Duhuo Jisheng Decoction-containing serum promotes proliferation of interleukin-1β-induced chondrocytes through the p16-cyclin D1/CDK4-Rb pathway

GUANGWEN WU[1], HUAILING FAN[2], YUANPENG HUANG[3], CHUNSONG ZHENG[1], JINXIA YE[1] and XIANXIANG LIU[1]

Academy of [1]Integrative Medicine and [2]Traditional Chinese Medicine, Fujian University of Traditional Chinese Medicine, Fujian, Fuzhou 350122; [3]Fujian Sports Vocational Education and Technical College, Fujian, Fuzhou 350003, P.R. China

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Abstract. Duhuo Jisheng Decoction (DHJSD) is a traditional Chinese herbal medicine that has multiple uses, including as a treatment for osteoarthritis (OA). However, the molecular mechanisms underlying the therapeutic effects of DHJSD on OA remain unknown. In the present study, a serum pharmacological method was applied to investigate the effects of DHJSD on the proliferation of chondrocytes treated with interleukin-1β (IL-1β) *in vitro*. This is a cell model commonly used to reproduce the mechanisms involved in degenerative arthropathies, including OA. The most effective intervention conditions of DHJSD serum were examined by MTT assay. The degenerative chondrocyte model was established by IL-1β-culture for 24 h, and was verified by optical microscopy and immunohistochemical analyses. Following the successful establishment of the degenerative chondrocyte model, the chondrocytes were subsequently randomly divided into two groups: The blank serum group and the DHJSD treatment group. Subsequent to treatment with the corresponding serum, cell proliferation was detected by MTT assay and DNA staining followed by FACS analysis, and the mRNA and protein expression levels of cyclin D1, cyclin-dependent kinase 4 (CDK4), retinoblastoma tumor suppressor protein (Rb) and p16 were measured by reverse transcription polymerase chain reaction and western blotting, respectively. The results indicated that the most effective condition for the promotion of chondrocyte proliferation was 10% concentration of DHJSD 2-h serum, and the degenerative chondrocyte model was successfully reproduced by IL-1β-treatment for 24 h. The mRNA and protein expression levels of cyclin D1, CDK4 and Rb in the DHJSD serum-treated cells were significantly increased compared with those in the blank serum group, whereas p16 expression was significantly downregulated. These results indicate that treatment of cells with DHJSD-containing serum is able to promote IL-1β-induced chondrocyte proliferation by promoting G_1/S phase transition via modulating the expressions of cyclin D1, CDK4, Rb and p16, which contribute to the clinical efficacy of DHJSD in OA.

Correspondence to: Dr Xianxiang Liu, Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, 1 Huatuo Road Shangjie Minhou, Fujian, Fuzhou 350122, P.R. China
E-mail: liuxianxiang@163.com

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Introduction

Osteoarthritis (OA) is a prevalent articular disease in the elderly (1,2), and is characterized by a series of pathological changes in the structure and function of the joints, mainly due to a degenerative process that takes place in the articular cartilage (3). The chondrocyte is the only cell type present in mature cartilage, and is responsible for extracellular signaling and the maintenance of cartilage homeostasis. Changes in chondrocyte function are critical for the degradation of articular cartilage and serve an important function in the pathogenesis of OA (4,5). Several studies have reported that there is reduced proliferative activity in osteoarthritic chondrocytes and thus promoting chondrocyte proliferation may be an efficient strategy to treat or delay the progression of OA (6,7).

Due to the fact that the incidence of knee osteoarthritis in individuals aged >65 years old is 60-70%, with the incidence rate reaching 85% in the population of those aged >75 years (8), OA has had a major economic and social impact on populations and health-care systems worldwide (9,10). Although non-steroidal anti-inflammatory drugs (NSAIDs) have been widely prescribed to reduce joint pain and stiffness, the inflammatory component of OA is usually minimal. Thus, the requirement for the anti-inflammatory effect of NSAIDs in OA is controversial (11). Hyaluronic acid is easily applied by intra-articular injection; however, it has a short half-life and repeated intra-articular injections increase the chances of joint infection (12). Advanced OA is currently only managed by surgical replacement of the joints, however, there remain difficulties regarding the degree of invasion, cost and long-term prognosis (13). These disadvantages call for an evaluation of the risks and benefits of the therapies for

OA and the requirement for less toxic options. An increasing number patients suffering from OA turn to complementary and alternative medicine treatments, including Chinese herbal medicine (14).

Chinese herbal medicine, a major modality in traditional Chinese medicine (TCM) that has been practiced for thousands of years in China and other countries in Asia, has advanced in the treatment of OA, including improving clinical findings and inhibiting inflammatory reactions and cartilage degeneration (15,16). *In vivo* and *in vitro* studies have also indicated that Chinese herbal formulas produce multiple comprehensive effects against OA (17-19). Duhuo Jisheng Decoction (DHJSD), initially documented in the book *Bei Ji Qian Jin Yao Fang* (20), is composed of the following ingredients: *Angelica pubescens*; *Saposhnikovia divaricata*; *Ligusticum chuanxiong*; *Achyranthes bidentata*; *Loranthus parasiticus*; *Gentiana macrophylla*; *Eucommia ulmoides*; *Angelicae sinensis*; *Poria cocos*; *Codonopsis pilosula*; radix *Rehmannia preparata*; radix *Paeoniae alba*; *Asarum sieboldii*; *Glycyrrhiza uralensis*; and *Cinnamomum cassia*. It has been widely used for treating OA (21), and a previous study indicated that DHJSD contains drug- and lead-like compounds with potential synergy and polypharmacology against OA (22). An *in vivo* study demonstrated that treatment with DHJSD promotes the progression of chondrocytes from G_1 to S phase (23), and this may be one of the mechanisms underlying its use in OA. In addition, previous studies have reported that chondrocytes treated with IL-1β produce a particularly effective cell model of the mechanisms involved in degenerative arthropathies (24).

In order to further elucidate the precise mechanism of DHJSD in OA, a serum pharmacological method was employed to investigate its effects on the proliferation of IL-1β-induced chondrocytes *in vitro* in the current study.

Materials and methods

Reagents. Fetal bovine serum, Dulbecco's modified Eagle's medium and trypsin were purchased from Hyclone Laboratories, Inc. (Logan, UT, USA). A cyclin D1 antibody was purchased from Abcam (Cambridge, MA, USA). Cyclin-dependent kinase 4 (CDK4), Rb and p16 antibodies were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-collagen type II antibody was obtained from Merck Millipore (Darmstadt, Germany). The WesternBreeze Chemiluminescent Immunodetection kit was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Cyclin D1, CDK4, Rb, p16 and β-actin primers were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). Table I displays the primer sequences. IL-1β and collagenase II were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Animals. A total of 144 healthy Sprague Dawley rats of average gender (two-month-old, 230-250 g) and 60 Sprague Dawley rats of either gender (four-week-old, 90-110 g) were purchased from Shanghai SLAC Laboratory Animal Co., Inc., Shanghai, China and raised in a sterile environment. Experiments involving the animals complied with the Guidance Suggestions for the Care and Use of Laboratory Animals (2006) by the Ministry of Science and Technology, China (25).

