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Original Research Article

Protective effect of buganjianyao decoction against IL-1β-induced degeneration of endplate chondrocytes in rats via NF-κB signaling pathway

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Abstract

Purpose: *To investigate the protective effect of buganjianyao decoction (BJD) against IL-1β-induced degeneration of endplate chondrocytes in a rat model, and the underlying mechanism of action.*
Methods: *Rat endplate chondrocytes were cultured in 6-well tissue culture plates. Two types of serum were used: normal serum and BJD-containing serum. The endplate chondrocytes were grouped as follows: blank group given 0.5 % FBS, induced group treated with 0.5 % volume fraction of IL-1β (10 μg/L), normal serum groups treated with 5, 10 and 20 % volume fractions of normal serum, and BJD serum groups treated with 5, 10 and 20 % volume fractions of BJD-containing serum. Cell Counting Kit-8 (CCK-8) assay was used to measure the proliferation of endplate chondrocytes. The expressions of aggrecan and matrix metalloproteinase-3(MMP-3) were determined by ELISA, while reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to assay the mRNA expressions of IκB kinaseα (IKKα) and NF-κB p65. Western blotting was used measure the protein expressions of IKKα and NF-κB p65.*
Results: *Compared with normal serum group treated with a similar volume fraction, the proliferation capacity and aggrecan expression of BJD serum group increased at 24 h and 48 h post-treatment, while expressions of MMP-3, IKKα and NF-κB p65 decreased. The effects were more pronounced in the 20 % volume fraction of BJD serum group than in the other groups.*
Conclusion: *BJD exerts protective effect against IL-1β-induced degeneration of endplate chondrocytes via inhibition of the NF-κB signaling pathway. This finding provides an experimental basis for the potential development of BJD for the treatment of DDD.*

Keywords: *Buganjianyao decoction, Endplate chondrocytes, Aggrecan, Matrix metalloproteinase-3, Nuclear factor-kappa B*

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INTRODUCTION

Degenerative disc disease (DDD) is a major cause of low back pain, a frequently-occurring disease in orthopedics. In China's ageing society, DDD appears to be rapidly increasing year by year. The disease is characterized by protracted course of illness and repeated attacks, and some patients may lose the ability to work, leading to great economic and mental pressures.

Previous studies have shown that endplate cartilage is an important component of the intervertebral disc and its main channel of nutrition. Degeneration of endplate chondrocytes leads to degeneration of intervertebral disc nucleus pulposus, eventually resulting in a series of symptoms such as lumbar disc degeneration and lumbar disc herniation [1,2]. Therefore, endplate cartilage is crucial for maintenance of the normal function of intervertebral disc, and its degeneration is closely related to degeneration of the intervertebral disc [3,4]. As a result, it is very important to reduce endplate cartilage degeneration in order to prevent and treat DDD.

It has been reported that BJB is effective in the treatment for DDD, and it results in good clinical efficacy [5]. Previous studies have found that BJD has a good repair effect on lumbar intervertebral disc degeneration [6-11]. However, the mechanisms associated with the effect of BJD on degenerated endplate chondrocytes are poorly understood, and warrant further investigation. In this study, an *in vitro* model of IL-1β-induced degeneration of endplate chondrocytes was established, and the effect of BJD on proliferation of endplate chondrocytes, as well as the mRNA and protein expressions of NF-κB signaling pathway-related factors such as aggrecan, MMP-3expression, IKKα, NF-κB p65, were investigated.

EXPERIMENTAL

Animals

Three-month-old male Sprague-Dawley (SD) rats (180-220 g), and 3-week-old male SD rats (100-140 g) were supplied by Hunan Academy of Chinese Medical Science, China. The animals were maintained at a mean temperature of 22 ± 1 ºC, 50 ± 1% humidity and a photo period of 12-h light/12-h dark cycle. The biological license number of the experimental animals was SCXK (Xiang) 2015-0008. The study received approval from the ethics committee of Hunan Academy of Chinese Medical Science (approval no.2018-0002), and the study was carried out in line with international guidelines for animal studies [12].

