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Article

Duhuo Jisheng Decoction inhibits SDF-1-induced inflammation and matrix degradation in human degenerative nucleus pulposus cells *in vitro* through the CXCR4/NF-κB pathway

Zong-chao LIU[1, *, #], Zhen-long WANG[1, #], Chen-yi HUANG[1], Zhi-jiang FU[1], Yong LIU[1], Zhang-chao WEI[1], Shi-gui LIU[1], Chuan MA[1, *], Jie-liang SHEN[2], Dayue Darrel DUAN[3, 4]

[1]*Department of Orthopedic Surgery, the Traditional Chinese Medicine Affiliated Hospital of Southwest Medical University, Luzhou 646000, China;* [2]*Department of Orthopedic Surgery, the First Affiliated Hospital of Chongqing Medical University, Chongqing 400016, China;* [3]*Center for Phenomics of Traditional Chinese Medicine, Southwest Medical University, Luzhou 646000, China;* [4]*Laboratory of Cardiovascular Phenomics, Department of Pharmacology, University of Nevada Reno School of Medicine, Reno, NV 89557, USA*

Abstract

Lower back pain (LBP) is the most common disease in orthopedic clinics world-wide. A classic Fangji of traditional Chinese medicine, Duhuo Jisheng Decoction (DHJSD), has been proven clinically effective for LBP but its therapeutic mechanisms remain unclear. We hypothesized that DHJSD might relieve LBP through inhibiting the exaggerated proinflammatory cytokines and extracellular matrix (ECM) degradation. Thus, we studied the effects of DHJSD on stromal cell-derived factor-1 (SDF-1)-induced inflammation and ECM degradation in human nucleus pulposus cells (hNPCs). The primary hNPCs were isolated from either degenerated human intervertebral disc (HID) of LBP patients or normal HID of lumbar vertebral fracture patients, and cultured *in vitro*. The cells were treated with SDF-1 (10 ng/mL) and subsequently with different concentrations (100–500 μg/mL) of DHJSD for 24 h, respectively. We found that application of DHJSD significantly antagonized the SDF-1-induced production of proinflammatory cytokines and reduction of aggrecan and type II collagen in the hNPCs. DHJSD also markedly reduced the SDF-1-induced increase of CXCR4 and p-p65 and inhibited the nuclear translocation of p65 in the hNPCs. DHJSD, CXCR4-siRNA, and NF-κB inhibitor (BAY11-7082) caused the same inhibition of exaggerated proinflammatory cytokines in the SDF-1-treated hNPCs. These results provided compelling evidence that DHJSD may inhibit the generation of proinflammatory mediators and ECM degradation of HID through an orchestrated targeting at multiple molecules in the SDF-1/CXCR4/NF-κB pathway, thus offered novel mechanistic insights into the clinical effectiveness of DHJSD on LBP.

Keywords: duhuojisheng decoction; traditional Chinese medicine; SDF-1; NF-κB; degenerative intervertebral disc; lower back pain

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Introduction

Lower back pain (LBP) is the most common disease in orthopedic clinics and causes a huge financial burden worldwide[1, 2]. About 40% of the patients with LBP are caused by intervertebral disc degeneration[3]. But the causative and molecular mechanisms of LBP have not yet been fully elucidated. It has been found that the exaggerated expressions of proinflammatory factors such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) is positively correlated with intervertebral disc degeneration[4]. An increase of proinflammatory factors promoted the extracellular matrix (ECM) degradation, which is a key cause of intervertebral disc degeneration[5]. Also involved in the inflammation and degradation are a small family of soluble chemically attractive cytokines that regulate the recruitment of peripheral response cells[5, 6]. One of the most widely studied chemokines is stromal cell-derived factor-1 (SDF-1)[5, 6]. The main function of SDF-1 is to guide cell migration and to further stimulate the chemotaxis of inflammatory cells and induce cell surface to produce integrins. Our previous studies have shown that the SDF-1 expression and apoptosis in degeneration of human

[#]These authors contributed equally to this work.
[*]To whom correspondence should be addressed.
E-mail langmanzhishen2008@163.com (Chuan MA);
565409672@qq.com (Zong-chao LIU)
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intervertebral disc (HID) tissues were significantly higher than in normal HID tissues[6]. It has also been reported that SDF-1 promotes the metabolism of chondrocytes by stimulating the release of matrix metalloproteinase-1 (MMP-1) and -13 (MMP-13) in osteoarthritis[7]. SDF-1activates various cells by binding to the G-protein-coupled receptor (GPCR), C-X-C motif chemokine receptor 4 (CXCR4)[7, 8]. CXCR4 has been recognized as the only receptor for SDF-1 and is the only regulator of SDF-1-mediated biological activity[9]. SDF-1α/CXCR4 promote inflammation of the vessel wall[9, 10]. The SDF-1α / CXCR4 axis inhibitor AMD3100 can reduce the expression of inflammatory factors in lung tissue and airway inflammation as well as airway hyperresponsiveness[9, 11]. In recent years, nuclear factor-kappa B (NF-κB) has been found to be involved in SDF-1 mediated signaling pathways[12-14]. NF-κB is a transcription factor, which has an important effect on the response of the cell to injury, stress, and inflammatory reaction. It has been also reported that NF-κB plays a significant role in disc degeneration[15-17].

Current treatments of LBP, either surgical or non-surgical, have been usually ineffective and often limited by side effects[2]. However, a widely used classic fangji of traditional Chinese medicine (TCM), duhuojisheng decoction (DHJSD), has been proven very effective clinically in the treatment of LBP for thousands of years[18-37]. In TCM, LBP is defined as a part of "feng-han-shi" "Bi Zheng" caused by deficiency in both "Gan" and "Shen" or "Qi" and "Xue". DHJSD is an effective eliminator of "feng-han-shi" through boosting "Gan" and "Shen" functions and therefore can reinforce the bones and tendons and relieve pain of "Bi Zheng"[38]. But the detailed molecular mechanisms for the effectiveness of DHJSD on LBP remain unclear. Although some modern pharmacological studies have demonstrated that DHJSD may have effects of analgesia, immune function regulation, blood vessel dilation, anti-inflammation, and antiplatelet aggregation[20, 22, 23, 26, 39, 40], the mechanisms of the therapeutic effect of DHJSD on HID degeneration are unknown. In this study we used the unique model of SDF-1-treated human nucleus pulposus cells (hNPCs) to test our hypothesis that DHJSD may retard HID degeneration and relieve the LBP through an orchestrated inhibition of the exaggerated production of multiple proinflammatory cytokines and ECM degradation in HID.