Preparation of DHJSD-containing serum. The 144 two-month-old rats were randomly divided into two groups: The DHJSD group (n=108) treated with a dose of 9.3 g/kg/day DHJSD, which is the equivalent dosage used clinically for humans (26) and the blank group (n=36) treated with an equivalent dose of saline. All drugs and saline were administered via gastric gavage twice a day in the morning and afternoon for 7 consecutive days. The two doses were given 2 h apart on the seventh day. The animals were anesthetized by intraperitoneal injection of 2 ml/kg 2% pentobarbital sodium (Sigma-Aldrich), and arterial blood of the DHJSD group was collected from the abdominal aorta at 1, 2 and 3 h after the final dose in the DHJSD group and at 2 h in the blank group. The collected blood was placed in a 37˚C thermostatic water bath for 30 min and centrifuged at 3,000 r/min for 15 min. The serum fraction was isolated, heat inactivated in a 56˚C thermostatic water bath for 30 min and then filtered through a 0.22 μm filter. The resulting drug-containing serum was then aliquoted and stored at -20˚C.

Isolation, culture and verification of chondrocytes. The chondrocytes were isolated, cultured and verified as previously described (27,28). The cells used in the current experiments were successfully verified and counted with a hemocytometer and adjusted to 10^5 cells/ml.

Determination of chondrocyte viability by MTT assay. Cell viability was assessed by MTT colorimetric assay. The second-generation chondrocytes were seeded into 96-well plates at a density of $1.0x10^5$ cells/ml in 0.1 ml medium. The cells were treated with a range of concentrations (10-30%) of 1, 2 and 3-h DHJSD serum for 24, 36, 48, 60 and 72 h. At the end of the treatment, 100 μl 0.5 mg/ml MTT was added to each well and the samples were incubated for an additional 4 h at 37˚C. The purple/blue MTT formazan precipitate was dissolved in 100 μl dimethylsulfoxide (Sigma-Aldrich) and the optical density (OD) value of each well was measured at 490 nm wavelength using a microplate reader (BioTek, Winooski, VT, USA).

IL-1β-induced degenerative chondrocyte model. The degenerative chondrocyte model was established as previously described (29,30). Briefly, third generation chondrocytes were exposed to 10 ng/ml IL-1β for 24 h, and then washed with 1X phosphate-buffered saline (PBS). The successful establishment of the degenerative chondrocyte model was verified by optical microscopy and immunohistochemical analyses.

Observation of cellular morphological changes. The second generation chondrocytes were seeded into 6-well plates at a density of $1.0x10^5$ cells/ml in 2 ml medium. The cells were treated with 10 ng/ml IL-1β for 24 h. The changes in cell morphology were observed using a phase-contrast microscope (Olympus Corporation, Tokyo, Japan) and images were captured at a magnification of x100.

Immunohistochemical assay. The second-generation chondrocytes were cultured on glass coverslips in 6-well plates

Table I. Primer sequences.

Gene	Primer sequence	Amplicon length (bp)	Annealing temperature (˚C)
Cyclin D1	sense, 5'-GAC ACC AAT CTC CTC AAC GAC-3' antisense, 5'-AGA CAA GAA ACG GTC CAG GTA G-3'	216	55
CDK4	sense, 5'-CCT ACG GAC ATA CCT GGA CAA-3' antisense, 5'-GAG GCA ATC CAA TGA GAT CAA-3'	404	55
Rb	sense, 5'-CTT TAT TGG CCT GTG CTC TTG-3' antisense, 5'-ATT CCA TGA TTC GAT GCT CAC-3'	225	55
p16	sense, 5'-GCT CTC CTG CTC TCC TAT GGT-3' antisense, 5'-AGA AGT TAT GCC TGT CGG TGA-3'	268	55
β-actin	sense, 5'-GGG AAG TGC TGG ATA G-3' antisense, 5'-GTG ATG TTT CGG ATG G-3'	453	55

at a density of $1.0x10^5$ cells/ml in 2 ml medium. Following treatment with 10 ng/ml IL-1β for 24 h, the cells were washed with PBS three times for 5 min and fixed in 4% paraformaldehyde (Sigma-Aldrich) for 20 min. The antigen retrieval buffer (10 mM sodium citrate; pH 6.0) was preheated to 95˚C in a coverglass staining jar placed in a water bath at 95˚C. The coverslips were heated at 95˚C for 10 min. Endogenous peroxidase activity of the sections was quenched by incubation in PBS containing 3% H_2O_2 for 10 min following three washes in PBS. Immunohistochemical staining was performed using the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) according to the manufacturer's instructions. Briefly, following blocking with normal serum in PBS, the coverslips were treated with the anti-collagen type II antibody at a concentration of 1:250 overnight at 4˚C. The coverslips were incubated with a biotinylated anti-rabbit IgG antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) for 60 min and then treated with the ABC reagent for 60 min. Next, the cultures were treated with DAB (Vector Laboratories, Inc.) for 3 min and subsequently dehydrated with increasing concentrations of ethanol solutions, cleared with xylene (Sigma-Aldrich) and mounted on a coverslip using neutral gum.

Cell treatment and grouping. Following IL-1β induction for 24 h, the chondrocytes were randomly divided into two groups: The DHJSD group and the blank serum group. After treatment with the appropriate serum, cell proliferation levels were detected using MTT assay and DNA staining, followed by fluorescence-activated cell sorting (FACS) analysis. The mRNA and protein levels of cyclin D1, CDK4, Rb and p16 were measured by reverse transcription (RT) followed by semi-quantitative polymerase chain reaction (PCR) analysis and western blotting, respectively.

Determination of viability of IL-1β-induced chondrocytes by MTT assay. Following treatment with 10% DHJSD 2-h serum for 24, 36, 48, 60 and 72 h, the viability of IL-1β-induced chondrocytes was assessed by MTT colorimetric assay. The protocol was as in the previous description.

Detection of cell cycle distribution in IL-1β-induced chondrocytes by flow cytometric analysis. Subsequent to treatment, the cell cycle distribution of the IL-1β-induced chondrocytes was determined by flow cytometric analysis by FACS with a BD FACSCalibur cytometer (BD Biociences, Franklin Lakes, NJ, USA) and a cell cycle assay kit. Propidium iodide staining was performed according to the manufacturer's instructions. The percentage of cells in the different phases was calculated by ModFit LT software, version 3.0 (Verity Software House, Inc., Topsham, ME, USA), and the numbers of cells in the G_0/G_1, S and G_2/M phases were determined.

RT and semi-quantitative PCR analysis. Following treatment with 10% DHJSD 2-h serum for 24, 48 and 72 h, the IL-1β-induced chondrocytes were washed with PBS and total RNA was isolated with TRIzol® reagent (Invitrogen Life Technologies). Oligo (dT) primers (1 μg) were used for the reverse transcription of the RNA template using SuperScript II reverse transcriptase (Invitrogen, Grand Island, NY, USA) according to the manufacturer's instructions. The obtained cDNA was used to determine the relative expression of cyclin D1, CDK4, Rb and p16 by PCR using Taq DNA polymerase (Thermo Fisher Scientific, Pittsburgh, PA, USA) and β-actin was used as an internal control. The primer sequences and the annealing temperature used in the reactions are listed in Table I. The amplified products were analyzed by 1.5% agarose gel electrophoresis. Optical density ratios for cyclin D1, CDK4, Rb and p16 to β-actin were used for the semi-quantitative analyses.