Drug

Buganjianyao decoction (BJD) comprised the following Chinese herbs: Danggui (*Angelica sinensis,* 15g); *chuanxiong* (*ligustici wallichii*, 12g); *baishao* (radix paeoniae alba, 30g); *shudihuang* (rehmannia rehmanniae, 25g); *chaozaoren* (fried *jujube* benevolence, 10g); *duzhong* (*Eucommia ulmoides,* 15g); *yanhusuo* (*Corydalis yanhusuo*, 10g); *wugong* (centipede, 4g); quanxie (scorpion, 3g); and *gancao* (licorice, 5g. All drugs were provided by the TCM pharmacy of Affiliated Hospital of Hunan Academy of Chinese Medical Science. The raw components of the BJD were combined and steeped in distilled water (10:1, v:w) and boiled for 30 min. The drug was decocted once again following the same steps. Finally, the boiled liquids from the first and second decoctions were mixed and concentrated to obtain a crude drug of concentration 2.322g/mL.

Reagents

Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone (USA), while fetal bovine serum (FBS) was purchased from Gibco (USA). Counting Kit-8 (CCK-8, catalog number NM641) was supplied by Dojindo Laboratories, while IL-1β (catalog number 110719) and ELISA kits for aggrecan and MMP-3 (catalog numbers Z12036801 and Z12036802, respectively, were purchased from Wuhan Huamei Biological Engineering Co. Ltd. Tris (catalog number #SLBW1297), Trizol (catalog no.01761/30324) and UltraSYBR Mixture (catalog no.40352) were bought from Beijing Kangwei Century Biotechnology Co. Ltd. IκB Kinase (IKK) and NF-κB p65 mRNA primer were products of Shanghai Shenggong Biological Engineering Technology Service Co. Ltd), while antibodies for IKKα, NF-κB p65 and β-actin were purchased from Santa Cruz Group Inc. and Proteintech Group Inc (catalog numbers were SC-7218, 10745-1-AP, and 60008-1-Ig, respectively).

Preparation of BJD-containing serum and normal serum

After adaptive feeding for 5 days, 20 SD male rats aged 3 months were randomly assigned to 2 groups: drug-containing serum group and blank control group (10 rats/group), according to equivalent dose conversion coefficients of different animals [13]. Rats in drug-containing serum group were intragastrically administered the human dose of BJD i.e. 1ml/200 g, twice daily, while rats in blank control group were intragastrically administered an equivalent volume of physiological saline. On day 8, 1 h after the last intragastric dose, the rats were anesthetized after which blood samples were drawn from the abdominal aorta. *Buganjianyao* decoction (BJD)-containing serum and normal serum were prepared. After sterilization, the serum samples were kept in a -20℃ refrigerator after incubation in a water bath at 56 ℃ for 30 min in order to inactivate serum complements and antibodies. These were subsequently filtered

and the serum samples were sterilized again prior to use.

Isolation and culture of endplate chondrocytes

Five 4-week-old SD rats were sacrificed by decapitation. Then, the L1-L6 lumbar endplate cartilage was harvested to isolate endplate chondrocytes using the following procedure: digestion with 0.2 % trypsin in a 37 ℃ incubator for 20 min, followed by digestion with 0.02 % collagenase at 37 ℃ for 24 h. The chondrocytes were filtered through a 100-μm cell sieve and washed in phosphate buffered saline (PBS), followed by seeding in a culture bottle at a density of 2 x 10^4/cm^2 in DMEM containing 10 % fetal bovine serum and 1 % penicillin and streptomycin. The cells were cultured in a 37 ℃ incubator containing 5 % CO_2 and 95 % air. Endplate chondrocytes were identified using collagen type immunofluorescence (Figure 1).

