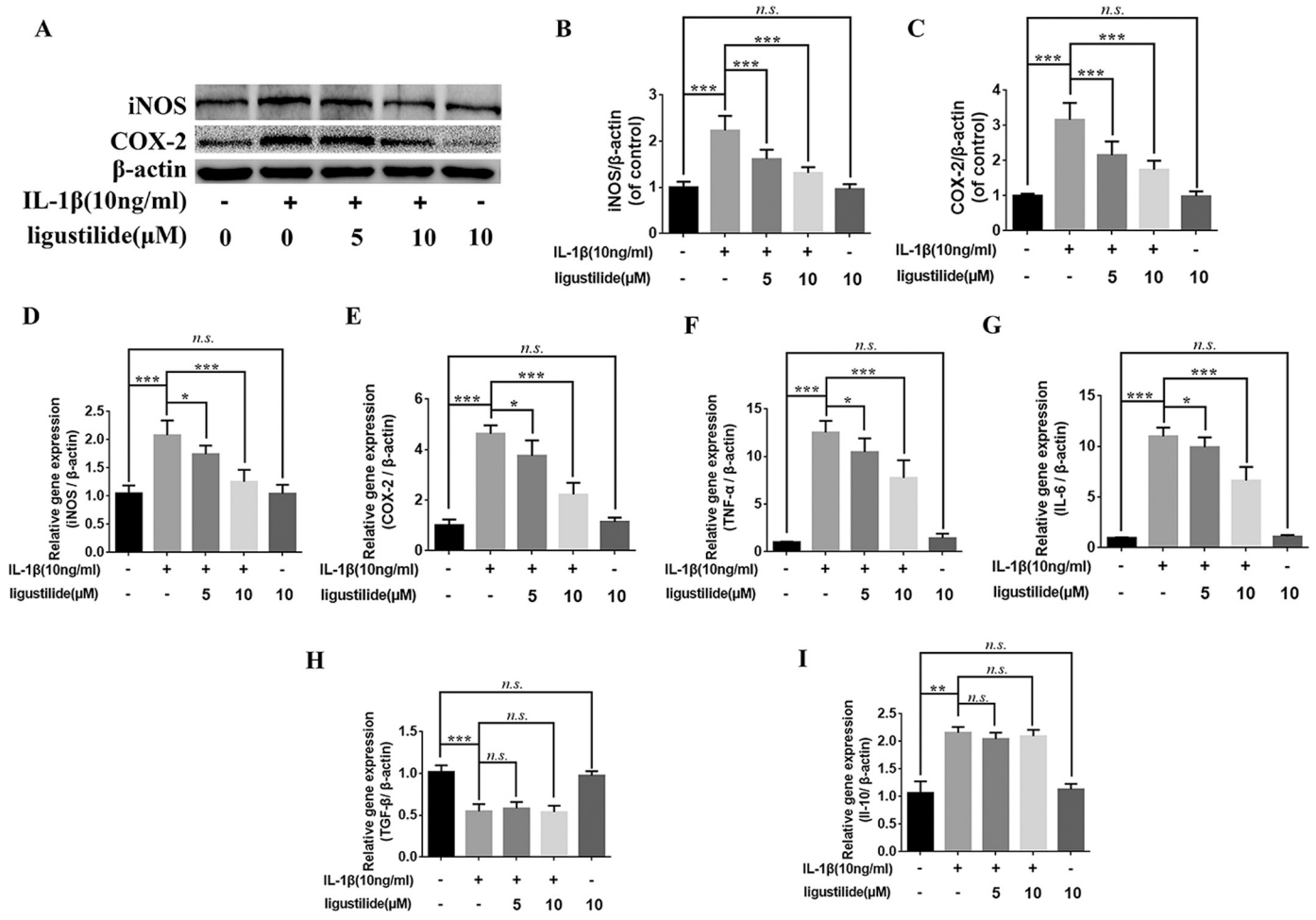

Fig. 2. Effects of ligustilide on IL-1β-induced inflammation in NP cells.

The NP cells were incubated with various concentration of ligustilide for 24 h and IL-1β for 12 h. (A) The protein expression of iNOS, COX-2 in NP cells were evaluated by western blots; (B, C) quantification of immunoblots of iNOS, COX-2. (D, E) The mRNA expression of iNOS, COX-2 in NP cells were evaluated by Real-time PCR. (F, G) The generation of TNF-α and IL-6 were determined by Real-time PCR. (H, I) The mRNA expression of IL-10 and TGF-β in NP cells were evaluated by Real-time PCR. The experiment was repeated three times, with a representative example shown; The values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as ***P < 0.001, **P < 0.01, *P < 0.05, *ns* P > 0.05.