Western blot analysis. Following treatment with 10% DHJSD 2-h serum for 24, 48 and 72 h, the IL-1β-induced chondrocytes were lysed with mammalian cell lysis buffer containing protease and phosphatase inhibitor cocktails (EMD Millipore Corporation, San Diego, CA, USA), and the lysates were separated by 12% SDS-PAGE gel under a reducing condition at 100 V for 1 h. Subsequent to electrophoresis, proteins were transferred to polyvinylidine fluoride membranes (Sigma-Aldrich) in 5% w/v non-fat dry milk using a semidry

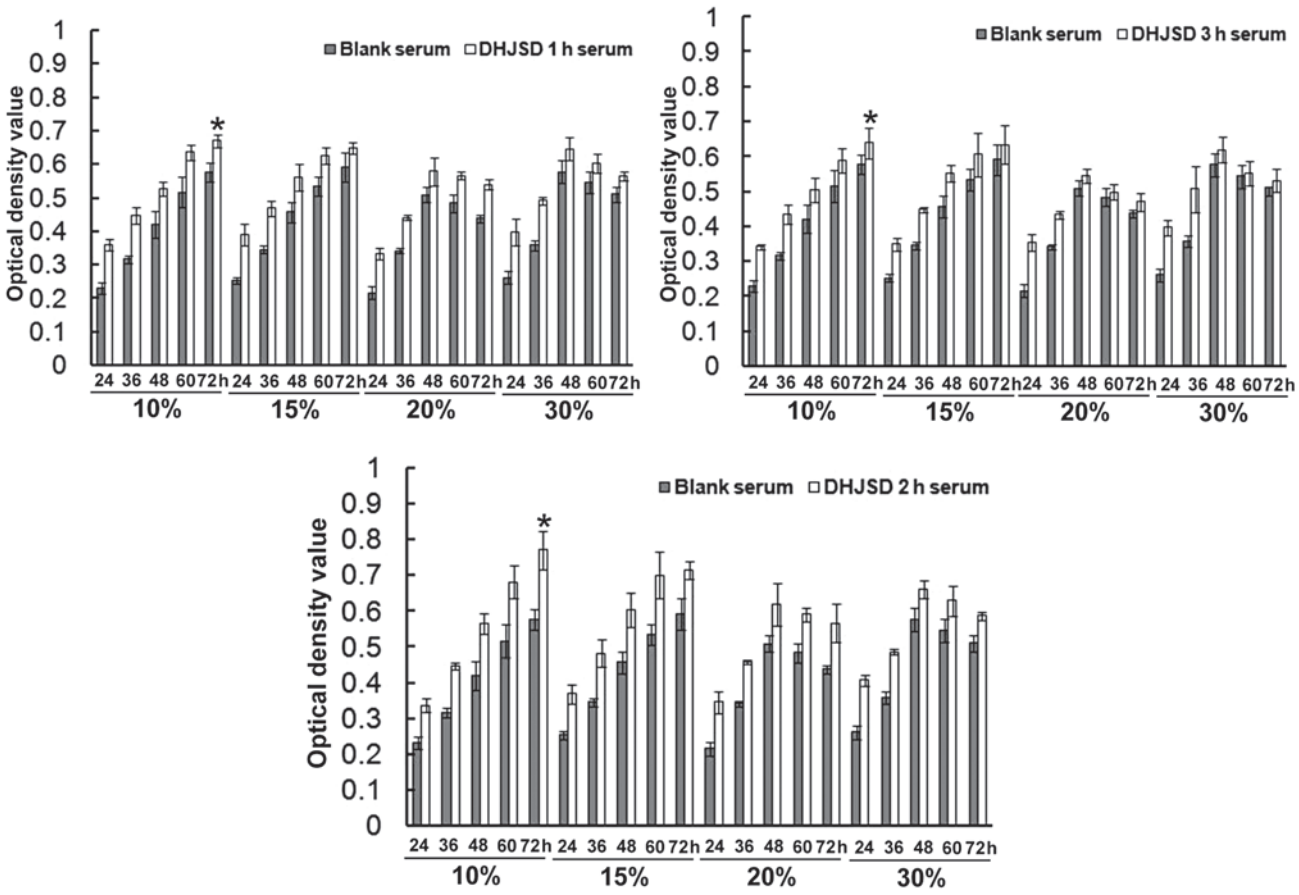

Figure 1. Effect of DHJSD serum on chondrocyte viability. Following treatment with a range of concentrations (10-30%) of DHJSD 1, 2 and 3-h serum for 24, 36, 48, 60 and 72 h, cell viability was determined by MTT assay. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01 vs. blank serum group. DHJSD, Duhuo Jisheng Decoction.

blotting system. The membranes were blocked for 30 min with agitation at room temperature in SuperBlock T20 (TBS) blocking buffer (Thermo Fisher Scientific Inc., Rockford, IL, USA). The membranes were washed in Tris-buffered saline with 0.25% Tween-20 (TBST) (Baoman Biotechnology, Shanghai, China) and exposed to primary antibodies against cyclin D1 (1:400), CDK 4 (1:400), pRb (1:500) and p16 (1:600) overnight at 4˚C. β-actin (1:1,000) was also measured as an internal control for protein loading. The membranes were then washed in TBST, and incubated with secondary horseradish peroxidase-conjugated antibodies (Beijing Zhongshan Golden Bridge Biotechnology, Beijing, China) at 1:2,500 dilution for 1 h at room temperature and the membranes were washed again in TBST. Finally, the antibody-bound protein bands were detected with enhanced chemiluminescence, and images were captured using a ChemiDoc XRS+ (Bio-Rad Laboratories, Hercules, CA, USA). The grayscale value ratio of the target protein to the internal control was used to measure the relative concentration of cyclin D1, CDK4, pRb and p16.

Statistical analysis. The data were analyzed using SPSS, version 13.0 (SPSS, Inc., Chicago, IL, USA). Statistical data are expressed as the mean ± standard deviation. Statistical analysis of the data was performed with Student's t-test and one-way analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Effect of DHJSD serum on chondrocyte viability. As presented in Fig. 1, the 10 and 15% concentrations of all DHJSD sera were able to promote the proliferation of chondrocytes in a time-dependent manner. The 20 and 30% concentrations of the three DHJSD sera also promoted the proliferation of chondrocytes, but reached a peak at 48 h and reduced at 72 h. The proliferation was most significant subsequent to 10% DHJSD sera treatments for 72 h (P<0.01, compared with the blank serum groups). When comparing the different sampling time DHJSD sera, it was identified that the optimum proliferation was produced by the groups treated with the 2-h DHJSD serum. Therefore, the 10% concentration of the DHJSD 2-h serum was used in the following experiments.

Degenerative chondrocyte model verification. As presented in Fig. 2A and B, compared with the normal chondrocytes, the IL-1β-induced chondrocytes were larger with finger-like protrusions at the edge, and the cell membrane and cytoplasm was not clear. In addition, the IL-1β-induced cells were polygonal in shape, and had declining refractive indices. Type II collagen, a protein specific to chondrocytes, was stained brown/yellow in the chondrocyte cytoplasm by immunocytochemical staining. As indicated in Fig. 2C and D, the staining became paler in the IL-1β-induced group.