Materials and methods

DHJSD aqueous extract preparation

The origin, medicinal composites, and processing technology of DHJSD were standardized based on marker compounds to achieve quality control according to the Chinese Pharmacopoeia 2015 (Chinese Pharmacopeia Commission: Pharmacopoeia of the People's Republic of China. Chinese Medical Science and Technology Press; Beijing, China, 2010), the same as previously described and published[20, 22, 23, 26, 39, 40]. Briefly, the 15 components of DHJSD, namely 9 g of Duhuo (Radix Angelicae Pubescentis), 6 g of Jisheng (Herba Taxilli), Qinjiao (Radix Gentianae Macrophyllae), Fangfeng (Radix Saposhnikoviae), Xixin (Herba Asari), Rougui (Cortex Cinnamomi), Fuling (Poria Cocos), Chuanxiong (Rhizoma Chuanxiong), Danggui (Radix Angelicae Sinensis), Niuxi (Radix Achyranthis Bidentatae), Dihuang (Radix Rehmanniae Preparata), Baishao (Radix Paeoniae Alba), Duzhong (Cortex Eucommiae Ulmoidis), Renshen (Panax ginseng) and Gancao (Radix Glycyrrhizae)[20, 22, 23, 26, 39, 40], were mixed and soaked in distilled water, rotary evaporator for rotary steam and the solution was filtered and concentrated[41]. After boiled for 30 min twice the DHJSD solution was filtered (filter 0.22 μm) and then dissolved in DMEM/F12 medium containing 15% fetal bovine serum at a final concentration of 10 mg/mL. The solution was subsequently filtered again through a filter (0.22 μm) and stored at 4 °C as a stock solution before use. In the rest of the experiments, the stock DHJSD solution (10 mg/mL) was further dissolved into the medium with final corresponding concentrations of 100, 200, 300, 400 and 500 μg/mL of DHJSD.

Primary hNPC isolation and culture

The study protocol of using human tissues was approved by the Ethics Committee of Southwest Medical University (Luzhou, China). Written informed consent was obtained from all tissue donors before their surgeries. All degenerative NP tissues were acquired from lumbar spine surgery patients. The relatively normal HID tissues as control were obtained from 5 volunteers [2 females and 3 males aged 20–28 (24.4±3.3) years old] with lumbar vertebral fracture (LVF) who did not have a documented medical history of LBP. The human IDD tissues were obtained from 15 patients with lumbar disc herniation (LDH) [5 females and 10 males aged 49–72 (58.2±7.3) years old]. The degree of intervertebral disc degradation (IVDD) was assessed according to Pfirrmann classification by preoperative magnetic resonance imaging scans[42]. The IVDD group exhibited Pfirrmann grades III–V. The human NP tissues were microscopically isolated from IVDD tissues by a scalpel under sterile conditions. Then the tissues were washed twice with PBS and cut into 1 mm^3 fragments. The fragments of NP tissues were digested in 0.25% trypsin solution for 30 min followed by 0.2% type II collagenase for 3–4 h at 37 °C. Tissue debris was removed by passing through a 200-μm filter and then the NP cells were resuspended in DMEM/F12 containing 15% FBS and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. When the cells grew to confluence of 80%–90%, they were digested by 0.25% trypsin solution and subcultured in culture flasks. The third generation of hNPCs was used for all experiments.

Cell viability assay

Cell viability of hNPCs was determined by the Cell Counting Kit-8 (CCK-8) assay according to the manufacturer's instruction (Dojindo Laboratories, Kumamoto, Japan). Briefly, hNPCs were seeded in a 96-well plate (100 μL/well) at a density of 1×10^4 cells/mL and cultured for 24 h. They were subsequently treated with or without 10 ng/mL SDF-1 (Sigma-Aldrich, St Louis, MO, USA) and various concentrations of DHJSD (100, 200, 300, 400 and 500 μg/mL) for 24 h, respectively. After

24 h incubation, 10 μL of the WST-8 reagent [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfonyl)-2*H*-tetrazolium] was added to each well, and the cells were incubated at 37°C for 2 h. The absorbance of the wells was measured at 450 nm by a Bio-Rad 680 microplate reader (Bio-Rad, Hercules, CA, USA). Each experiment was performed in three replicate wells.

Cell treatments

After the cell viability assay with CCK-8, the hNPCs were treated with control, SDF-1 (10 ng/mL), or DHJSD + SDF-1 for 24 h. Double-stranded small interfering RNA (siRNA) targeting CXCR4 (CXCR4-siRNA) was transfected into the cultured cells using PepMutesiRNA Transfection Reagent (Signa Gen Laboratories, Rockville, MD, USA) according to the manufacturer's protocol (Santa Cruz Biotechnology, Inc, Dallas, TX, USA; cat. no. Sc-35422). After transfection with 50 nmol/L CXCR4-siRNA for 72 h, the cultured cells were treated with or without SDF-1 (10 ng/mL) and DHJSD (300 μg/mL) for 24 h at 37°C.

Fluorescence immunocytochemistry

After each group of cells were treated, cells cultured in 24-well plates were washed by PBS for several times and then were fixed with 4% paraformaldehyde for 10 min. the cells were blocked with normal goat serum for 1 h, and then were incubated with p65 antibody (1:100) at 4°C overnight. Finally, Nuclear counterstaining was incubated with 4',6-diamidino-2-phenylindole. After washed three times with PBS, the cells were observed through a fluorescence microscope.

Real-time PCR analysis

The total RNA was extracted from human NP tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. An ultraviolet spectrophotometer (Olympus, Japan) was used to measure the purity and concentration of RNA. Next, 1 μg of mRNA was reverse transcribed to cDNA. The cDNA samples were amplified by performing real-time PCR in ABI Prism 7500 (ABI, USA) by using SYBR® Green Real-Time PCR Master Mix (TOYOBO, QPK-201). Relative expression levels of the indicated genes were calculated using $2^{-\Delta\Delta Ct}$ method. All primers were synthesized by TaKaRa (TaKaRa, China). The Primers for human genes were in Table 1.