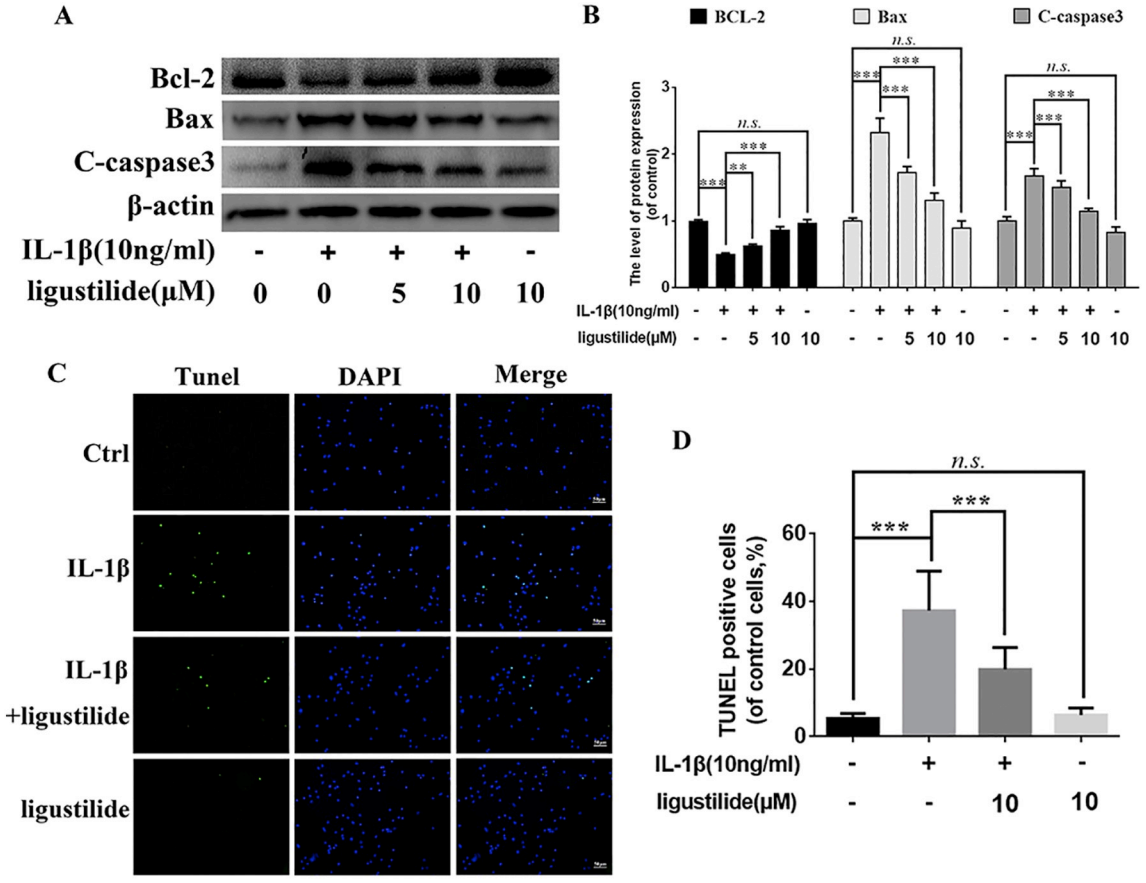

Fig. 3. Effects of ligustilide on IL-1β-induced apoptosis in NP cells.
(A) The protein expression of Bcl-2, Bax, Cleaved-caspase3 in NP cells were evaluated by western blots; (B) quantification of immunoblots of Bcl-2, Bax, Cleaved-caspase3. (C) TUNEL assay was measured in NP cells as treated above (original magnification ×200, scale bar: 50 μm); (D) three images were randomly selected and the number of cells with green fluorescence was quantified; the experiment was repeated three times, with a representative example shown; the values presented are the means ± S.D. of three independent experiments. Significant differences between groups are indicated as ***P < 0.001, **P < 0.01, *P < 0.05, *ns* P > 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

1β, but downregulated with the treatment of ligustilide in a dose dependent manner (Fig. 4C). In addition, we measured the expression of Collagen II and MMP13 through immunofluorescence staining, Collagen II was labeled by Collagen II specific antibody and MMP 13 was stained by MMP 13 specific antibody. As shown in Fig. 4E, the fluorescence intensity of Collagen II was decreased in NP cells after IL-1β stimulation, whereas ligustilide prevented the decrease in NP cells. Meanwhile, the fluorescence intensity trend of mmp13 is opposite to Collagen II in NP cells with the same treatment. The mRNA expression of Collagen II in NP cells was decreased and the mRNA expression of MMP13 in NP cells was upregulated after IL-1β treatment, and the mRNA expression of Collagen II and MMP13 in NP cells was reversed after ligustilide treatment in a dose dependent manner. Therefore, ligustilide alleviated IL-1β induced extracellular matrix degradation in NP cells.

3.5. Ligustilide inhibited the NF-κB pathway activation in IL-1β-induced nucleus pulposus cells

In order to investigate the potential mechanism about the anti-inflammation function of ligustilide in NP cells. In this study, nuclear and cytosol protein from NP cells was isolated measured by western blot to compare the expression of IκBα in nuclear and the expression of p65 in cytoplasm of NP cells. We observed that IL-1β upregulated the expression of p65 in nuclear of NP cells, but the expression of p65 was decreased by ligustilide in a dose dependent manner (Fig. 5A, B and C). However, there was no clear different expression of p65 in nuclear and IκBα in cytoplasm of NP cells when ligustilide alone treatment comparing to the control group. In addition, the expression of IκBα in the cytoplasm was descending after IL-1β treatment, but ligustilide could inhibit the degradation of IκBα in NP cells after IL-1β stimulation. Besides, the results of immunofluorescence staining of p65 in NP cells shown that IL-1β promoted p65 translocated to nuclear of NP cells, but the translocation of p65 to nuclear was inhibited by ligustilide (Fig. 5D). These dates suggest that ligustilide inhibit the inflammation response in NP cells through NF-κB pathway.

3.6. Ligustilide ameliorate the progression of intervertebral disc degeneration in Rats puncture model

Based on the protective effect of ligustilide on NP cells *in vitro*, we further explore whether ligustilide could delay the progression of intervertebral disc degeneration in punctured rat model *in vivo*. We established the punctured rat IDD model by using 26G needle to stab the whole layer of the annulus fibrosus (AF) of SD rats through the tail skin for 1 mins. After surgery, the rats in the ligustilide treatment group were intraperitoneally injected with ligustilide of 10 mg/kg per day, and the control group and the IDD group were given the same amount of normal saline. MRI images were taken at 8 weeks after the disc puncture surgery, and rats were sacrificed for histological analysis at 8 weeks after surgery. As show in Fig. 6A, there was a decrease of signal intensity of punctured discs (the white arrow) in IDD group compared to control group, suggesting that punctured intervertebral disc has undergone degeneration according to MRI. However, the signal