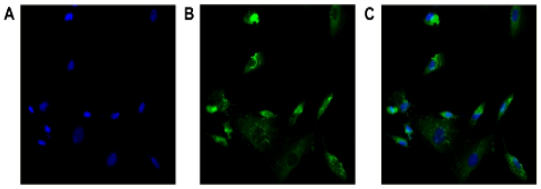

Figure 1: Immunofluorescence results of collage type II. (A) Nucleus (blue); **(B)** collage type II (green); **(C)** merged image

Experimental grouping and intervention

The endplate chondrocytes were cultured in 6-well tissue culture plates. Experimental grouping and intervention were as follows: blank group (0.5% FBS), induced group treated with 0.5% volume fraction of IL-1β (10 μg/L), normal serum groups treated with 5, 10 and 20 % volume fractions of normal serum, and BJD serum groups treated with 5, 10 and 20 % volume fractions of BJD-containing serum. There were six wells for each group, with 100 μL in each well. The well plates were cultured for 24 and 48 h, and the following indices were determined:

Determination of proliferation of endplate chondrocytes

A total of 10 μl CCK-8 assay reagent was added to each culture plate, and culturing was continued at 37 ℃ for 4 h. Then, the absorbance (OD) of each well was read at 450 nm and the results were analyzed as index of proliferation of endplate chondrocytes according to the manufacturer's protocols.

Assay of expressions of aggrecan and MMP-3

The expressions of aggrecan and MMP-3 in endplate chondrocytes were assayed with ELISA after cell supernatants were collected from cell culture medium through centrifugation.

Assay of mRNA expressions of IKKα and NF-κB p65

Real-time quantitative polymerase chain reaction (RT-qPCR) was used to measure the mRNA expression levels of IKKα and NF-κB p65 in endplate chondrocytes. Total RNA was extracted from each culture well with TRIzol reagent, and RT-qPCR was performed after RNA reverse transcription. The amplification conditions were 40 cycles of 95 ℃ for 10 min, 95 ℃ for 10 sec, and 60 ℃ for 60 s. The melting curve acquisition range was 60 – 95 ℃. The relative gene expressions were estimated with the $2^{-\triangle\triangle Ct}$ procedure. The sequences of primers employed are indicated in Table 1.

Table 1: Primers for RT-qPCR

Target gene	Primer	Length
IKKα	Forward: ACTTAAAGAGAGCCAGATCCTT Reverse: ATCGACATCTTTGGCATAACCC	175bp
NFKB-p65	Forward: ACTATGGATTTCCTGCTTACGG Reverse: GCACAATCTCTAGGCTCGTT	118bp
Actin	Forward: TGGTGATGGAGGAGGTTTAGTAAGT Reverse: AACCAATAAAACCTACTCCTCCCTTAA	133bp

Assay of protein expressions of IKKα and NF-κB p65

Western blotting was used to assay the protein expressions of IKKα and NF-κB p65 in endplate chondrocytes. The endplate chondrocytes were washed with PBS, and the supernatants were removed after centrifugation at 3000 rpm for 2 min. The supernatant was then incubated with 100 uL of RIPA lysis buffer for 10 min, and centrifuged at 12000 rpm for 15 min at 4 ℃. Protein content was determined in supernatant with BCA assay kit. Then, equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis and electroblotted to PVDF membranes. The membranes were blocked by

incubation with 5 % BSA solution for 1 h, after which they were incubated overnight at 4 ℃ with the following primary antibodies: rabbit anti-IKKα, rabbit anti-NF-κB p65, and mouse anti-β-actin (Santa Cruz Group,Inc, and Proteintech Group, Inc) at dilutions of 1:2000, 1:2000 and 1:5000, respectively. Thereafter, the membranes were rinsed thrice with Tris-buffered saline containing Tween (TBST), each rinse for 15 min, followed by incubation with HRP-conjugated mouse anti-rabbit secondary antibody (1:5000 dilution, Proteintech Group) for 90 min at room temperature. Thereafter, the membranes were washed three times with TBST (15 min for each rinse), followed by visualization using enhanced chemiluminescence reagent, and exposure to X-ray film. Grayscale analysis of expression signals relative to β-actin was done with Quantity One software.