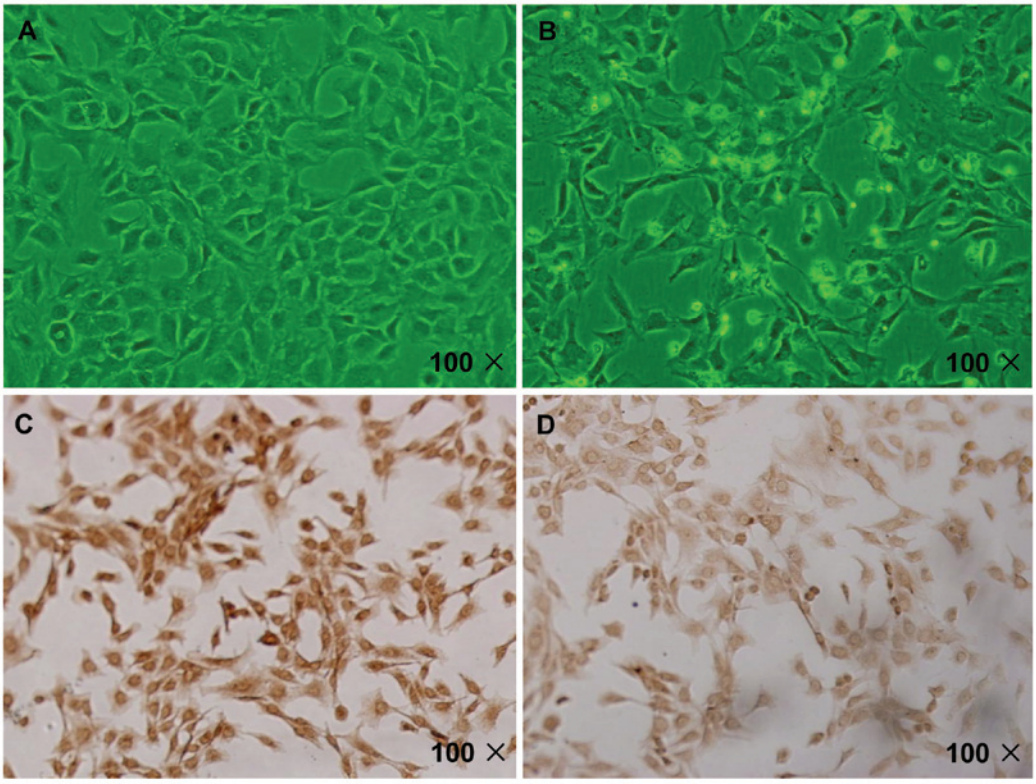

Figure 2. Verification of degenerative chondrocyte model. The degenerative chondrocyte model was established by exposing the third-generation chondrocytes to 10 ng/ml IL-1β for 24 h and verified by (A and B) optical microscopy and (C and D) immunohistochemical analyses. The photographs are representative images taken at a magnification of x100. A and C, normal chondrocytes; B and D, IL-1β induced chondrocytes. IL-1β, interleukin-1β.

DHJSD 2-h serum promotes the growth of IL-1β-induced chondrocytes. The effect of DHJSD 2-h serum on the viability of IL-1β-induced chondrocytes was determined by MTT assay. As displayed in Fig. 3, treatment with DHJSD 2-h serum and blank serum led to a gradual increase in cell viability in a time-dependent manner. The difference between the optical densities of the blank serum and the DHJSD 2-h serum groups at all measured time-points (24, 36, 48, 60 and 72 h) were significant (P<0.01); the promotional effect was greater in the DHJSD 2-h serum group (P<0.01, compared with the blank serum group). These results suggest that DHJSD 2-h serum promotes the growth of IL-1β-induced chondrocytes in a time-dependent manner.

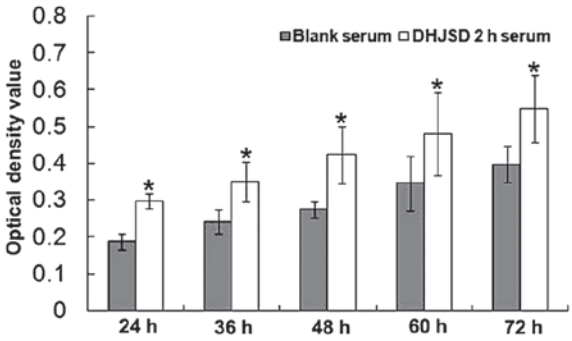

Figure 3. Effect of DHJSD 2-h serum on the viability of IL-1β-induced chondrocytes. After the degenerative chondrocytes were treated with 10% DHJSD 2-h serum and blank serum for 24, 36, 48, 60 and 72 h, cell viability was determined by MTT assay. Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01 vs. blank serum group. DHJSD, Duhuo Jisheng Decoction; IL-1β, interleukin-1β.

FACS analysis. To further verify the previous results, the effect of DHJSD 2-h serum on the cell cycle in IL-1β-induced chondrocytes was evaluated. As presented in Fig. 4 and Table II, the percentage of G_0/G_1 phase cells reduced in a time-dependent manner in the DHJSD 2-h serum group and the blank serum group. In the DHJSD 2-h serum group, the percentages of G_0/G_1 phase cells were 72.97±1.13, 65.13±1.35 and 59.18±2.12% following treatment for 24, 48 and 72 h, respectively. These were significantly lower than the corresponding percentages in the blank serum group (79.59±1.69, 71.33±1.11 and 63.86±2.32%, respectively; P<0.01 or P<0.05). However, the percentages of S phase cells exhibited the opposite trend, and increased in a time-dependent manner. In the DHJSD 2-h serum group, the percentages of S phase cells were 16.91±0.64, 22.28±2.45 and 28.05±2.63% following treatment for 24, 48 and 72 h, respectively. These were significantly higher than the corresponding percentages of the blank serum group (09.92±2.00, 17.02±2.96 and 24.20±1.05%, respectively; P<0.01 or P<0.05). The proliferation indices in the DHJSD 2-h serum group were 27.03±1.13, 34.87±1.35 and 40.82±2.11% following treatment for 24, 48 and 72 h, respectively. These were higher than the corresponding indices of the blank serum group (20.41±1.69, 28.67±1.11 and 36.14±2.32, respectively). These data demonstrate that DHJSD serum has the ability to promote proliferation by promoting G_1/S phase transition.

Effect of DHJSD 2-h serum on the mRNA expression of cyclin D1, CDK4, Rb and p16 in IL-1β-induced chondrocytes.

Table II. Cell cycle distribution detected by fluorescence-activated cell sorting (%).

Group	h	G_0/G_1	S	G_2/M	Proliferation index
Blank serum	24	79.59±1.69	09.92±2.00	10.49±0.33	20.41±1.69
	48	71.33±1.11[a]	17.02±2.96[a]	11.65±2.08	28.67±1.11
	72	63.86±2.32[b]	24.20±1.05[b]	11.94±1.51	36.14±2.32
DHJSD 2-h serum	24	72.97±1.13[a]	16.91±0.64[a]	10.13±1.57	27.03±1.13
	48	65.13±1.35[b,e]	22.28±2.45[c,f]	12.59±1.11	34.87±1.35
	72	59.18±2.12[d,g]	28.05±2.63[d,h]	12.76±1.83	40.82±2.11

[a]P<0.01 vs. the blank serum 24 h group; [b]P<0.01 and [c]P<0.05 vs. the blank serum 48 h; [d]P<0.05 vs. the blank serum 72 h; [e]P<0.01 and [f]P<0.05 vs. the DHJSD 2-h serum 24 h group; [g]P<0.01 and [h]P<0.05 vs. the DHJSD 2-h serum 48 h group. DHJSD, Duhuo Jisheng Decoction.