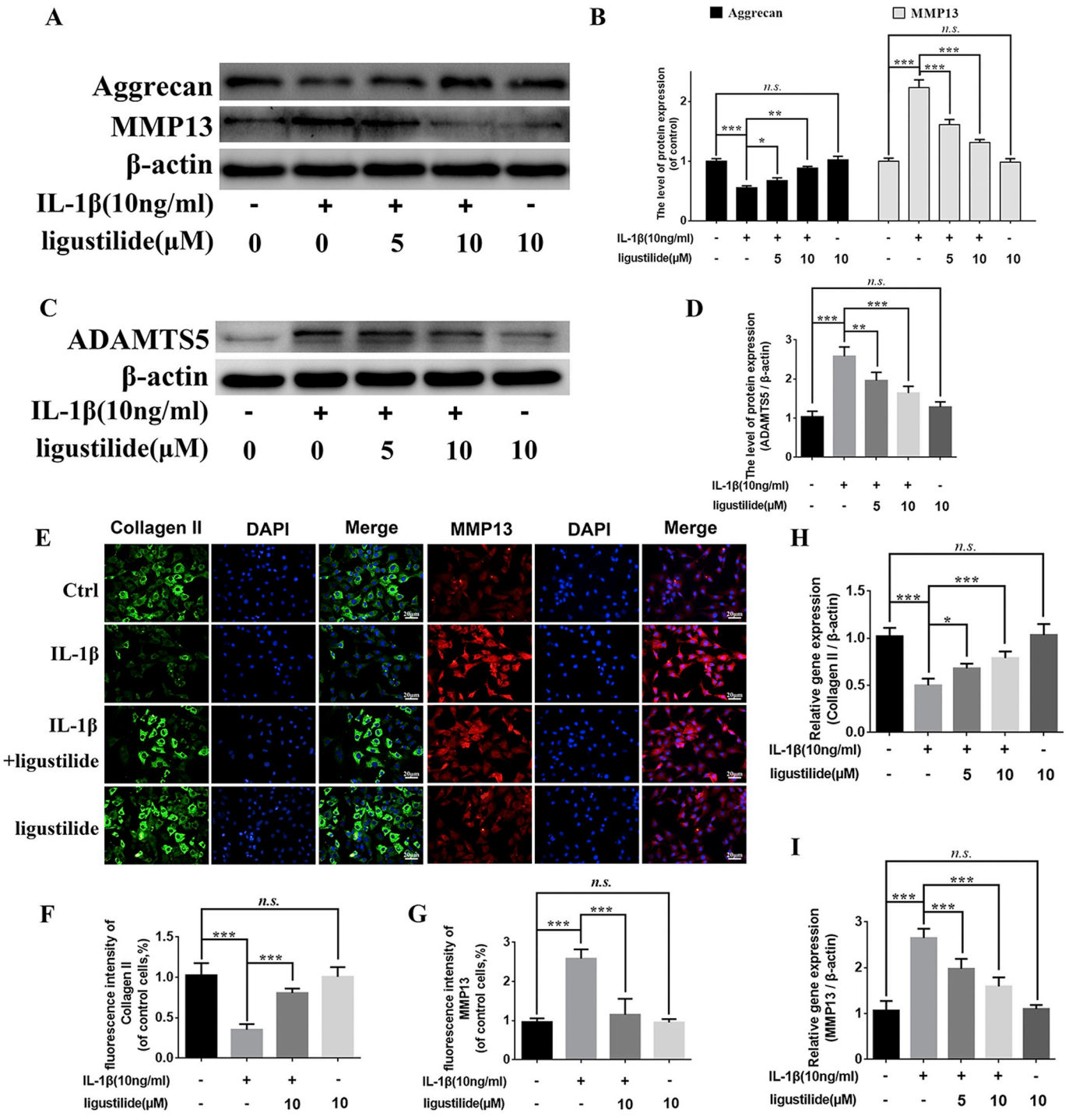

Fig. 4. Effects of ligustilide on IL-1β-induced extracellular matrix degradation in NP cells.
(A) Protein expressions of aggrecan, MMP13 in NP cells treated as above were evaluated by western blot; (B) quantification of immunoblots of aggrecan, MMP13; (C) protein expressions of ADAMTS5 in NP cells treated as above were evaluated by western blot; (D) quantification of immunoblots of ADAMTS5; (E) the representative collagen II (Green) and MMP13 (Red) were detected by the immunofluorescence combined with DAPI staining for nuclei (original magnification ×400, scale bar: 20 μm); (F, G) the fluorescence intensity of Col II (Green) and MMP13 (Red) were analyzed by image J. (H, I) The mRNA expression of Collagen II and MMP13 were measured by real-time Q-PCR; All experiments were performed at least three times and the data in the figures represent the mean ± S.D. Significant differences between groups are indicated as ***P < 0.001, **P < 0.01, *P < 0.05, *ns* P > 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

intensity reduction of the intervertebral disc in ligustilide group was much lower than that in the IDD group (Fig. 6A). The phenomenon was also confirmed by the quantitative evaluation using the Pfirrmann MRI grading system (Fig. 6B).

As shown in Fig. 6C, Safranin O (SO) staining is a method to stain proteoglycans and glycosaminoglycans. Compared with the control group, we found that the NP tissue of the IDD group was almost disappeared at 8 weeks after surgery, but the NP tissue of the ligustilide group was better retained compared to IDD group. Not only the structure of NP tissues was better preserved in ligustilide group, but also the