Statistical analysis

Data were evaluated with SPSS 16.0 statistical software (IBM, Armonk, NY, USA). Measurement data are presented as mean ± standard deviation (SD). When the measurement data met the requirements for normality and homogeneity of variance, one-way analysis of variance (LSD method) was used, while non-parametric test was used for inconsistent data. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Endplate chondrocytes

Compared with normal serum group treated with similar volume fraction, the endplate cartilage cell proliferation capacity in BJD serum group increased 24 h and 48 h after treatment ($p < 0.05$). Higher cell proliferation was observed in the 20 % volume fraction BJD serum group than in the 5 % and 10 % volume fraction BJD serum groups ($p < 0.05$). These results which are shown in Figures 2 and 3, suggest that BJD increased the proliferation of IL-1β-induced degenerated endplate chondrocytes.

Effect of BJD on the expressions of aggrecan and MMP-3

Compared with normal serum group treated with similar volume fraction, increased expressions of aggrecan were observed in BJD serum group 24 h and 48h after treatment, while the MMP-3 expression decreased ($p < 0.05$; Figure 4). Higher expression of aggrecan and lower expression of MMP-3 were observed in the 20 % volume fraction of BJD serum group, when compared to 5 % and 10% volume fraction BJD serum groups ($p < 0.05$). These results suggest that BJD upregulated aggrecan expression and decreased MMP-3 expression in IL-1β-induced degenerated endplate chondrocytes.

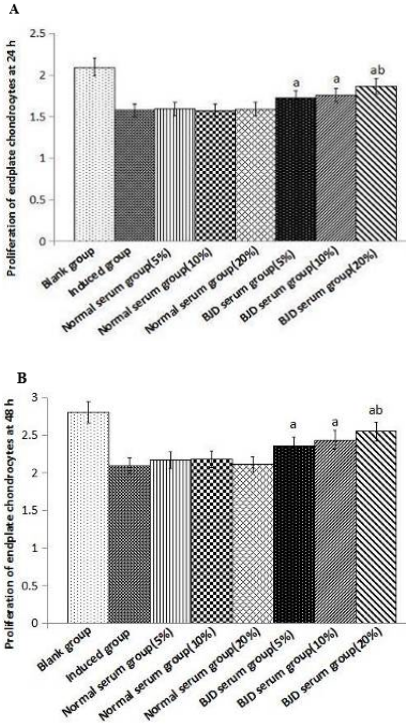

Figure 2: Effect of BJD on the proliferation of endplate chondrocytes in each group at 24 h (A) and 48 h (B) after treatment. $^{a}P < 0.05$, vs normal serum group with corresponding volume fraction for the same duration; $^{b}p < 0.05$, vs 5 % and 10% volume fraction BJD serum groups

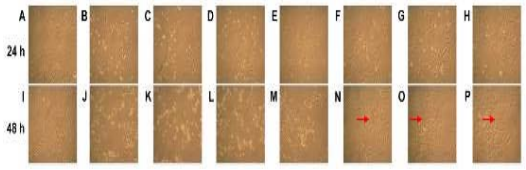

Figure 3: Appearance of endplate chondrocytes under inverted phase contrast microscope. **A**, I: blank group; **B, J: i**nduced group; **C, K:** 5 % normal serum group; **D, L:** 10 % normal serum group; **E, M:** 20 % normal serum group; **F, N:** 5 % BJD serum group; **G, O:** 10 % BJD serum group; **H, P:** 20 % BJD serum group

Effect of BJD on mRNA expressions of IKKα and NF-κB p65

Compared with normal serum group treated with similar volume fraction, decreased mRNA expressions of IKKα and NF-κB p65 were observed in BJD serum group 24 h and 48 h after treatment ($p < 0.05$). In addition, the mRNA expressions of IKKα and NF-κB p65 were significantly lower in the 20 % volume fraction

BJD serum group than in 5 % and 10 % volume fraction BJD serum groups ($p < 0.05$; Figure 5). These results suggest that BJD decreased the mRNA expressions of IKKα and NF-κB p65 in IL-1β-induced degenerated end plate chondrocytes.