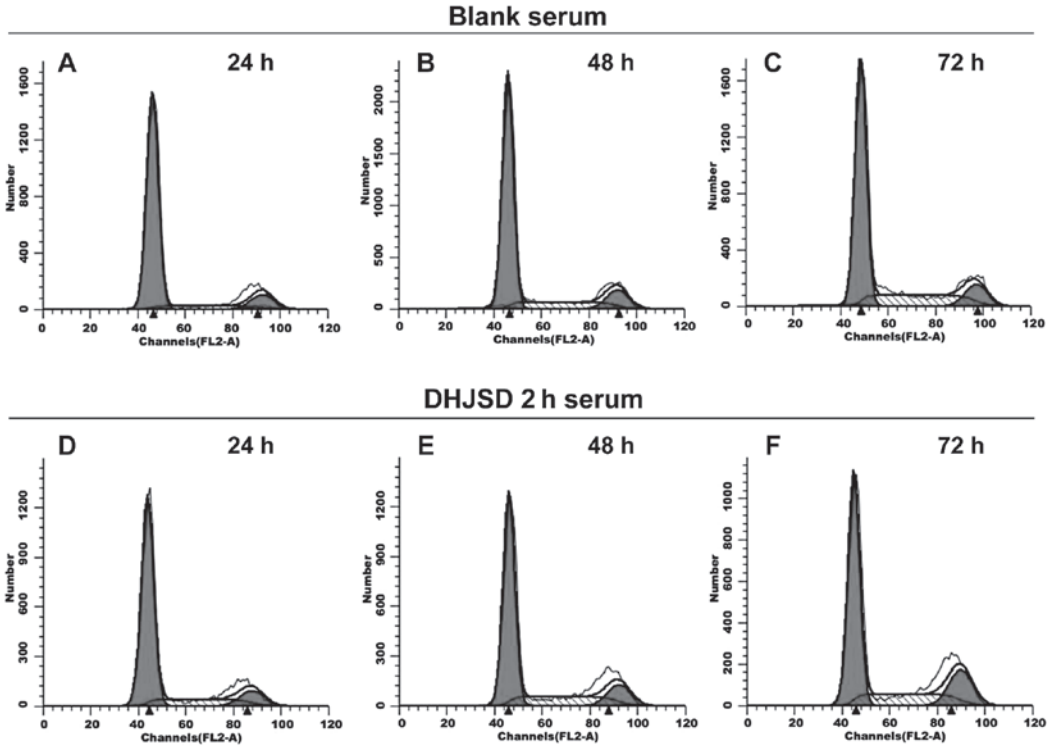

Figure 4. Effect of DHJSD 2-h serum on the cell cycle of IL-1β-induced chondrocytes. Following treatment with (A-C) blank serum or (D-F) 10% DHJSD 2-h serum for 24 h, 48 h and 72 h, cells were collected and stained with PI followed by FACS analysis. DHJSD, Duhuo Jisheng Decoction; IL-1β, interleukin-1β; PI, propidium iodide; FACS, fluorescence-activated cell sorting.

As presented in Fig. 5A and B, the mRNA expression levels of cyclin D1, CDK4 and Rb gradually increased, while those of p16 reduced, in a time-dependent manner in the DHJSD 2-h and blank serum group. A significant difference was indicated between the DHJSD and blank serum groups at all time points (P<0.01). In the DHJSD 2-h serum group, the expression levels of cyclin D1, CDK4 and Rb mRNA were higher than those in the blank serum group following treatment for 24, 48 or 72 h (P<0.01 or P<0.05). However, the expression of p16 exhibited the opposite trend, and its expression level was lower in the DHJSD 2-h serum group compared with the blank serum group at all time points (P<0.01 or P<0.05).

Effect of DHJSD 2-h serum on the protein expression of cyclin D1, CDK4, pRb and p16 in IL-1β-induced chondrocytes. The protein expression patterns of cyclin D1, CDK4, pRb and p16 were similar to their respective mRNA levels (Fig. 6A and B). The protein expression levels of cyclin D1, CDK4 and pRb were increased, and those of p16 were reduced in a time-dependent manner in both the DHJSD 2-h and blank serum groups. However, the protein expression levels of cyclin D1, CDK4 and pRb in DHJSD 2-h serum group were significantly higher, and those of p16 were significantly lower, as compared with the blank serum group at all the time periods (P<0.01 or P<0.05).

Discussion

OA is a major degenerative disease affecting millions of individuals. The ability of articular cartilage to self-repair is

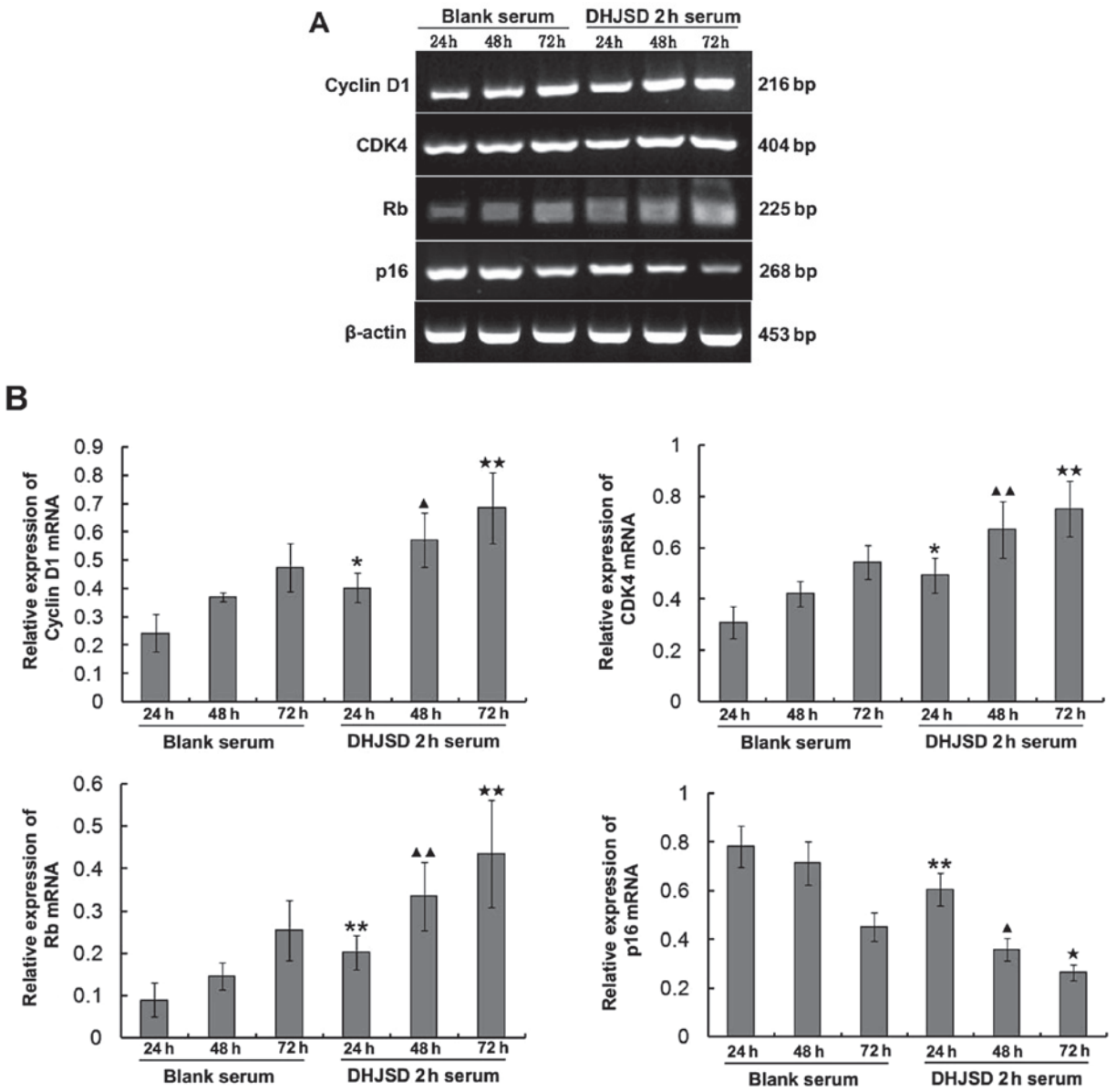

Figure 5. Effect of DHJSD 2-h serum on the mRNA expression of cyclin D1, CDK4, Rb and p16 in IL-1β-induced chondrocytes. Following treatment with 10% DHJSD 2-h serum and blank serum for 24, 48 and 72 h, the cells were collected and the mRNA levels of cyclin D1, CDK4, Rb and p16 were determined by RT-PCR. β-actin was used as the internal control. (A) The images are representative blots. (B) Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01 and **P<0.05 vs. blank serum 24 h; ▲P<0.01, ▲▲P<0.05 vs. blank serum 48 h; ★P<0.01, ★★P<0.05 vs. blank serum 72 h. DHJSD, Duhuo Jisheng Decoction; CDK4, cyclin-dependent kinase 4; Rb, retinoblastoma tumor suppressor protein; IL-1β, interleukin-1β; RT-PCR, reverse transcription-polymerase chain reaction.