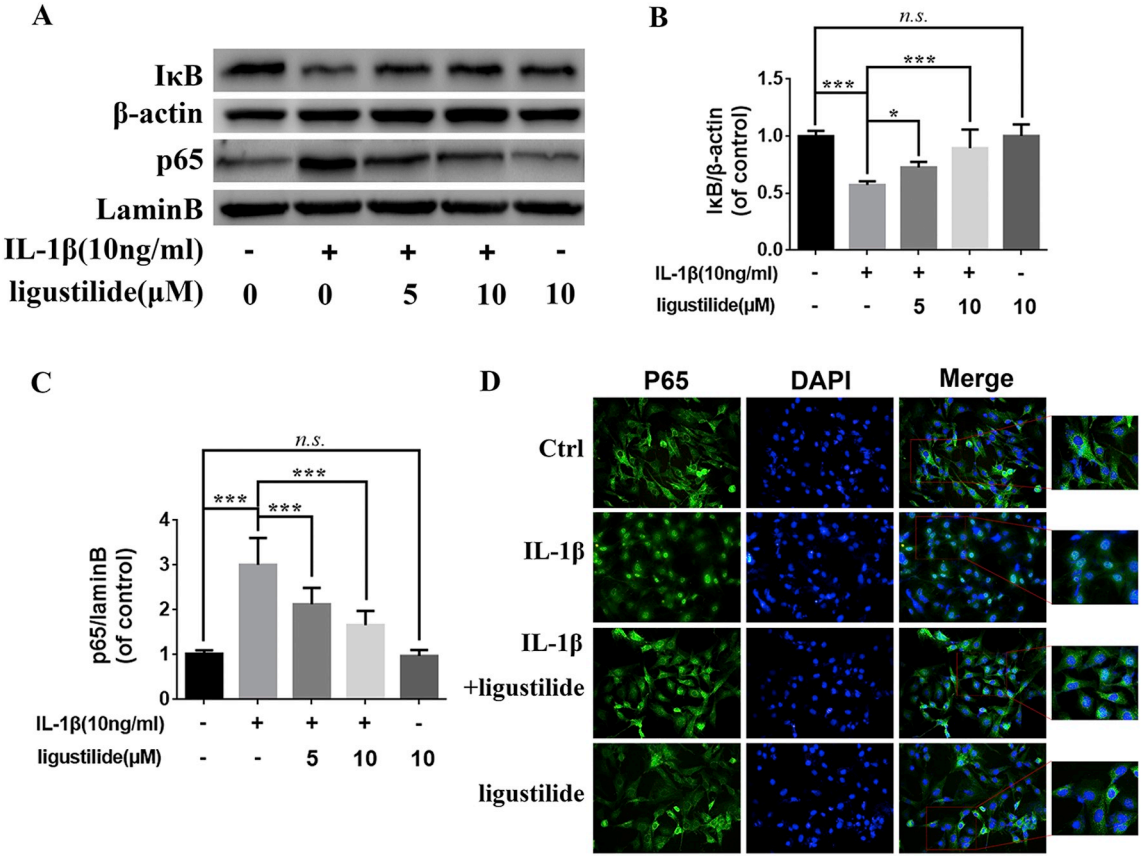

Fig. 5. Effects of ligustilide on IL-1β induced NF-κB activated in NP cells.
(A) The expressions of IκBα in cytoplasm and p65 in nuclear in NP cells treated as above were evaluated by western blot; (B, C) quantification of immunoblots of IκBα and p65; (D) the nuclei translocation of p65 was detected by the immunofluorescence combined with DAPI staining for nuclei (original magnification ×400, scale bar: 25 μm). All experiments were performed at least three times and the data in the figures represent the mean ± S.D. Significant differences between groups are indicated as ***P < 0.001, **P < 0.01, *P < 0.05, *ns* P > 0.05.

extracellular matrix of NP tissues was better preserved compared to IDD group (Fig. 6C). Histology scores evaluation also confirm this phenomenon above. These results suggesting that ligustilide may be beneficial for NP tissues.

Besides, apoptosis of NP cells *in vivo* was also measured by TUNEL assay. As shown in Fig. 6E, TUNEL positive cells in NP tissues was significantly increased in IDD group, whereas ligustilide prevented the increment of TUNEL positive cells in NP tissues (Fig. 6E, F).

In a conclusion, ligustilide may ameliorate the progression of IDD through inhibit the ECM degradation and apoptosis of NP cells *in vivo*.

4. Discussion

Since the high morbidity of IDD and the limitation of currently treatment modalities, there is an urgent need to develop a treatment method that delays or even reverses IDD and preserves the physiological function of the intervertebral disc. Due to the upregulation of IL-1β in IDD and its function of acceleration of IDD [25,26], IL-1β is used to simulate the process of intervertebral disc degeneration *in vitro*. In this study, we demonstrated for first time that ligustilide, a traditional herbal formula, may play a crucial role in treatment for IDD. The results of this study showed that ligustilide could inhibit IL-1β induced apoptosis and activation of NF-κB in NP cells, which subsequently suppressed the ECM degradation in the presence of IL-1β. In addition, *in vivo* study suggests that ligustilide attenuated the apoptosis and ameliorate the progression of IDD in punctured rat IDD model.

As well all know, intervertebral disc degeneration (IDD) is a complicated disorder, which is the potential leading cause for Low back pain. With the rapid accumulation of knowledge on the pathology of IDD, many factors that cause and accelerate the progression of IDD have been recognized, especially inflammation response, apoptosis of NP cells and ECM degradation [27]. In fact, the dysfunction of NP tissue is recognized as the initiating factor of IDD. And NP not only buffers the external stress on the spine, but also produces the extracellular matrix, secretes pro-inflammatory cytokines [7]. According to recent research, IL-1β, TNF-α and IL-6, the upregulated pro-inflammatory cytokines in degenerated NP tissues, contribute to the acceleration of progression of IDD [7]. Accumulation of evidence showed that IL-1β can upregulate zinc-based matrix degrading enzymes, especially matrix metalloproteinases, which may lead to the degradation of ECM and subsequently resulted in IDD [28,29]. Besides, report suggests that TNF-α promotes degeneration of the intervertebral disc by promoting the loss of extracellular matrix in NP cells. Consistent with the conclusion above, we detected that the levels of TNF-α and IL-6 secreted by NP cells are significantly elevated under IL-1β stimulation (Fig. 2). Recent study showed that IL-10 and TGF-β may ameliorate the progression of IDD [30,31], but we found out that ligustilide have no effect on these anti-inflammatory cytokines. We also found out that iNOS and COX-2 increased in NP cells under IL-1β stimulation [31] (Fig. 2). iNOS, originates from the guanidine nitrogen of L-arginine, could promoted the generation of NO, which stimulated the secretions of MMPs and inhibit the synthesis of collagen II and proteoglycan. And COX-2 could also accelerate the ECM degradation by stimulating generation of MMPs and ADAMTS5. The increment of iNOS, COX-2, TNF-α and IL-6 in NP cells finally result in IDD development. In our study, we demonstrated that ligustilide can inhibit the generation of iNOS, COX-2 and related