Table 1. Primers for PCR.

Gene	Primer Sequence
GAPDH	Sense: 5'-GCACCGTCAAGGCTGAGAAC-3'
	Antisense: 5'-TGGTGAAGACGCCAGTGGA-3'
IL-1β	Sense: 5'-GAAATGATGGCTTATTACAGTGGC-3'
	Antisense: 5'-GCCACTGTAATAAGCCATCATTTC-3'
TNF-α	Sense: 5'-TCATCTACTCCCAGGTCCTCTTCA-3'
	Antisense: 5'-TGAAGAGGACCTGGGAGTAGATGA-3'
MMP-3	Sense: 5'-ATTCCATGGAGCCA GGCTTTC-3'
	Antisense: 5'-CATTTGGGTCAAACTCCAACTGTG-3'
MMP-13	Sense: 5'-TTGATGATGATGAAACCTGGACAAG-3'
	Antisense: 5'-TTGCCGGTGTAGGTGTAGATAGGAA-3'

ELISA

After each group of cells were treated for 24 h, the supernatants were collected and centrifuged to remove cell fragments. The levels of TNF-α and IL-1β in the supernatant were measured using an ELISA kit (R&D Systems, Inc. Minneapolis, MN, USA) and a microplate reader (Omega Bio-Tek, Inc) according to the manufacturer's instructions.

Western blot analysis

Total protein was extracted using radio-immunoprecipitation assay (RIPA) lysis buffer containing a mixture of protease inhibitor, and protein concentration was measured by Enhanced BCA Protein Assay Kit (Beyotime, P0010S). Protein samples were mixed with sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample loading buffer, boiled for 5 min. After electrophoresed in 6%–12% SDS-PAGE gels, protein samples were transferred to polyvinylidene difluoride membrane (PVDF, 0.45 or 0.22 μm). The membranes were blocked with 5% nonfat dry milk in tris-buffered saline (TBST) for 1 h and incubated with primary antibodies rabbit anti-p-p65 and anti-p65 (Cell Signaling Technology, Inc, Beverly, MA, USA), rabbit anti-MMP-3 and anti-MMP-13 (Epitomics), rabbit anti-CXCR4 (Cell Signaling Technology, Inc, Beverly, MA, USA), mouse anti-TNF-α (OriGene, Herford, Germany), mouse anti-IL-1β (OriGene) and mouse anti-β-actin (Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) overnight at 4°C. After washed three times for 10 min in TBST, the membranes were incubated in secondary antibody for 2 h. Finally, the membranes were treated with ECL plus reagent (Invitrogen, USA) and the results were analyzed by the accompanied software.

Statistical analysis

All measurements were performed in triplicate, and results were expressed as mean±standard deviation (SD). Statistical analysis was performed by one-way analysis of variance (ANOVA) or Student's *t*-test using SPSS 19.0 (IBM, Corp, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Basal expression of TNF-α, IL-1β, MMP-3 and MMP-13 in human NP tissues

The expression levels of TNF-α, IL-1β, MMP-3 and MMP-13 were significantly increased in the degenerative human NP tissues compared with the normal NP tissues as revealed by PCR (Figure 1A) and Western blot (Figure 1B) analyses. These results suggest that the increase of multiple proinflammatory factors and extracellular matrix (ECM) degradation may play a critical role in the process of human IVDD.

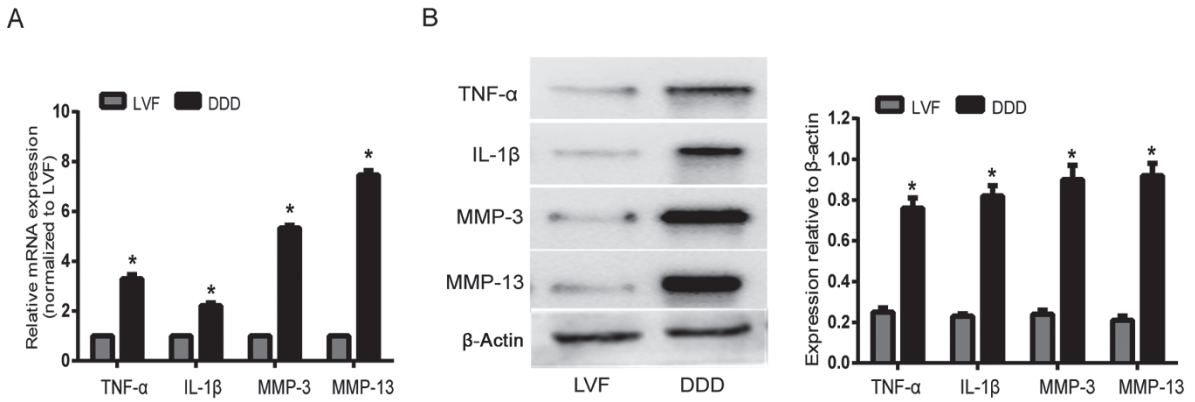

Figure 1. Expression of proinflammatory factors in human nucleus pulposus cells (hNPCs). Real-time PCR (A) and Western blot (B) analyses for TNF-α, IL-1β, MMP-3, and MMP-13 expression in hNPCs from patients with lumbar vertebra fracture (LVF) and disc degenerative disease (DDD). Data represents mean±SD, $^{*}P<0.05$ *vs* LVF group.

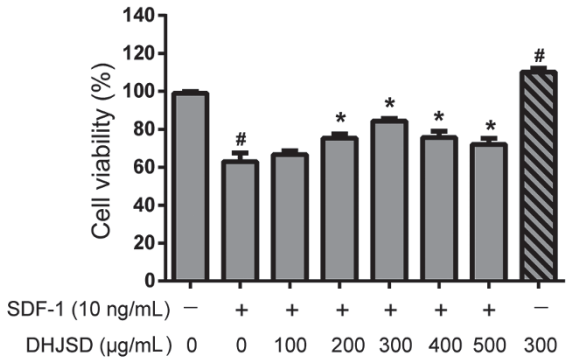

Figure 2. Duhuo Jisheng Decoction (DHJSD) on cell viability of hNPCs in the absence and presence of SDF-1. Cells were treated with SDF-1 (10 ng/mL) and SDF-1 plus various DHJSD concentrations (100, 200, 300, 400 and 500 μg/mL) as well as 300 μg/mL DHJSD alone (without SDF-1) for 24 h. $^{\#}P<0.05$ compared to untreated group (without SDF-1 or DHJSD); $^{*}P<0.05$ compared with SDF-1 treated group.