limited by a low tissue turnover rate and the avascular nature of the cartilage, meaning that OA is an irreversible disease (31). With regards to therapeutic strategies for OA, there are a large number of active research and drug discovery programs aimed at identifying structure-modifying methods of inhibiting joint destruction in OA. Current drug therapies are only able to reduce symptoms; however, none of these approaches have significant efficacy as a disease-modifying anti-OA treatment (32). Chinese herbal medicine is a major modality in TCM, which has been practiced for thousands of years in China and other Asian countries, and is used for the treatment of arthritis and related disorders such as Bi syndrome (33,34). DHJSD is a traditional Chinese herbal formula which has been widely used for OA treatment; however, the molecular mechanisms underlying the therapeutic effects of DHJSD on OA remain unknown. Thus, the present study was designed to investigate whether the treatment of OA with DHJSD affected the proliferation of degenerative chondrocytes, and the possible underlying molecular mechanism. It was demonstrated that DHJSD-containing serum promoted IL-1β-induced chondrocytes proliferation through the p16-cyclin D1/CDK4-Rb pathway.

Serum pharmacology, suggested in a study by Tashino (35), is a novel method to study traditional Chinese herbs. It allows one to avoid the various disadvantages of adding drugs directly to cells (36). Various ingredients are absorbed into the blood through the gastrointestinal tract and transformed into bioactive ingredients following oral administration of traditional Chinese herbs. Cells treated with serum containing traditional Chinese medicines *in vitro* are in a similar condition to cells

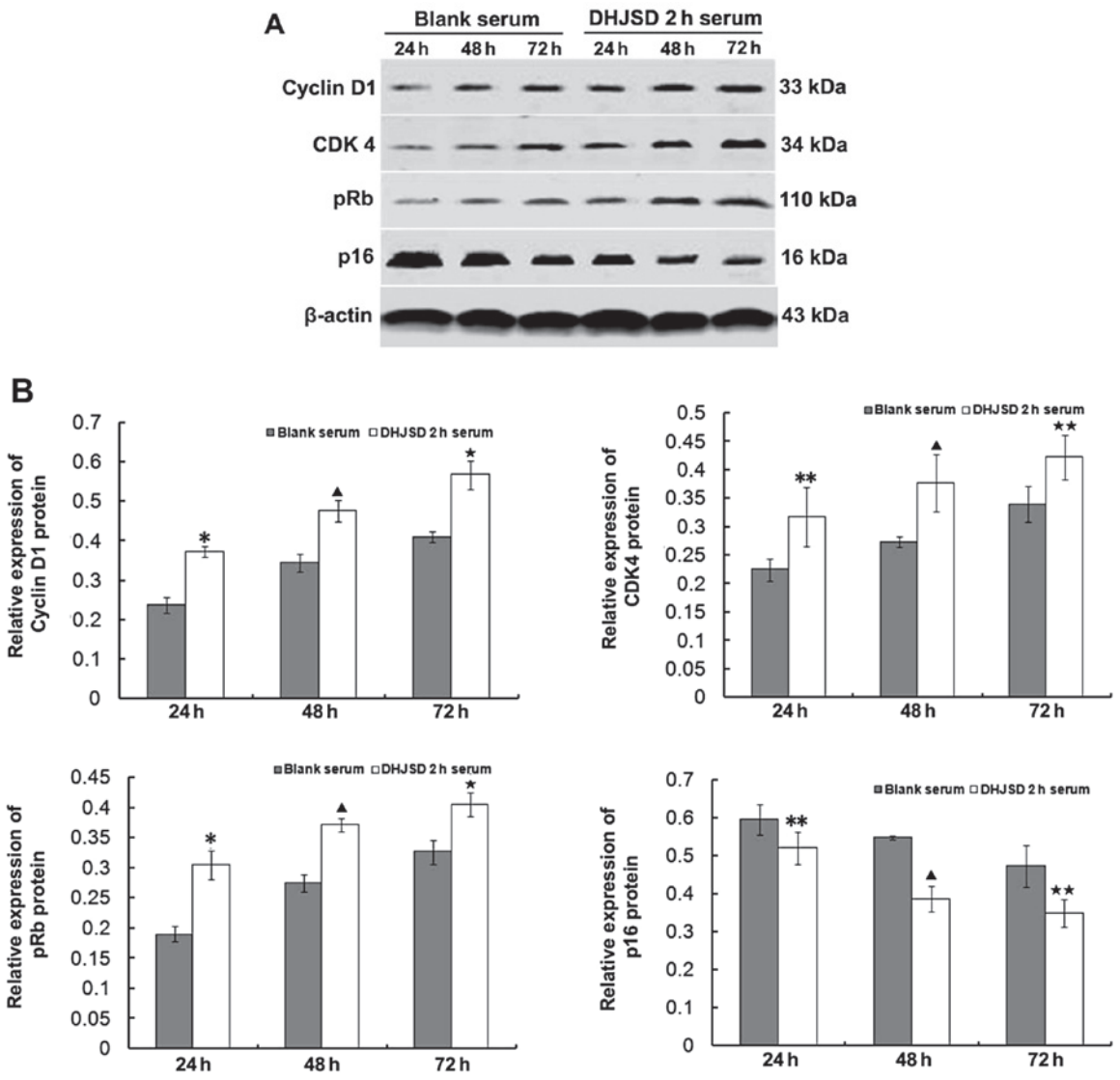

Figure 6. Effect of DHJSD 2-h serum on the protein expression of cyclin D1, CDK4, pRb and p16 in IL-1β-induced chondrocytes. Following treatment with 10% DHJSD 2-h serum and blank serum for 24, 48 and 72 h, the cells were collected. The protein levels of cyclin D1, CDK4, pRb and p16 were determined by western blotting. β-actin was used as the internal control. (A) Representative images of western blots. (B) Data are presented as the mean ± standard deviation of three independent experiments. *P<0.01, **P<0.05 vs. blank serum 24 h; ▲P<0.01 and ▲▲P<0.05 vs. blank serum 48 h; ★P<0.01 and ★★P<0.05 vs. blank serum 72 h. DHJSD, Duhuo Jisheng Decoction; CDK4, cyclin-dependent kinase 4; pRb, phosphorylated retinoblastoma tumor suppressor protein; IL-1β, interleukin-1β; RT-PCR, reverse transcription-polymerase chain reaction.

in vivo (37). Therefore, serum pharmacology experiments on Chinese herbal medicines may produce reliable consistency with corresponding experiments *in vivo* (38). Based on the pharmacokinetics, it is well-established that drugs have different effects on the body over different time periods of treatment. Thus, in the present study, arterial blood was collected from the abdominal aorta at 1, 2 and 3 h subsequent to the final dose of DHJSD. In order to identify the ideal intervention conditions, the proliferation of chondrocytes was detected by MTT assay following treatment with a range of concentrations of the DHJSD 1, 2 and 3-h sera for 24, 36, 48, 60 and 72 h. The results demonstrated that the ideal condition was the 10% concentration of 2-h DHJSD serum, which was used in the proceeding experiments.