Effect of BJD on protein expressions of IKKα and NF-κB p65

As shown in Figure 6 and Figure 7, compared with normal serum group treated with similar volume fraction, the IKKα and NF-κB p65 protein expressions in BJD serum group decreased 24 h and 48 h after treatment ($p < 0.05$). Moreover, the protein expressions of IKKα and NF-κB p65 were significantly lower in the 20 % volume fraction BJD serum group than in 5 % and 10 % volume fraction BJD serum groups ($p < 0.05$). These findings indicate that BJD decreased the protein expressions of IKKα and NF-κB p65 in IL-1β-induced degenerated endplate chondrocytes.

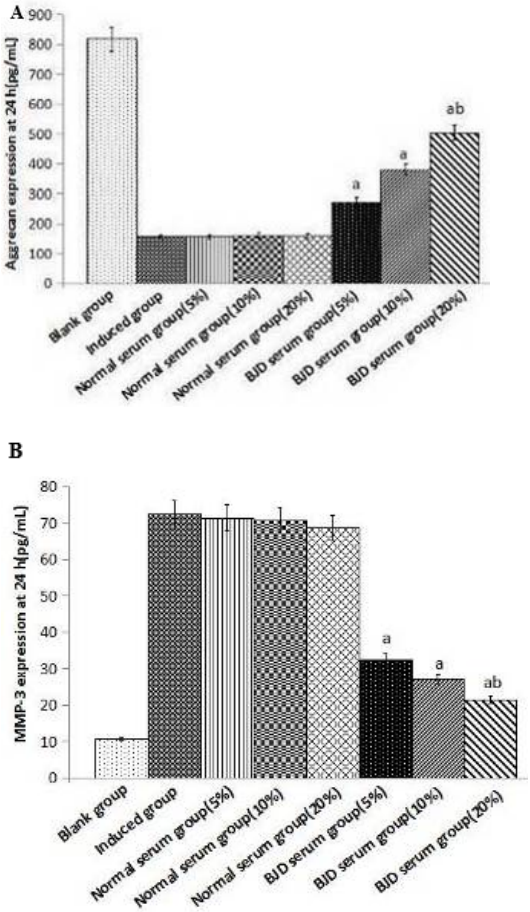

Figure 4: Effect of BJD on the expression of aggrecan (A) and MMP-3 (B) 24 h after treatment. [a]$P < 0.05$, vs normal serum group with similar volume fraction for the same duration; [b]$p < 0.05$, vs with 5 % and 10 % volume fraction BJD serum groups

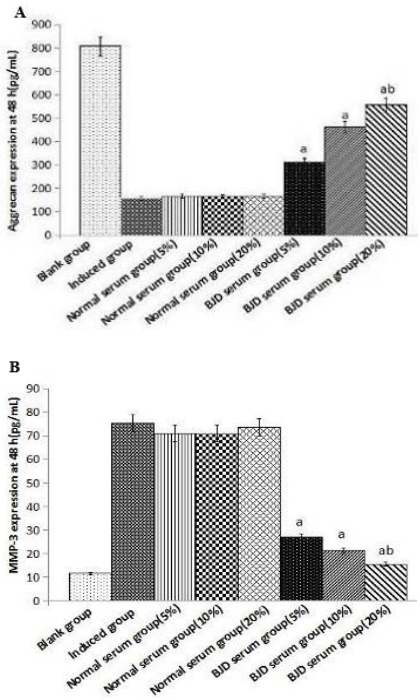

Figure 5: Effect of BJD on the expression of aggrecan (A) and MMP-3 (B) 48 h after treatment. [a]$P < 0.05$, vs normal serum group with similar volume fraction for the same duration; [b]$p < 0.05$, vs with 5 % and 10 % volume fraction BJD serum groups