Effects of DHJSD on cell viability of SDF-1-treated hNPCs

To investigate the effects of DHJSD on cell viability, hNPCs were treated with either SDF-1 (10 ng/mL) or SDF-1 plus various concentrations of DHJSD for 24 h, followed by analysis with the CCK-8 viability assay. As shown in Figure 2, SDF-1 significantly reduced the viability of hNPCs to 62.98%±4.48% ($P<0.05$ compared to the group of untreated cells) while DHJSD (300 μg/mL) alone promoted the cell viability of hNPCs to 110.23%±1.89% ($P<0.05$ compared to the group of untreated cells). The addition of various concentrations (100, 200, 300, 400 and 500 μg/mL) of DHJSD increased the cell viability of SDF-1-induced hNPCs. In the presence of SDF-1. the group of 300 μg/mL DHJSD had the highest viability of hNPCs among all concentrations of DHJSD, suggesting 300 μg/mL DHJSD may produce the maximal effect of enhancing the viability of SDF-1-induced hNPCs. Therefore, we decided to use 300 μg/mL of DHJSD in the rest of experiments to study the effects of DHJSD on the 10 ng/mL of SDF-1 induced reduction in cell viability of hNPCs.

Effects of DHJSD on the generation of TNF-α and IL-1β in hNPCs

As shown in Figure 3, SDF-1 significantly increased the mRNA (Figure 3A) and protein (Figure 3B) levels of TNF-α and IL-1β compared to that in the control groups. However, this SDF-1 induced increase in the expression of TNF-α and IL-1β mRNA and protein were markedly antagonized by DHJSD ($P<0.05$ SDF-1+DHJSD *vs* SDF-1).

Effects of DHJSD on SDF-1 induced hNPCs matrix degradation

To determine the effect of DHJSD on human IVD degeneration, the mRNA and protein levels of various matrix-degrading enzymes were examined by real-time PCR (Figure 4A) and Western blot (Figure 4B). SDF-1 treatment significantly increased the mRNA and protein levels of multiple MMPs (MMP-3 and MMP-13), but decreased the mRNA and protein levels of aggrecan and collagen II (protein:aggrecan, $P<0.01$; collagen II, $P<0.01$ *vs* control group). However, DHJSD treatment markedly decreased the mRNA and protein levels of multiple MMPs (MMP-3 and MMP-13) and increased the mRNA and protein levels of aggrecan and collagen II (protein: aggrecan, $P=0.03$; collagen II, $P<0.01$ *vs* SDF-1 group), indicating that the SDF-1-induced hNPC matrix degradation could be significantly inhibited by DHJSD.

Effects of DHJSD on the proinflammatory factors generation and matrix degradation of HID

To understand the mechanisms for the DHJSD inhibition of the SDF-1-induced inflammation and degeneration of hNPCs we further studied the effects of DHJSD on SDF-1-induced proinflammatory factors and matrix degradation. As shown in Figure 5A, the protein levels of CXCR4 and p-p65 were markedly increased in the SDF-1 treated hNPCs group when compared with the control group (CXCR4, $P<0.01$; p-p65, $P=0.02$). However, DHJSD markedly reduced the levels of

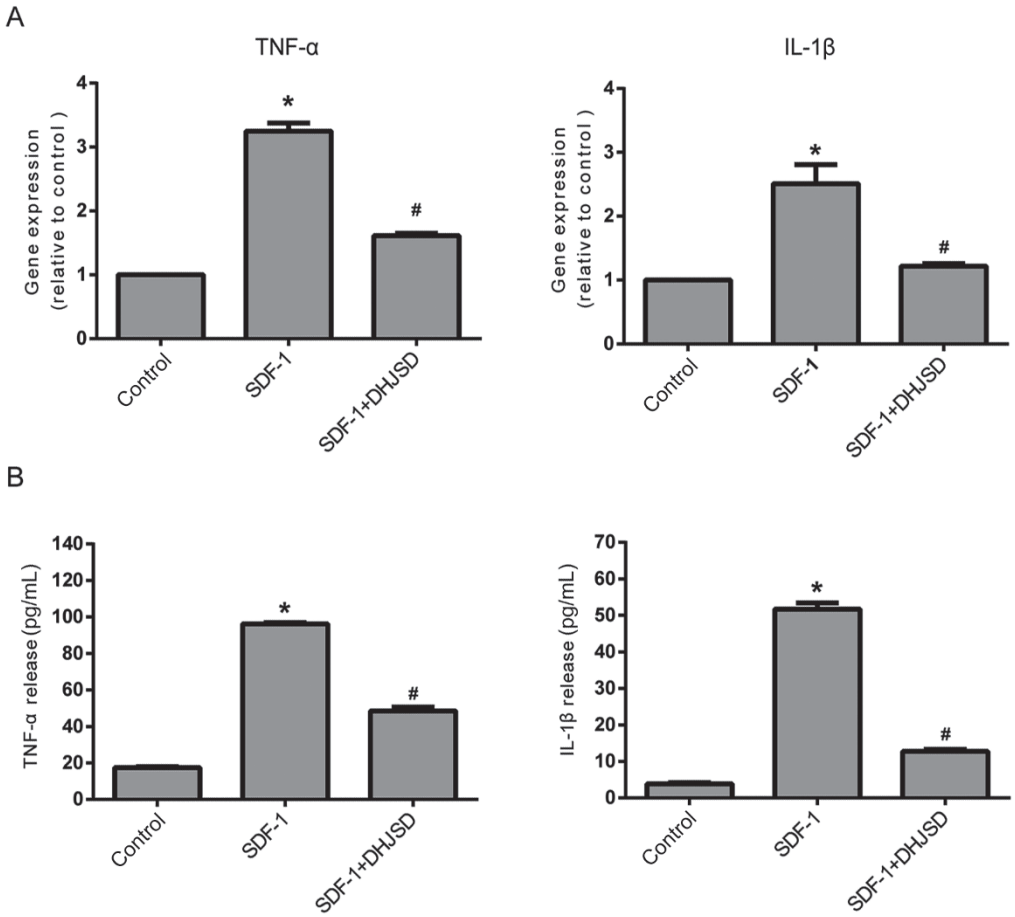

Figure 3. DHJSD on SDF-1 induced expression of TNF-α and IL-1β in human nucleus pulposus cells (hNPCs). (A) The mRNA expressions of TNF-α and IL-1β were determined by real-time PCR. (B) The secretion of TNF-α and IL-1β from hNPCs into the culture medium were determined by ELISA. $^{*}P<0.05$ compared with control group. $^{\#}P<0.05$ compared with SDF-1 group.