The mechanisms that lead to cartilage degradation primarily involve the excessive production of matrix metalloproteinases (MMPs), including collagenases and stromelysins (39). Chondrocytes largely contribute to this enhancement by secreting high levels of MMPs in response to cytokines, primarily IL-1β. These cytokines synergically enhance pro-MMP secretion and modulate the activation or inhibition systems, leading to an increase in the proteolytic activity of cartilage (40). *In vitro*, IL-1β modifies the normal metabolic functions of chondrocytes, and provokes an imbalance between the catabolic and anabolic events, leading to an excess of cartilage resorption (40,41). Park *et al* (42) also reported that the IL-1β-treated construct can be a simplified model in a closed system to simulate pathological OA cartilage. Therefore, IL-1β was used in the current study to reproduce the degenerative chondrocyte model, and a concentration of 10 ng/ml IL-1β was selected for use, as in a previous study (29,30). In the present study, the cells displayed typical morphologies of degenerative chondrocytes following IL-1β stimulation; numerous chondrocytes became larger with finger-like protrusions at the edges, and the cell membrane and

cytoplasm became unclear. It was also demonstrated that the expression of the chondrocyte-specific protein type II collagen was reduced, which is in accordance with previous findings (43). In view of these results, the IL-1β-stimulated chondrocyte model was indicated to provide a suitable context in which to study the effects of DHJSD 2-h serum on the proliferation of degenerative chondrocytes.

In the present study, it was demonstrated that DHJSD 2-h serum and blank serum treatment led to a time-dependent increase in the viability of IL-1β-induced chondrocytes, and the potentiating effect was significantly greater in the DHJSD 2-h serum group ($P<0.01$, vs. the blank serum group). These results suggest that DHJSD 2-h serum promotes IL-1β-induced chondrocyte growth in a time-dependent manner. Notably, FACS analysis, which measures the DNA content of cells and is more sensitive to cell cycle changes than the MTT method, was utilized in the current study. The percentages of G_0/G_1 phase cells reduced in a time-dependent manner in the DHJSD 2-h serum group. The percentage of S phase cells exhibited the opposite trend, and increased in a time-dependent manner. These data demonstrate that DHJSD 2-h serum promotes IL-1β-induced chondrocyte proliferation by promoting G_1/S phase transition.

Previous studies have indicated that the p16-cyclin D1/CDK4-Rb pathway serves a central function in the G_1/S phase transition; this feedback-regulating network determines the process of the cell cycle (44). Briefly, extracellular signals induce the expression of cyclin D1 in cells entering the cell cycle and this binds to and activates CDK4 (45,46). The ensuing complexes in turn lead to the phosphorylation of retinoblastoma (Rb), resulting in its dissociation from transcription factors, which are predominant members of the E_2F family, and activate a number of genes required for the progression of the cell cycle to the S phase (45). p16, a member of the INK4 family of CDK inhibitors, inhibits CDK4, maintaining Rb in its unphosphorylated E_2F-associated state, and thereby preventing G_1/S phase transition (47,48). In the current study, it was demonstrated that DHJSD 2-h serum enhanced cyclin D1, CDK4 and Rb, and reduced p16 mRNA expression levels in IL-1β-induced chondrocytes, indicating that DHJSD 2-h serum promotes the progression of chondrocytes from the G_1 to the S phase by influencing cyclin D1, CDK4, Rb and p16. In order to further confirm the results, the effects of DHJSD 2-h serum on the protein expression of cyclin D1, CDK4, pRb and p16 were determined by western blotting analysis. The results revealed that the protein expression of cyclin D1, CDK4 and Rb were increased, and the expression level of p16 was reduced following DHJSD serum treatment, which is in accordance with the observed patterns of mRNA expression.

In conclusion, the current study demonstrated that DHJSD-containing serum of rats has the ability to promote proliferation in IL-1β-induced chondrocytes, through the promotion of G_1/S transition via modulating the expressions of cyclin D1, CDK4, Rb and p16. These data provide a better understanding of the effects and mechanisms of DHJSD in the treatment of OA. However, it is unclear which of the composites of this classic herbal medicine contributes to the pro-proliferative effect. Therefore, further study of the individual components of DHJSD is required in future, in order to clarify these mechanisms.

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References

1. Felson DT: Clinical practice. Osteoarthritis of the knee. N Engl J Med 354: 841-848, 2006.
2. Zhang W, Nuki G, Moskowitz RW, *et al*: OARSI recommendations for the management of hip and knee osteoarthritis: part III: Changes in evidence following systematic cumulative update of research published through January 2009. Osteoarthritis Cartilage 18: 476-499, 2010.
3. Qiu G: Osteoarthritis diagnosis and treatment guidelines. Chinese J Joint Surgery 1: 281-285, 2007.
4. Shortkroff S and Yates KE: Alteration of matrix glycosaminoglycans diminishes articular chondrocytes' response to a canonical Wnt signal. Osteoarthritis Cartilage 15: 147-154, 2007.
5. Chan BY, Fuller ES, Russell AK, *et al*: Increased chondrocyte sclerostin may protect against cartilage degradation in osteoarthritis. Osteoarthritis Cartilage 19: 874-885, 2011.
6. Huang JG, Xia C, Zheng XP, *et al*: 17β-Estradiol promotes cell proliferation in rat osteoarthritis model chondrocytes via PI3K/Akt pathway. Cell Mol Biol Lett 16: 564-575, 2011.
7. Kashiwagi A, Schipani E, Fein MJ, Greer PA and Shimada M: Targeted deletion of Capn4 in cells of the chondrocyte lineage impairs chondrocyte proliferation and differentiation. Mol Cell Biol 30: 2799-2810, 2010.
8. Sarzi-Puttini P, Cimmino MA, Scarpa R, *et al*: Osteoarthritis: an overview of the disease and its treatment strategies. Semin Arthritis Rheum 35 (Suppl 1): 1-10, 2005.
9. Badley EM, Rasooly I and Webster GK: Relative importance of musculoskeletal disorders as a cause of chronic health problems, disability, and health care utilization: findings from the 1990 Ontario Health Survey. J Rheumatol 21: 505-514, 1994.
10. Felson DT, Lawrence RC, Dieppe PA, *et al*: Osteoarthritis: new insights. Part 1: the disease and its risk factors. Ann Intern Med 133: 635-646, 2000.
11. Bradley JD, Brandt KD, Katz BP, Kalasinski LA and Ryan SI: Comparison of an antiinflammatory dose of ibuprofen, an analgesic dose of ibuprofen, and acetaminophen in the treatment of patients with osteoarthritis of the knee. N Engl J Med 325: 87-91, 1991.
12. Lohmander LS, Dalén N, Englund G, *et al*: Intra-articular hyaluronan injections in the treatment of osteoarthritis of the knee: a randomised, double blind, placebo controlled multicentre trial. Ann Rheum Dis 55: 424-431, 1996.
13. Berenbaum F: New horizons and perspectives in the treatment of osteoarthritis. Arthritis Res Ther 10 (Suppl 2): S1, 2008.
14. Ahmed S, Anuntiyo J, Malemud CJ and Haqqi TM: Biological basis for the use of botanicals in osteoarthritis and rheumatoid arthritis: a review. Evid Based Complement Alternat Med 2: 301-308, 2005.
15. Parkman CA: Alternative therapies for osteoarthritis. Case Manager 12: 34-36, 2001.
16. Teekachunhatean S, Kunanusorn P, Rojanasthien N, *et al*: Chinese herbal recipe versus diclofenac in symptomatic treatment of osteoarthritis of the knee: a randomized controlled trial [ISRCTN70292892]. BMC Complement Altern Med 4: 19, 2004.
17. Setty AR and Sigal LH: Herbal medications commonly used in the practice of rheumatology: mechanisms of action, efficacy, and side effects. Semin Arthritis Rheum 34: 773-784, 2005.
18. Chun SC, Jee SY, Lee SG, Park SJ, Lee JR and Kim SC: Anti-inflammatory activity of the methanol extract of moutan cortex in LPS-activated Raw264.7 cells. Evid Based Complement Alternat Med 4: 327-333, 2007.
19. Wu M and Gu Z: Screening of bioactive compounds from moutan cortex and their anti-inflammatory activities in rat synoviocytes. Evid Based Complement Alternat Med 6: 57-63, 2009.
20. Sun SM: Bei Ji Qian Jin Yao Fang. Vol 8. 1st edition. People's Medical Publishing Press, Beijing, pp166-167, 1982.
21. Lai JN, Chen HJ, Chen CC, Lin JH, Hwang JS and Wang JD: Duhuo jisheng tang for treating osteoarthritis of the knee: a prospective clinical observation. Chin Med 2: 4, 2007.