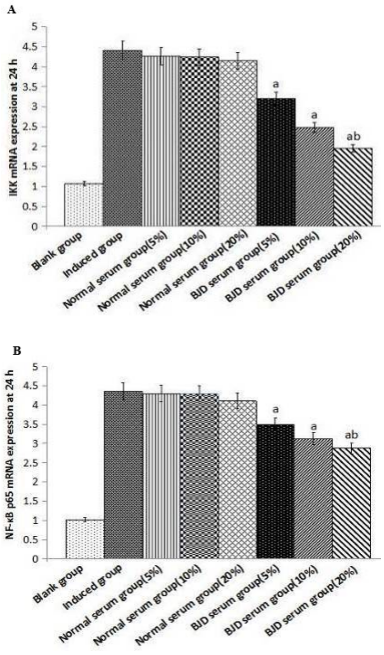

Figure 6: Effect of BJD on the mRNA expressions of IKKα (A) and NF-κB p65 (B) in each group 24 h after treatment. [a]$P < 0.05$, vs normal serum group treated with similar volume fraction for the same duration; [b]$p < 0.05$, vs 5 % and 10 % volume fraction BJD serum groups

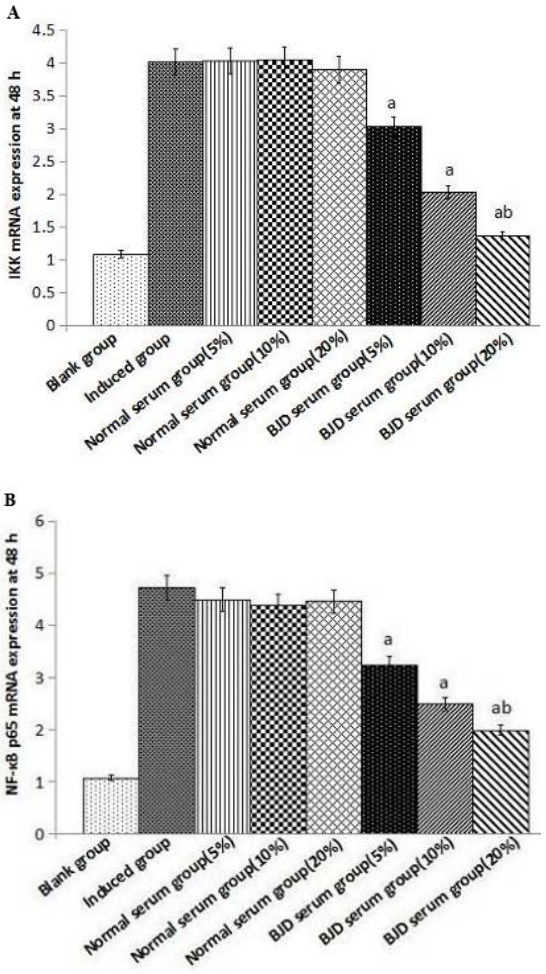

Figure 7: Effect of BJD on the mRNA expressions of IKKα (A) and NF-κB p65 (B) in each group 48 h after treatment. [a]*P* < 0.05, vs normal serum group treated with similar volume fraction for the same duration; [b]*p* < 0.05, vs 5 % and 10 % volume fraction BJD serum groups