CXCR4 and p-p65 in the SDF-1 treated hNPCs group (CXCR4, $P<0.01$; p-p65, $P=0.02$). Interestingly, DHJSD caused the same down regulation of TNF-α, IL-1β, MMP-3 and MMP-13 expression as the CXCR4-siRNA or NF-κB inhibitor (BAY11-7082) in the SDF-1-treated hNPCs (Figure 5). Furthermore, as shown in Figure 6, DHJSD treatment or down regulation of the CXCR4 expression by CXCR4-siRNA transfection all inhibited the nuclear translocation of p65 in the SDF-1 treated hNPCs. These results strongly suggest that DHJSD may inhibit the generation of human intervertebral disc proinflammatory factors and ECM degradation via targeting at multiple molecules in the SDF-1/CXCR4/NF-κB pathway in a well-orchestrated and integrated fashion.

Discussion

The major findings in the current study include the pharmacological anti-inflammation and anti-degeneration effects of DHJSD on IVDD through the inhibition of SDF-1-induced proinflammatory factors and matrix degradation via targeting multiple molecules in the SDF-1/CXCR4/NF-κB pathway in hNPCs. It provides novel insights into the molecular mechanisms of DHJSD on human disc degeneration and has significant impact on the validation of clinical application of DHJSD, a classic TCM fangji, for the treatment of LBP caused by human IVDD.

The exaggerated inflammation with the increase of proinflammatory factors in intervertebral disc cells and ECM degradation are considered the major pathologic processes that cause disc degeneration[43]. Therefore, inhibition of the proinflammatory factors and ECM degradation in the intervertebral disc may be a very effective mechanism to retard the disc degeneration (Figure 2). IVDD is characterized by an increase in the expression levels of proinflammatory cytokines, including IL-1β, which induce ECM degradation, chemokine production and changes in cell phenotype[43, 44]. The release of chemokines promotes the infiltration and activation of immune cells, which amplifies the inflammatory cascade. SDF-1 is highly expressed in inflamed tissues, where it attracts activated $CXCR4^{+}$ T cells, thus enhancing local inflammatory responses[45]. We have previously reported that SDF-1/CXCR4 axis induces apoptosis of degenerative human nucleus pulposus cells via the NF-κB pathway[6]. In this study, we further found

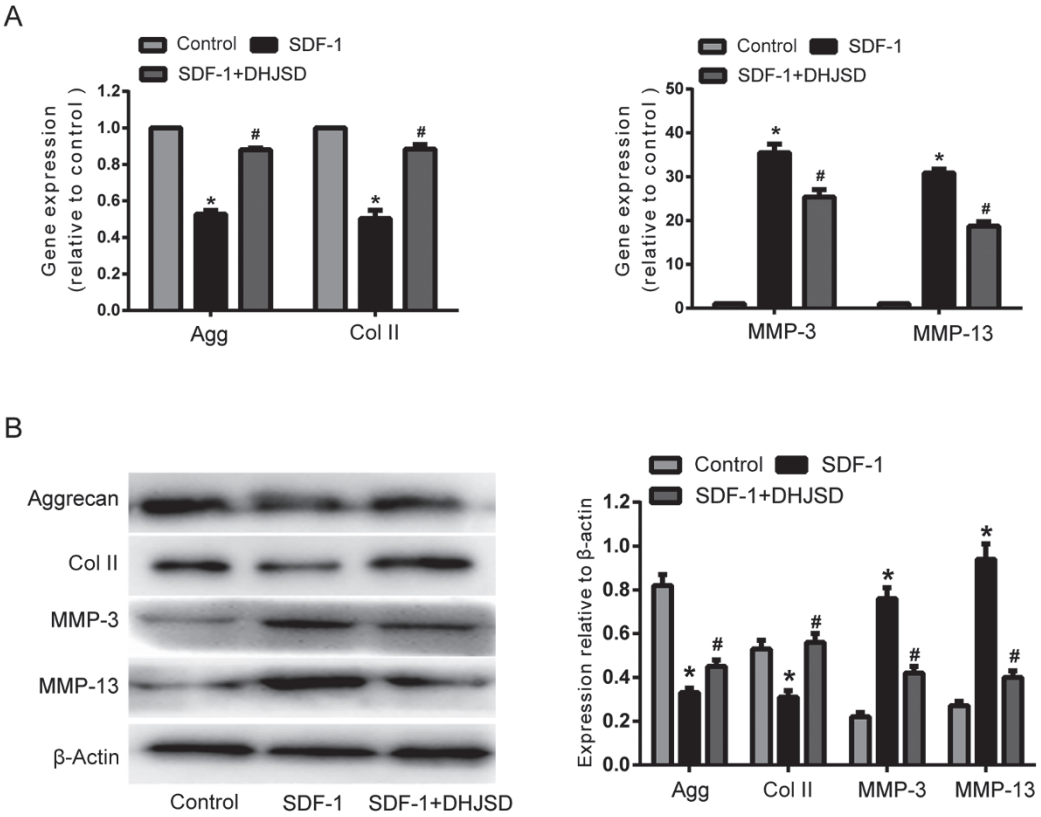

Figure 4. DHJSD on SDF-1-induced matrix degradation. (A) The mRNA expressions of aggrecan, collagen II, MMP-3 and MMP-13 were measured by real-time PCR analysis. (B) The protein expressions of aggrecan, collagen II, MMP-3 and MMP-13 were measured by Western blot analysis. $^{*}P<0.05$ when compared with control group. $^{\#}P<0.05$ when compared with SDF-1 group.

that proinflammatory cytokines and ECM degradation were markedly upregulated in SDF-1-induced human NPCs. Anti-inflammatory strategy with agents or gene therapy has been proven to be effective for delaying disc degeneration *in vitro* studies, but its clinical efficacy remains to be further investigated[46, 47]. In this study, we provided compelling evidence that the treatment of IDD with DHJSD significantly reduced the generation of proinflammatory factors and ECM degradation. A screening method was used in the present study to measure SDF-1-induced human NPCs viability by CCK-8 analysis. We found that the minimum dose for DHJSD to reverse SDF-1-induced decrease in NPCs viability was 300 μg/mL. DHJSD significantly reversed the SDF-1-induced upregulation of proinflammatory factors and key molecules in ECM degradation, including TNF-α, IL-1β, aggrecan, collagen II, MMP-3 and MMP-13, in human NPCs.