22. Zheng CS, X XJ, Ye HZ, Wu GW, Li XH, Huang SP and Liu XX: Computational approaches for exploring the potential synergy and polypharmacology of Duhuo Jisheng Decoction in the therapy of osteoarthritis. Mol Med Report 7: 1812-1818, 2013.
23. Wu G, Chen W, Fan H, *et al*: Duhuo Jisheng Decoction promotes chondrocyte proliferation through accelerated G1/S transition in osteoarthritis. Int J Mol Med 32: 1001-1010, 2013.
24. Panico AM, Cardile V, Garufi F, Puglia C, Bonina F and Ronsisvalle G: Effect of hyaluronic acid and polysaccharides from *Opuntia ficus indica* (L.) cladodes on the metabolism of human chondrocyte cultures. J Ethnopharmacol 111: 315-321, 2007.
25. The Ministry of Science and Technology of the People's Republic of China: Guidance Suggestions for the Care and Use of Laboratory Animals, 2006.
26. Huang J, Huang X, Chen Z, Zheng Q and Sun R: Dose conversion among different animals and healthy volunteers in pharmacological study. Chin J Clin Pharmacol Ther 9: 1069-1072, 2004 (In Chinese).
27. Li XH, Du M, Liu XX, *et al*: Millimeter wave treatment inhibits NO-induced apoptosis of chondrocytes through the p38MAPK pathway. Int J Mol Med 25: 393-399, 2010.
28. Li XH, Wu MX, Y HZ, Chen WL, Lin JM, Zheng LP and Liu XX: Experimental study on the suppression of sodium nitroprussiate-induced chondrocyte apoptosis by tougu xiaotong capsule-containing serum. Chin J Integr Med 17: 436-443, 2011 (In Chinese).
29. Sanchez C, Mathy-Hartert M, Deberg MA, Ficheux H, Reginster JY and Henrotin YE: Effects of rhein on human articular chondrocytes in alginate beads. Biochem Pharmacol 65: 377-388, 2003.
30. Wu SQ, Otero M, Unger FM, *et al*: Anti-inflammatory activity of an ethanolic *Caesalpinia sappan* extract in human chondrocytes and macrophages. J Ethnopharmacol 138: 364-372, 2011.
31. Mankin HJ: The response for articular cartilage to mechanical injury. J Bone Joint Surg Am 64: 460-466, 1982.
32. Brandt KD and Mazzuca SA: Lessons learned from nine clinical trials of disease-modifying osteoarthritis drugs. Arthritis Rheum 52: 3349-3359, 2005.
33. Bensky D, Gamble A and Stöger E (eds): Chinese Herbal Medicine: Materia Medica. 3rd edition. Eastland Press, Seattle, WA, 2004.
34. Ho LJ and Lai JH: Chinese herbs as immunomodulators and potential disease-modifying antirheumatic drugs in autoimmune disorders. Curr Drug Metab 5: 181-192, 2004.
35. Tashino S: 'Serum pharmacology' and 'serum pharmaceutical chemistry': from pharmacology of Chinese traditional medicines to start a new measurement of drug concentration in blood. Ther Drug Monit Res 5: 54-64, 1988.
36. Liu N, Liu JT, Ji YY and Lu PP: Dahuang zhechong pill containing serum inhibited platelet-derived growth factor-stimulated vascular smooth muscle cells proliferation by inducing G1 arrest partly via suppressing protein kinase C α-extracellular regulated kinase 1/2 signaling. Chin J Integr Med 18: 371-377, 2012 (In Chinese).
37. Dang XY, Dong L, Shi HT and Zou BC: Effects of serum containing Chinese medicine Sanpi Pingwei formula on proliferation and apoptosis of human SGC-7901 cells. Chin J Integr Med 19: 119-126, 2013.
38. Wang WJ, Li DJ, Li J and Zhou WJ: An in vitro study on neuroprotective effects of serum containing Gengnianchun decoction and its main monomers against amyloid beta protein-induced cellular toxicity. J Chin Integr Med 8: 67-73, 2010 (In Chinese).
39. Henrotin Y, Sanchez C and Reginster JY: The inhibition of metalloproteinases to treat osteoarthritis: reality and new perspectives. Expert Opin Ther Patents 12: 29-43, 2002.
40. Tetlow LC, Adlam DJ and Woolley DE: Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage. Arthritis Rheum 44: 585-594, 2001.
41. Martel-Pelletier J, Di Battista J and Lajeunesse D: Biochemical factors in joint articular tissue degradation in osteoarthritis. In: Osteoarthritis: Clinical and Experimental Aspects. Reginster J-YL, Pelletier JP, Martel-Pelletier J, Henrotin YE (eds). Springer, Berlin pp156-187, 1999.
42. Park K, Hoffmeister B, Han DK and Hasty K: Therapeutic ultrasound effects on interleukin-1beta stimulated cartilage construct in vitro. Ultrasound Med Biol 33: 286-295, 2007.
43. Tan L, Peng H, Osaki M, Choy BK, Auron PE, Sandell LJ and Goldring MB: Egr-1 mediates transcriptional repression of COL2A1 promoter activity by interleukin-1beta. J Biol Chem 278: 17688-17700, 2003.
44. Gibson SL, Dai CY, Lee HW, *et al*: Inhibition of colon tumor progression and angiogenesis by the Ink4a/Arf locus. Cancer Res 63: 742-746, 2003.
45. Massagué J: G1 cell-cycle control and cancer. Nature 432: 298-306, 2004.
46. Musgrove EA, Caldon CE, Barraclough J, Stone A and Sutherland RL: Cyclin D as a therapeutic target in cancer. Nat Rev Cancer 11: 558-572, 2011.
47. Li J, Poi MJ and Tsai MD: Regulatory mechanisms of tumor suppressor P16(INK4A) and their relevance to cancer. Biochemistry 50: 5566-5582, 2011.
48. Witkiewicz AK, Knudsen KE, Dicker AP and Knudsen ES: The meaning of p16(ink4a) expression in tumors: functional significance, clinical associations and future developments. Cell Cycle 10: 2497-2503, 2011.