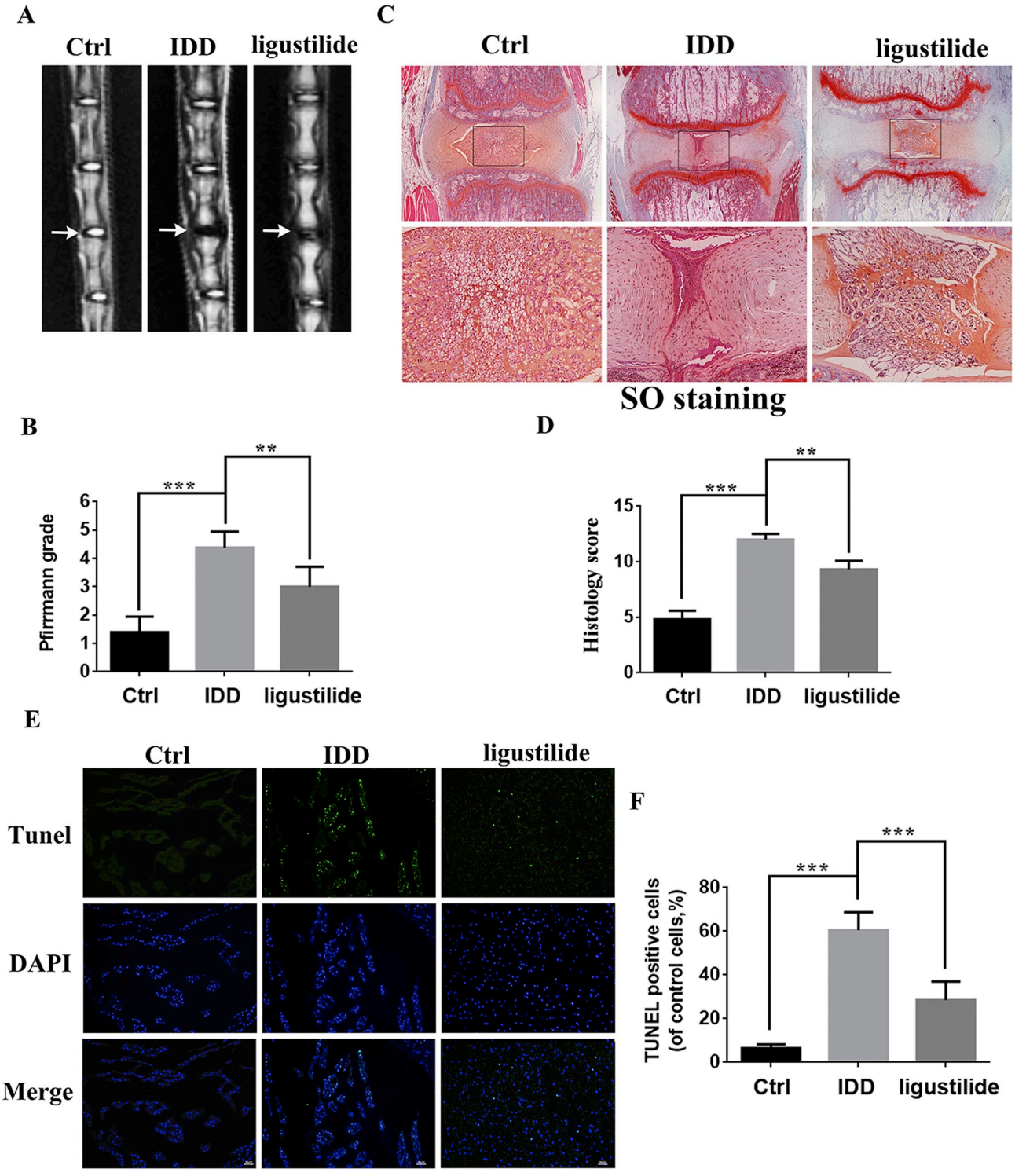

Fig. 6. Ligustilide ameliorate the progression of IDD in punctured rat model.
Rat IDD model was established by stabbing the whole layer of annulus fibrosus (AF) through the tail skin using needles (26G) for 1 min. Ligustilide IDD group rats were injected intraperitoneally with ligustilide (10 mg/kg/day) every day. 8-week degenerated discs were taken MRI and stained with Safranin O; (A) T2-weighted MRI of a rat tail with a needle-punctured disc at 8 weeks post-surgery (white arrow: location of the needle-puncture disc); (B) the Pfirrmann MRI grade scores in three groups at week 8 (6 rats at each time point for each group); (C) representative SO staining of disc samples from different experimental groups at 8 weeks post-surgery (original magnification × 40, scale bar: 100 μm). Three sections were randomly selected for quantification, with a representative example shown; (D) the histological grades evaluated at 8 weeks post-surgery in three groups (6 rats per group). (E) TUNEL assay was used to evaluate the apoptosis of NP cells in degenerated discs in different group. (F) Three images were randomly selected and the number of cells with green fluorescence was quantified; all experiments were performed at least three times and the data in the figures represent the mean ± S.D. Significant differences between groups are indicated as ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, *ns* $P > 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

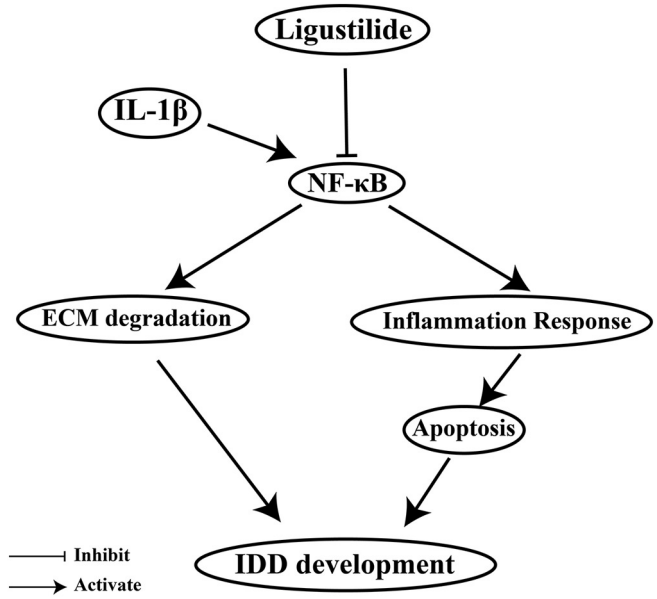

Fig. 7. Potential molecular mechanism involved in ligustilide treatment in NP cells.
Ligustilide attenuated IL-1β induced inflammation, apoptosis and ECM degradation in NP cells *via* suppressing NF-κB pathway.

cytokines including TNF-α and IL-6 in NP cells, and finally prevented the degradation of ECM under IL-1β stimulation (Fig. 4).