The SDF-1/CXCR4 axis has been previously associated with the pathogenesis of chronic inflammatory diseases, including osteoarthritis[6] and rheumatoid arthritis[48]. Our previous studies have demonstrated that the SDF-1/CXCR4 axis is involved in the IVDD process[49]. The results from the current study provided novel evidence that the SDF-1/CXCR4 axis also regulates many inflammatory responses and ECM degradation in IVDD. The NF-κB pathways have been reported to play important roles in the regulation of inflammatory response and ECM degradation[50, 51]. Here, for the first time in literature, we found that DHJSD treatment had same inhibitory effects as the CXCR4 knock down by CXCR4-siRNA and NF-κB inhibition by BAY11-7082 on the SDF-1-induced expression of TNF-α, IL-1β, MMP-3, MMP-13, CXCR4 and p-p65, and simultaneously suppressed p65 nucleus translocation. These results strongly suggest that DHJSD exerts its anti-inflammation and anti-degeneration effects through an orchestrated and integrated targeting at multiple key molecules in the SDF-1/CXCR4/NF-κB pathway. This may explain the unique effectiveness of the TCM fangji DHJSD and its advantages over the conventional non-steroid anti-inflammatory drugs (NSAIDs) in the treatment of LBP.

It is important to understand that due to the uniqueness of the composition theory of effective TCM fangji the study of TCM pharmacology needs a revolutionary paradigm shift in terms of rationales and approaches. It is now the time to consider a new knowledge network for deep understanding of an integrated and orchestrated combination of multi-targets for precision medicine, instead of an accumulation of single molecular target. In TCM, LBP is diagnosed as a "feng-han-shi"-caused "Bi Zheng", which usually attacks patients with dual deficiencies in both "Gan" and "Shen"[52]. Here, we

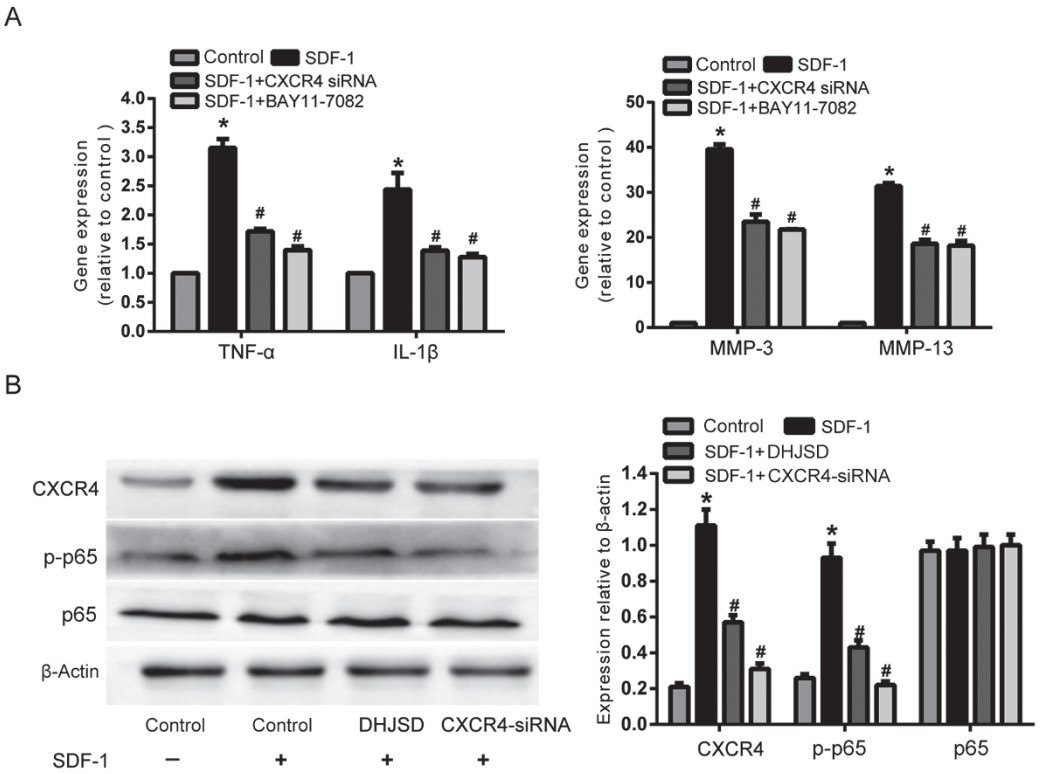

Figure 5. Effects of DHJSD on human intervertebral disc proinflammatory factors and ECM degradation through the SDF-1/CXCR4/NF-κB pathway. (A) Effects of NF-κB inhibitor, BAY11-7082, and CXCR4 siRNA in the presence of SDF-1 on the expression of TNF-α, IL-1β, MMP-3 and MMP-13 measured by real-time PCR analysis. (B) Western blot analysis of DHJSD and CXCR4-siRNA on the protein expression of CXCR4, p-p65 and p65 in the hNPCs after treated with SDF-1. Data are presented as the mean± standard deviation from three independent experiments. $^{*}P<0.05$ *vs* the control group. $^{\#}P<0.05$ *vs* SDF-1 group.

prefer to use the original TCM definition of "Gan" and "Shen" instead of the commonly used but confusing terms of "liver" and "kidney", respectively, because they are totally different in terms of organs and functions. Similarly, the TCM terms of "Qi" and "Xue" are not the same as "air" and "blood".