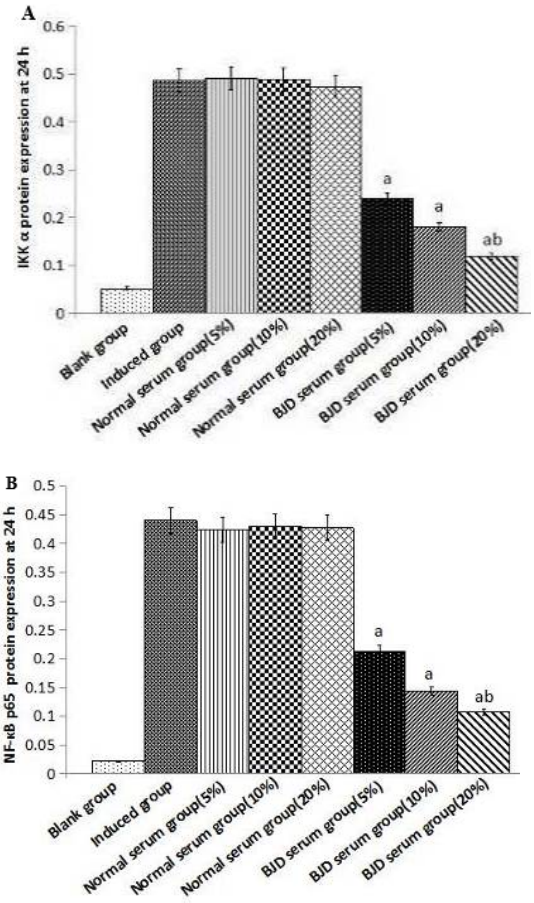

Figure 8: Effect of BJD on protein expressions of IKKα (A) and NF-κB p65 (B) in each group 24 h after treatment. [a]*P* < 0.05, vs normal serum group treated with similar volume fraction for the same duration; [b]*p* < 0.05, vs 5 % and 10 % volume fraction BJD serum groups

DISCUSSION

In this study, it was found that BJD exerted protective effect against IL-1β-induced degeneration of endplate chondrocytes via inhibition of the NF-κB signaling pathway. Thus, it promoted the expression of aggrecan and reduced MMP-3 expression by regulating the mRNA and protein expressions of IKKα and NF-κB p65. This might serve as a mechanism for the prevention and treatment of degenerative disc diseases. The primary nutrient supply to the intervertebral disc is activated by adjacent endplate cartilage. Thus, an intact endplate cartilage is crucial for maintaining normal function of the intervertebral disc. Therefore, once the endplate cartilage degenerates, its porosity and permeability are significantly reduced, thereby reducing the nutrient supply to the intervertebral disc, a situation which results in degenerative changes in intervertebral disc. The protein, IL-1β, which is produced by chondrocytes, osteoclasts and other cells, disturbs the balance between anabolism and catabolism, and induces degeneration of endplate chondrocytes. Therefore, IL-1β is often used for *in vitro* induction of degeneration of endplate chondrocytes in experiments [14]. The NF-κB signaling pathway has an important role in degeneration of endplate chondrocyte [15-18]. In the present study, the NF-κB signaling pathway was activated in the pathogenesis of DDD. The expressions of IL-6, MMP-3 and other downstream products were increased, while aggrecan expression was decreased. The NF-κB signaling pathway is activated by exogenous or endogenous stimulants such as IL-1,

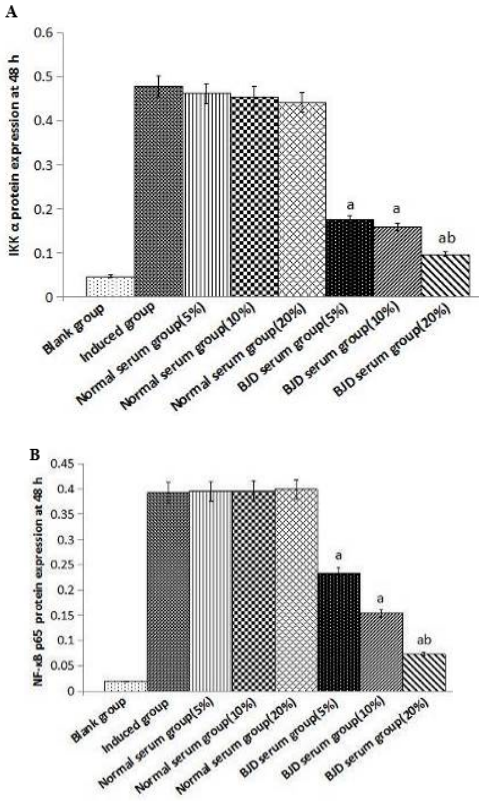

Figure 9: Effect of BJD on protein expressions of IKKα (A) and NF-κB p65 (B) in each group 48 h after treatment. [a]*P* < 0.05, vs normal serum group treated with similar volume fraction for the same duration; [b]*p* < 0.05, vs 5 % and 10 % volume fraction BJD serum groups