Moreover, apoptosis, a very important type of IVD cell death, has been considered to play a crucial role in the process of degeneration. In addition, accumulation evidences showed that inflammation was involved in the apoptosis of various cells, including hepatocyte and NP cells [10,32]. And MAPK and AKT signaling pathway may involve in inflammation induced apoptosis in NP cells. Cao et al. [33] suggested that Simvastatin protects NP cells from apoptosis by MAPK signaling pathway, and Zhou et al. [32] demonstrated that 20(R)-Rg3 inhibited the apoptosis cells by AKT signaling pathway. Besides, a recent research demonstrated that ligustilide could also protect chondrocytes through AKT signaling pathway [34]. Therefore, MAPK and AKT signaling pathway may play a significant role in the protection of IDD, whereas more experiments should be performed to investigate whether ligustilide protects NP cells by MAPK or AKT signaling pathway. Ding et al. [6] reported that apoptosis induced cellular loss has been believed to contribute to the degradation of ECM which finally resulted in IDD. Recently study suggested that prolactin could inhibit the development of IDD through attenuating apoptosis of NP cells [35]. In addition, due to our previous study, metformin and spermidine could ameliorate the progression of IDD by protecting NP cells against apoptosis [18,36]. In our study, TUNEL and Western blots results showed that there were less positive apoptotic cells, which were recognized as marker for initiation of apoptosis, in ligustilide treatment group comparing to IL-β treatment group in *in vitro*. Besides, *in vivo* study showed that apoptotic cells was decreased after ligustilide treatment compared to IDD group. These results indicate that ligustilide delays degeneration of the intervertebral disc by inhibiting apoptosis of NP cells.

Moreover, previous studies reported that activation of NF-κB signaling pathway is involving in the pathophysiological progression of IDD [37,38]. As you know, the generation of catabolic proteinases and anabolic proteinases in NP cells could be regulated by NF-κB signaling pathway [39]. IL-1β stimulation triggers phosphorylation of IκBα, releasing p65 from the cytoplasm and transferring it to the nucleus, which then degraded in the cytoplasm. In the nucleus, NF-κB is highly activated in a variety of diseases and can promote the transcription of pro-inflammatory cytokines, chemokines, adhesion molecules, MMP, Cox-2 and inducible nitric oxide (iNOS), which subsequently promotes degradation of ECM [40,41]. In addition, NF-κB activation is also involved in apoptosis, although its role is not always straightforward. For instance, NF-κB activation may lead to induction of apoptosis in some cell types [42]. ligustilide may protect NP cells against apoptosis and inflammation response through directly suppressed NF-κB. To further investigate the relationship between ligustilide and the NF-kB pathway, we performed western blots and immunofluorescence staining to detect changes of p65 expression in clear and its location. The results of study reveal that ligustilide could inhibit NF-κB pathway and subsequently decreased the production of inflammatory mediators, cytokines and matrix-degrading proteases through NF-κB signaling pathway (Figs. 4A–F and 5A–D).

The punctured IDD model is used to demonstrate similarities between animal models and human IDD. Although, this model is interpreted and extrapolated to human, IDD model exhibits similar changes such as losing of NP cells, calcification, ECM degradation, narrowing of intervertebral space and Biomechanical change [43,22]. We found that ligustilide protected against degradation of NP tissues and the ECM. In addition, we detected that ligustilide attenuated the apoptosis of NP cells in degenerated discs. This phenomenon is consistent with the *in vitro* results and suggests that ligustilide could ameliorate IDD *in vivo*.

In a conclusion, present study demonstrated that ligustilide could inhibit the IL-1β induced inflammation and ECM degradation through inhibiting the NF-κB pathway. In addition, ligustilide prevents IL-1β-induced apoptosis *in vivo* and *in vitro* to ameliorate the leakage of NP cells (Fig. 7). This finding may provide a potential therapeutic treatment for IDD.

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Conflict of interest

The authors declare no conflict of interest.

Contribution

Shengsun Ren and Jiaoxiang Chen contributed to the conception of the study; Ke Wang, Tingting Chen and Xiaozhou Ying contributed significantly to analysis and manuscript preparation; Zengjie Zhang, Zhenxuan Shao, Jialiang Lin performed the cell experiment, data analyses and wrote the manuscript; Tianzhen Xu, Yu chen performed the animal experiment, data analyses and wrote the manuscript.

References

[1] T.J. Freemont, C. LeMaitre, A. Watkins, J.A. Hoyland, Degeneration of intervertebral discs: current understanding of cellular and molecular events, and implications for novel therapies, Expert Rev. Mol. Med. 3 (11) (2004) 1–10.
[2] K. Luoma, H. Riihimaki, R. Luukkonen, R. Raininko, E. Viikari-Juntura, A. Lamminen, Low back pain in relation to lumbar disc degeneration, Spine 25 (4) (2000) 487–492.
[3] C.E. Jensen, A. Riis, K.D. Petersen, M.B. Jensen, K.M. Pedersen, Economic evaluation of an implementation strategy for the management of low back pain in general practice, Pain 158 (5) (2017) 891–899.
[4] M.A. Adams, P.J. Roughley, What is intervertebral disc degeneration, and what causes it? Spine 31 (18) (2006) 2151–2161.
[5] C.K. Kepler, R.K. Ponnappan, C.A. Tannoury, M.V. Risbud, D.G. Anderson, The molecular basis of intervertebral disc degeneration, Spine J. 13 (3) (2013) 318–330.
[6] F. Ding, Z.W. Shao, L.M. Xiong, Cell death in intervertebral disc degeneration, Apoptosis 18 (7) (2013) 777–785.
[7] M.V. Risbud, I.M. Shapiro, Role of cytokines in intervertebral disc degeneration: pain and disc content, Nat. Rev. Rheumatol. 10 (1) (2014) 44–56.
[8] M.-H. Eduardo, O. Sergio, M.-H. Francisco, G. Carmen, Induction of COX-2 and