While the TCM terms of "Gan", "Shen", "Qi", and "Xue" for these particular etiological problems are complicated to be interpreted in Western medicine terms, a corresponding TCM fangji, DHJSD, was specifically developed with a unique integrated combination strategy to orchestrate the targeting at the primary and secondary causatives according to the principles of "Jun, Chen, Zuo, and Shi"[31]. As shown in Figure 7, DHJSD has an overall effect to eliminate "feng-han-shi", respectively, and thus to relieve pain of "Bi Zheng" through promoting "Gan" and "Shen" function and therefore strengthen the bones and tendons[30]. It also improves circulation and remove stasis of "Qi" and "Xue", reduces tissue swelling and relieves pain[20, 22, 23, 26, 32, 39, 40]. Some pharmacological studies have demonstrated that DHJSD may have effects of analgesia, immune function regulation, blood vessel dilation, anti-inflammation, and antiplatelet aggregation[20, 22, 23, 26, 32, 39, 40]. In a study of the aging-related osteoarthritis, another common bone degenerative disease, Chen *et al* found that DHJSD may inhibit VEGF and HIF-1α to retard cartilage degradation in a rabbit model of osteoarthritis[53]. How to translate these findings into the mechanisms of DHJSD on the degeneration of human intervertebral disc remains elusive. DHJSD derives its name from two of the principal ingredients, duhuo (contains osthole) and jisheng as "Jun" (or chief) components to target the major etiological problems, which is characterized by the strong effect on chronic "Bi" pain and numbness by eliminating "feng-han-shi", in the lower part of the "three jiaos"[21]. Its pharmaceutical actions are antirheumatic, anti-inflammatory, and analgesic (pain relieving) and thus it is specific for rheumatic pain that is sensitive to cold, especially if it occurs in the lower body. Previous quality control studies found that osthole exhibited anti-inflammation[54, 55] and anti-bone resorption effect[56] and stimulated osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling[57]. Jisheng has dual functions: it strengthens the "Gan" and "Shen" while subsequently nourishing the bones and tendons and relieves the pain of arthritis. Other ingredients in the formula include the basic formulas to tonify both "Qi" and "Xue", which assist the remedy in returning strength, vitality, and strong immune function, making it especially useful for long-term use by elderly or weakened patients. The "chen" components all facilitate the effect

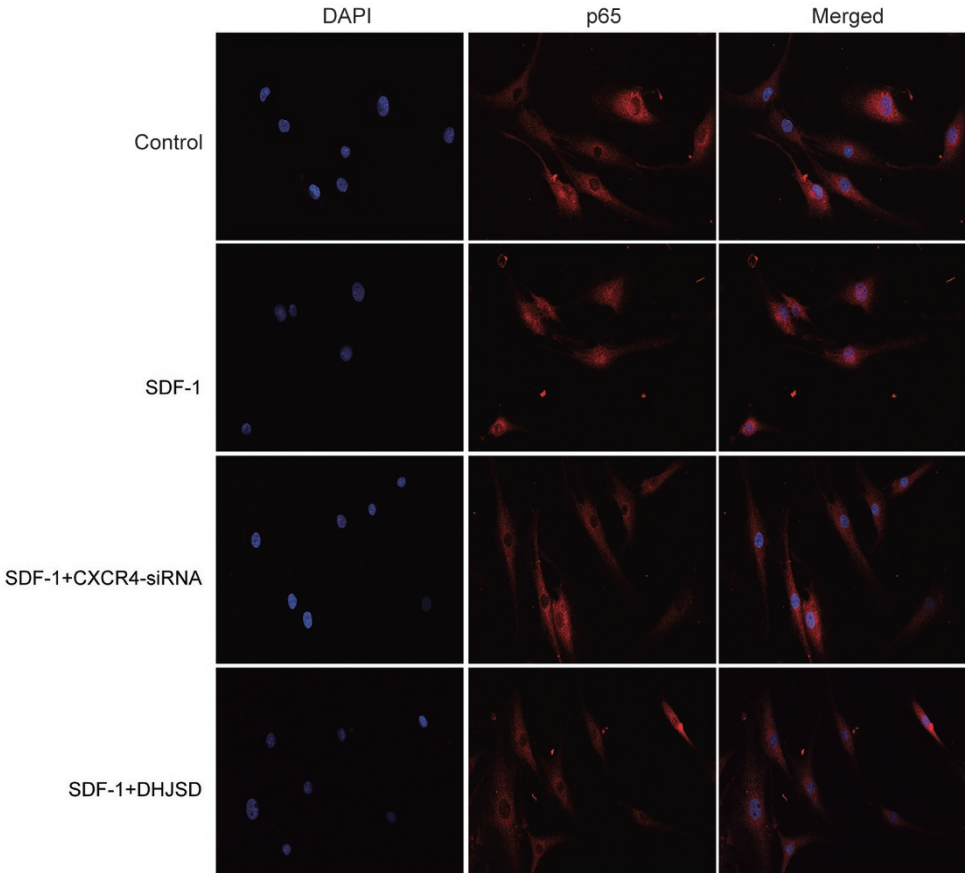

Figure 6. Effects of DHJSD on the nuclear translocation of p65 in the SDF-1 treated hNPCs. Fluorescence immunocytochemistry was used to detect nuclear translocation of p65.

of du-huo on "feng-han-shi" by targeting the secondary etiological problems, include fang-feng for systematic "feng-han-shi", xi-xin for "feng-han-shi" in the "Shen", rougui for improving circulation and eliminating "han", and finally qin-jiao for "feng-han-shi" in the joints. A very recent report found that qin-jiao had a significant protective effect on the rat model of osteoarthritis and the mechanisms may involve the inhibition of the Rho/NF-κB and TGF-β/smad-3 pathways[58]. The "Zuo" components include duzhong, niuxi, were used to promote function of "Gan" and "Shen" and strengthen the tendons and bones. Danggui, chuanxiong, dihuang, and baishao were used also as "Zuo" components to nourish and modulate function of "Xue". Renshen, fuling and gancao were used to promote function of "Pi" and "Qi". The integrated function of the DHJSD is therefore an effective "feng-han-shi" eliminator and LBP reliever. Indeed, previous quality control study of DHJSD suggested that this fangji comprises mainly osthole, gentiopicroside, loganic acid, and paeoniflorin[59, 60]. While osthole had anti-inflammation[54, 55] and anti-bone resorption effect[56] and could stimulate osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling[57], gentiopicroside has analgesic effects in the mice[61]. Loganic acid decreased proinflammatory cytokines in hypercholesterolemic rabbits[62, 63]. Paeoniflorin is able to suppress inflammation in experimental arthritis by inhibiting abnormal proliferation of lymphocytes and synoviocytes and the production of proinflammatory cytokines and chemokines, nitric oxide, vascular endothelial growth factor (VEGF), and GM-CSF by synoviocytes[19, 64-66]. Ferulic acid, isolated from chuanxiong and danggui[67] has antioxidative and anti-inflammatory effects[67, 68]. All the above study indicates that DHJSD has anti-inflammatory actions, consistent with the result in current study that DHJSD has an integrated anti-inflammatory effect and significant protective effect against the ECM degeneration of the SDF-1-treated hNPCs. While it warrants to further dissect the detailed mechanism of the synchronization of this classic TCM fangji in the treatment of LBP, we provided the very first compelling evidence at the molecular level that DHJSD may inhibit the generation of proinflammatory factors and ECM degradation of human intervertebral disc via targeting multiple key molecules in the SDF-1/CXCR4/NF-κB pathway in an orchestrated manner (Figure 7). These studies open a new field of research on the detailed mechanisms of TCM fangji of DHJSD through a Fangjiomics approach[69, 70].