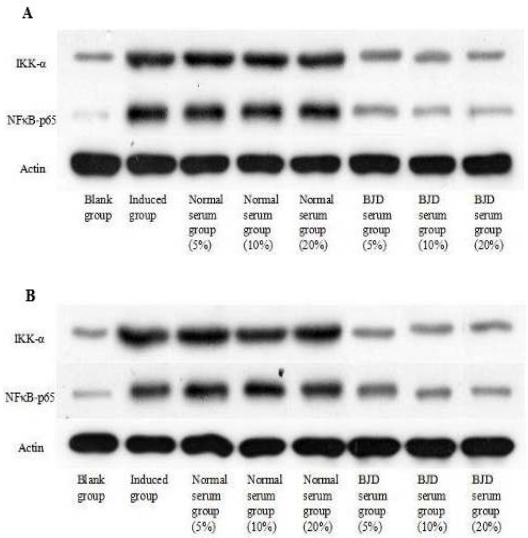

Figure 10: Effect of BJD on protein expressions of IKKα and NF-κB p65 in each group 24 h (A) and 48 h (B) after treatment

lipopolysaccharide (LPS), and various physical and chemical pressures [19,20]. The results of this study are consistent with those obtained in other studies [21,22]. Treatment of the end plate chondrocytes with IL-1β resulted in upregulation of the mRNA and protein expressions of IKKα and NF-κB p65, as well as the expression of MMP-3, a downstream factor. These events led to reduced expression of aggrecan, and ultimately to cell apoptosis.

Intervertebral disc belongs to the category of "jin" in traditional Chinese medicine (TCM). The liver *governs* the sinews, and *governs* blood capacity to meet physiological needs. In TCM, the state of the liver determines the condition of the tendons. The liver ensures that the tendons are unlocked and run smoothly, and it also directly affects blood supply to fibrocartilage ring and ligament. When the liver blood supply is insufficient, or when the liver does not contain blood, it becomes weak and broken, resulting in DDD which exacerbates the degeneration of tendons. This TCM theory gave rise to the proposal of "treating from the liver" according to the clinical characteristics of DDD. The BJD prescription is based on the *Bugan* decoction in *YizongJinjian* and *YixueLiuyao*. A comprehensive assessment of the prescription reveals that it can nourish blood and *soften* the liver, activate blood circulation and remove *blood stasis*, clear the *collaterals* and relieve pain, thereby mitigating the symptoms of waist pain, numbness, and DDD-related pain. Previous experiments have revealed that BJD produced good effect on lumbar intervertebral disc degeneration via its anti-inflammatory, analgesic and immunomodulatory properties [6-11]. However, the underlying mechanisms was not elucidated.

The results obtained in the current study clearly revealed that BJD produced a protective effect against IL-1β-induced degeneration of endplate chondrocytes. The serum worked by inhibiting the NF-κB signaling pathway, i.e. it promoted the expression of aggrecan and reduced the expression of MMP-3 via regulation of the mRNA and protein expressions of IKKα and NF-κB p65. Serum with 20 % volume fraction BJD was more effective than serum with lower BJD volume fractions. These findings have unraveled the mechanisms through which BJD mitigates lumbar intervertebral disc degeneration, thereby providing an important experimental basis for the treatment of DDD.

CONCLUSION

The results obtained in this study suggest that BJD exerts a protective effect against IL-1β-induced degeneration of endplate chondrocytes via inhibition of NF-κB signaling pathway, and

thus provides an experimental basis for the development of BJD for the treatment of DDD.

DECLARATIONS

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Competing interests

The authors have no competing interests with regard to this work.

Contributions of authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Xuyi Tan and Xincheng Zhang carried out the studies, participated in collecting data, and drafted the manuscript. Xiangzhong Qiu, Jie Qiu and Hao Tang performed the statistical analysis and participated in the design of the study. Shengchang Jiang, Fan Xue and Hao Deng drafted the manuscript. All authors read and approved the final manuscript.

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