PGE2 biosynthesis by IL-1β is mediated by PKC and mitogen-activated protein kinases in murine astrocytes, Br. J. Pharmacol. 131 (1) (2000) 152–159.
[9] P.P. Tak, G.S. Firestein, NF-κB: a key role in inflammatory diseases, J. Clin. Invest. 107 (1) (2001) 7–11.
[10] X. Wu, Y. Liu, X. Guo, W. Zhou, L. Wang, J. Shi, Y. Tao, M. Zhu, D. Geng, H. Yang, H. Mao, Prolactin inhibits the progression of intervertebral disc degeneration through inactivation of the NF-κB pathway in rats, Cell Death Dis. 9 (2) (2018) 98.
[11] C. Zhu, W. Jiang, Q. Cheng, Z. Hu, J. Hao, Hemeoxygenase-1 suppresses IL-1β-induced apoptosis through the NF-κB pathway in human degenerative nucleus pulposus cells, Cell. Physiol. Biochem. 46 (2) (2018) 644–653.
[12] N.M. Khan, A. Haseeb, M.Y. Ansari, P. Devarapalli, S. Haynie, T.M. Haqqi, Wogonin, a plant derived small molecule, exerts potent anti-inflammatory and chondroprotective effects through the activation of ROS/ERK/Nrf2 signaling pathways in human osteoarthritis chondrocytes, Free Radic. Biol. Med. 106 (2017) 288–301.
[13] E.S. Choi, J.J. Yoon, B.H. Han, D.H. Jeong, Y.J. Lee, D.G. Kang, H.S. Lee, Ligustilide attenuates vascular inflammation and activates Nrf2/HO-1 induction and, NO synthesis in HUVECs, Phytomedicine 38 (2018) 12–23.
[14] Y.-W. Su, W.-F. Chiou, S.-H. Chao, M.-H. Lee, C.-C. Chen, Y.-C. Tsai, Ligustilide prevents LPS-induced iNOS expression in RAW 264.7 macrophages by preventing ROS production and down-regulating the MAPK, NF-κB and AP-1 signaling pathways, Int. Immunopharmacol. 11 (9) (2011) 1166–1172.
[15] X. Kuang, H.J. Zhou, A.H. Thorne, X.N. Chen, L.J. Li, J.R. Du, Neuroprotective effect of ligustilide through induction of α-secretase processing of both APP and klotho in a mouse model of Alzheimer's disease, Front. Aging Neurosci. 9 (2017) 353.
[16] M.D. Zhu, L.X. Zhao, X.T. Wang, Y.J. Gao, Z.J. Zhang, Ligustilide inhibits microglia-mediated proinflammatory cytokines production and inflammatory pain, Brain Res. Bull. 109 (2014) 54–60.
[17] Z. Wu, H. Uchi, S. Morino-Koga, W. Shi, M. Furue, Z-ligustilide ameliorated ultraviolet B-induced oxidative stress and inflammatory cytokine production in human keratinocytes through upregulation of Nrf2/HO-1 and suppression of NF-κB pathway, Exp. Dermatol. 24 (9) (2015) 703–708.
[18] D. Chen, D. Xia, Z. Pan, D. Xu, Y. Zhou, Y. Wu, N. Cai, Q. Tang, C. Wang, M. Yan, J.J. Zhang, K. Zhou, Q. Wang, Y. Feng, X. Wang, H. Xu, X. Zhang, N. Tian, Metformin protects against apoptosis and senescence in nucleus pulposus cells and ameliorates disc degeneration in vivo, Cell Death Dis. 7 (10) (2016) e2441.
[19] Q. Tang, G. Zheng, Z. Feng, M. Tong, J. Xu, Z. Hu, P. Shang, Y. Chen, C. Wang, Y. Lou, D. Chen, D. Zhang, M. Nisar, X. Zhang, H. Xu, H. Liu, Wogonoside inhibits IL-1beta induced catabolism and hypertrophy in mouse chondrocyte and ameliorates murine osteoarthritis, Oncotarget 8 (37) (2017) 61440–61456.
[20] Q. Tang, Z. Feng, M. Tong, J. Xu, G. Zheng, L. Shen, P. Shang, Y. Zhang, H. Liu, Piceatannol inhibits the IL-1beta-induced inflammatory response in human osteoarthritic chondrocytes and ameliorates osteoarthritis in mice by activating Nrf2, Food Funct. 8 (11) (2017) 3926–3937.
[21] G. Zheng, Y. Zhan, Q. Tang, T. Chen, F. Zheng, H. Wang, J. Wang, D. Wu, X. Li, Y. Zhou, X. Wang, Y. Wu, Y. Zhou, H. Xu, N. Tian, X. Zhang, Monascin inhibits IL-1beta induced catabolism in mouse chondrocytes and ameliorates murine osteoarthritis, Food Funct. 9 (3) (2018) 1454–1464.
[22] B. Han, K. Zhu, F.C. Li, Y.X. Xiao, J. Feng, Z.L. Shi, M. Lin, J. Wang, Q.X. Chen, A simple disc degeneration model induced by percutaneous needle puncture in the rat tail, Spine 33 (18) (2008) 1925–1934.
[23] I. Castro-Mateos, R. Hua, J.M. Pozo, A. Lazary, A.F. Frangi, Intervertebral disc classification by its degree of degeneration from T2-weighted magnetic resonance images, Eur. Spine J. 25 (9) (2016) 2721–2727.
[24] C.W. Pfirrmann, A. Metzdorf, M. Zanetti, J. Hodler, N. Boos, Magnetic resonance classification of lumbar intervertebral disc degeneration, Spine 26 (17) (2001) 1873–1878.
[25] C.L. Le Maitre, A.J. Freemont, J.A. Hoyland, The role of interleukin-1 in the pathogenesis of human intervertebral disc degeneration, Arthritis Res. Ther. 7 (4) (2005) R732.