In conclusion, DHJSD inhibits the generation of SDF-1-induced human intervertebral disc proinflammatory factors and ECM degradation via the SDF-1/CXCR4/NF-κB pathway.

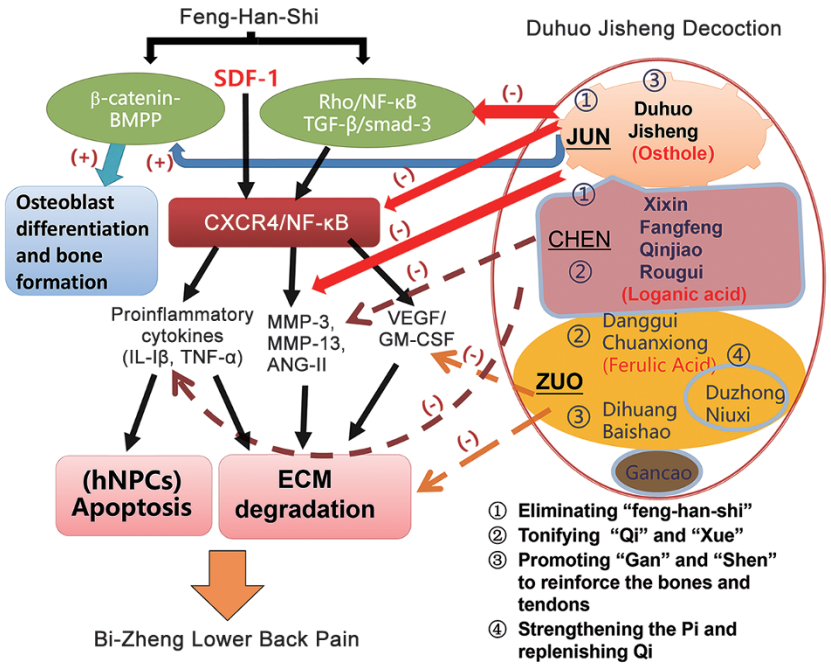

Figure 7. Schematic summary of molecular mechanisms for duhuo jisheng decoction (DHJSD) on the lower back pain (LBP) caused by Bi Zheng. In TCM, "Bi Zheng" LBP is caused by Feng-Han-Shi due to double deficiencies in "Gan" and "Shen" or in "Qi" and "Xue" [54]. DHJSD was specifically developed to orchestrate the therapeutic actions to target at the primary (Jun) and secondary (Chen and Zuo) causatives [31] and has an overall effect to eliminate "feng-han-shi" and thus relieves pain of "Bi Zheng" through promoting "Gan" and "Shen" function and reinforcing the bones and tendons [30]. It also improves circulation and removes stasis of "Qi" and "Xue", reduces tissue swelling, and relieves pain [20, 22, 23, 26, 32, 39, 40]. Duhuo (contains osthole) and jisheng target the major etiological problems as "Jun" (or chief) components by eliminating "feng-han-shi"[21]. Osthole exhibited anti-inflammation [56, 57] and anti-bone resorption effect [58] and stimulated osteoblast differentiation and bone formation by activation of beta-catenin-BMP signaling (indicated by the blue arrows) [59]. Jisheng has dual functions: it strengthens the "Gan" and "Shen" while subsequently nourishing the bones and tendons and relieves the pain of arthritis. DHJSD may inhibit VEGF and HIF-1α to retard cartilage degradation [55]. The "chen" components all facilitate the effect of du-huo on "feng-han-shi" by targeting at the secondary etiological problems through the inhibition of the Rho/NF-κB and TGF-β/smad-3 pathways[60]. The "Zuo" components include duzhong, niuxi, were used to promote function of "Gan" and "Shen" and strengthen the tendons and bones, danggui, chuanxiong, dihuang, and baishao to nourish and modulate function of "Xue"; renshen, fuling and gancao to promote function of "Pi" and "Qi". Therefore, the integrated function of the DHJSD is an effective "feng-han-shi" eliminator and LBP reliever and has anti-inflammation [56, 57] and anti-bone resorption effect [58] and can stimulate osteoblast differentiation and bone formation [59] and has an integrated anti-inflammatory effect and significant protective effect against the ECM degeneration of the SDF-1-treated hNPCs. ECM: extracellular matrix; hNPCs: human nucleus pulposus cells; VEGF: vascular endothelial growth factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; CXCR4: C-X-C motif chemokine receptor 4; MMP-3: metalloproteinase-3; MMP-13: metalloproteinase-13; ANG-II: angiotensin II; IL-1β: interleukin-1β; TNF-α: tumor necrosis factor-α; SDF-1: stromal cell-derived factor-1 (+) stimulation, (-) inhibition.

DHJSD may be used as an effective therapeutic agent for the treatment of IVDD-induced LBP.

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Author contribution

Chuan MA designed the experiments and overviewed the study; Zong-chao LIU and Zhen-long WANG conducted the experiments and wrote the manuscript; Zhi-jiang FU, Chen-yi HUANG, Yong LIU, Zhang-chao WEI, Shi-gui LIU, and Jie-liang SHEN performed part of the experiments; Zong-chao LIU evaluated the final results; Dayue Darrel DUAN participated in writing and finalizing the manuscript.

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