[26] L.J. Man, S.J. Ye, B. MinJung, J. Hye-Young, K. Haeyoun, H.I. Bo, K.Y. Do, S.D. Eun, Interleukin-1β induces angiogenesis and innervation in human intervertebral disc degeneration, J. Orthop. Res. 29 (2) (2011) 265–269.
[27] G. Fontana, E. See, A. Pandit, Current trends in biologics delivery to restore intervertebral disc anabolism, Adv. Drug Deliv. Rev. 84 (2014) 146–158.
[28] F. Fang, D. Jiang, IL-1beta/HMGB1 signalling promotes the inflammatory cytokines release via TLR signalling in human intervertebral disc cells, Biosci. Rep. 36 (5) (2016).
[29] K.L. Phillips, N. Jordan-Mahy, M.J. Nicklin, C.L. Le Maitre, Interleukin-1 receptor antagonist deficient mice provide insights into pathogenesis of human intervertebral disc degeneration, Ann. Rheum. Dis. 72 (11) (2013) 1860–1867.
[30] S. Wang, J. Wei, Y. Fan, H. Ding, H. Tian, X. Zhou, L. Cheng, Progranulin is positively associated with intervertebral disc degeneration by interaction with IL-10 and IL-17 through TNF pathways, Inflammation 41 (5) (2018) 1852–1863.
[31] L. Zheng, Y. Cao, S. Ni, H. Qi, Z. Ling, X. Xu, X. Zou, T. Wu, R. Deng, B. Hu, B. Gao, H. Chen, Y. Li, J. Zhu, F. Tintani, S. Demehri, A. Jain, K.M. Kebaish, S. Liao, C.A. Séguin, J.L. Crane, M. Wan, H. Lu, P.D. Sponseller, L.H. Riley, X. Zhou, J. Hu, X. Cao, Ciliary parathyroid hormone signaling activates transforming growth factor-β to maintain intervertebral disc homeostasis during aging, Bone Res. 6 (2018) 21.
[32] Y.D. Zhou, J.G. Hou, W. Liu, S. Ren, Y.P. Wang, R. Zhang, C. Chen, Z. Wang, W. Li, 20(R)-ginsenoside Rg3, a rare saponin from red ginseng, ameliorates acetaminophen-induced hepatotoxicity by suppressing PI3K/AKT pathway-mediated inflammation and apoptosis, Int. Immunopharmacol. 59 (2018) 21–30.
[33] J. Tu, W. Li, Y. Zhang, X. Wu, Y. Song, L. Kang, W. Liu, K. Wang, S. Li, W. Hua, C. Yang, Simvastatin inhibits IL-1beta-induced apoptosis and extracellular matrix degradation by suppressing the NF-kB and MAPK pathways in nucleus pulposus cells, Inflammation 40 (3) (2017) 725–734.
[34] X. Li, D. Wu, Z. Hu, J. Xuan, X. Ding, G. Zheng, Z. Feng, W. Ni, A. Wu, The protective effect of ligustilide in osteoarthritis: an in vitro and in vivo study, Cell. Physiol. Biochem. 48 (6) (2018) 2583–2595.
[35] X. Wu, Y. Liu, X. Guo, W. Zhou, L. Wang, J. Shi, Y. Tao, M. Zhu, D. Geng, H. Yang, H. Mao, Prolactin inhibits the progression of intervertebral disc degeneration through inactivation of the NF-kappaB pathway in rats, Cell Death Dis. 9 (2) (2018) 98.
[36] Z. Zheng, Z.G. Wang, Y. Chen, J. Chen, S. Khor, J. Li, Z. He, Q. Wang, H. Zhang, K. Xu, G. Fanghua, J. Xiao, X. Wang, Spermidine promotes nucleus pulposus autophagy as a protective mechanism against apoptosis and ameliorates disc degeneration, J. Cell. Mol. Med. 22 (6) (2018) 3086–3096.
[37] Z. Li, K. Zhang, X. Li, H. Pan, S. Li, F. Chen, J. Zhang, Z. Zheng, J. Wang, H. Liu, Wnt5a suppresses inflammation-driven intervertebral disc degeneration via a TNF-α/NF-κB-Wnt5a negative-feedback loop, Osteoarthr. Cartil. 26 (7) (2018) 966–977.
[38] S. Zhongyi, Z. Sai, L. Chao, T. Jiwei, Effects of nuclear factor kappa B signaling pathway in human intervertebral disc degeneration, Spine 40 (4) (2015) 224–232.
[39] M.P. Vincenti, C.E. Brinckerhoff, Transcriptional regulation of collagenase (MMP-1, MMP-13) genes in arthritis: integration of complex signaling pathways for the recruitment of gene-specific transcription factors, Arthritis Res. Ther. 4 (3) (2001) 157.
[40] Y.-J. Surh, K.-S. Chun, H.-H. Cha, S.S. Han, Y.-S. Keum, K.-K. Park, S.S. Lee, Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation, Mutat. Res. Fundam. Mol. Mech. Mutagen. 480–481 (2001) 243–268.
[41] K. Hess, A. Ushmorov, J. Fiedler, R.E. Brenner, T. Wirth, TNFα promotes osteogenic differentiation of human mesenchymal stem cells by triggering the NF-κB signaling pathway, Bone 45 (2) (2009) 367–376.
[42] < Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase..pdf > .
[43] A.C. Issy, V. Castania, M. Castania, C.E. Salmon, M.H. Nogueira-Barbosa, E.D. Bel, H.L. Defino, Experimental model of intervertebral disc degeneration by needle puncture in Wistar rats, Braz. J. Med. Biol. Res. 46 (3) (2013) 235–244.