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Original Article

Duhuo Jisheng Decoction inhibits the activity of osteoclasts in osteonecrosis of the femoral head via regulation of the RELA/AKT1 axis

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Abstract: Objective: To investigate the effect of Duhuo Jisheng Decotion (DHJSD) on the activity of osteoclasts in osteonecrosis of the femoral head (ONFH) and its underlying mechanism relating to the RELA/AKT1 axis. Methods: The TCMSP database was used to search for the effective ingredients and the targets of various Chinese medicines in DHJSD. Its targets were intersected with ONFH risk genes in DisGeNET and Malacards databases to obtain the potential target genes. qRT-PCR was used to detect the expression of potential target genes in ONFH tissues, and the ChIP experiment was used to verify the relationship between RELA and AKT1 promoter. An ONFH rat model was established and DHJSD was used for the treatment. The expressions of RELA and AKT1 in rats were intervened, and rats were grouped. qRT-PCR was applied to detect the expression levels of osteoclast markers ACP5, CTSK, and RANK in the tissues to evaluate the regulation of DHJSD on target genes and the mechanism of osteoclast differentiation. Results: A total of 231 effective targets of DHJSD were screened out in the TCMSP database. Intersection with ONFH risk genes yielded a total of 20 candidate genes. Protein-protein interaction analysis showed that AKT1 regulated other genes. KEGG functional enrichment analysis revealed that STAT1, AKT1, PPARG, PPARG, TNF and RELA were enriched in osteoclast differentiation pathway. Compared with normal tissues, the expression of STAT1 was decreased in ONFH tissues, and the expressions of AKT1, PPARG, TNF, and RELA were increased, among which, RELA and AKT1 are the most significantly increased genes (all $P<0.05$). ChIP experiment found that RELA had a binding relationship with AKT1 promoter. DHJST had the inhibitory effect on the expression of RELA and AKT1 in ONFH tissues, as well as the levels of ACP5, CTSK, and RANK. However, overexpression of RELA or AKT1 attenuated the inhibitory effect of DHJSD on the levels of ACP5, CTSK and RANK. Meanwhile, knocking down RELA partially reversed the effect of AKT1 on the effect of DHJSD. Conclusion: DHJSD inhibits the activity of osteoclasts in ONFH by inhibiting the RELA/AKT1 axis. This study further clarifies the potential specific mechanism of DHJSD to improve ONFH.

Keywords: Duhuo Jisheng Decoction, osteonecrosis of the femoral head, osteoclast, RELA, AKT1

Introduction

Osteonecrosis of the femoral head (ONFH), mainly occurring in middle-aged people, is a common painful disease of the hip joint, which can lead to hip dyskinesia [1]. ONFH has extremely complex pathogenesis, of which, heredity, drinking, autoimmunity, and hyperlipidemia are common risk factors [2]. In the early stage of the disease, non-surgical treatment can be used to prevent the collapse of the femoral head and subchondral fractures. Total hip replacement is usually required, under the circumstances of ineffective conservative treatment and the late stage of the disease [3]. At present, the pathogenesis of ONFH still remains unclear, and there is no effective treatment. Hence, it is urgent to explore the possible pathogenesis of ONFH and develop new treatment methods.

Duhuo Jisheng Decoction (DHJSD) is a classic ancient prescription for treating liver-and-kidney-deficiency-type arthritis. It consists of Du-

huo (Heracleum hemsleyanum Diels), Sangjisheng (Herba Taxilli), Duzhong (Eucommia ulmoides Oliv.), Niuxi (Achyranthes bidentata), Xixin (Asarum sieboldi Mig), Qinjiao (Gentiana macrophylla Pall), Fuling (Poria), Rouguixin (Cinnamomum cassia Presl), Fangfeng (Saposhnikovia divaricata (Turcz.) Schischk), Chuanxiong (Ligusticum striatum), Renshen (Panax ginseng), Gancao (Glycyrrhiza uralensis), Danggui (Chinese angelica), Shaoyao (Chinese herbaceous peony), and Gandihuang (dried Radix Rehmanniae), which are widely used in the treatment of bone diseases in clinical practice [4]. It has been found that DHJSD inhibited chondrocyte inflammation through Wnt/β-catenin signaling pathway [5]. DHJSD may inhibit cartilage destruction by reducing the content of MMP-13, TNF-α and other factors in the synovial fluid of rats, and has a repairing effect on cartilage in rats with knee osteoarthritis [6]. As the main functional cells of bone resorption, osteoclasts play an important role in bone growth and development, repair and reconstruction [7]. At present, the repair effect of DHJSD on cartilage cells has been well studied, but the effect on osteoclast differentiation has not been clear.

RELA proto-oncogene, a subunit of NF-κB, also known as P65, is a widespread transcription factor involved in a variety of biological processes [8]. The expression of NF-κB is significantly up-regulated in the articular cartilage of patients with ONFH [9]. AKT1 is a known oncogene that can be phosphorylated by PI3K, forming the well-known PI3K/AKT signaling pathway [10]. Total flavonoids can improve steroid-induced avascular necrosis of the femoral head through PI3K/AKT pathway [11]. It has been confirmed that RELA can directly target AKT1/MAPK3/1 and continuously activate NF-κB [12]. The interaction between RELA and AKT1 promoter will be further verified in this study.

Studies have found that IL-6, VEGFA, ALB, EGFR, CASP3, MAPK8, MYC may be the targets of DHJSD in the treatment of knee osteoarthritis [13]. This study connected DHJSD with the RELA/AKT1 axis through bioinformatics, to investigate the role of the DHJSD in regulating the reLA/AKT1 axis in osteoclast differentiation. Besides, this study also explored the potential targets of DHJSD in the treatment of ONFH, and provided a scientific basis for the later development of DHJSD combined drugs.

Materials and methods

Screening of effective ingredients in DHJSD

The TCMSP (https://www.tcmspw.com) was adopted to retrieve the active ingredients and targets of DHJSD (Supplementary Table 1). Oral bioavailability ≥30% and drug-like properties ≥0.18 were considered as the screening conditions. The target was presented by the name of the protein, which was standardized as Gene Symbol with the help of Uniprot database (https://www.uniprot.org/).

Construction of the target gene regulatory network of DHJSD in the treatment of ONFH

The risk genes for femoral necrosis were retrieved in the DisGeNET database (https://www.disgenet.org/search) with "femoral necrosis" as the key search term (Supplementary Table 2). The Hiplot website (https://hiplot.com.cn) was used to intersect the risk genes with the target genes of HJST. Cytoscape was applied to build a target gene regulation network for the treatment of femoral necrosis with DHJSD.

Screening of core genes

STRING (https://string-db.org/) was used to perform protein-protein interaction (PPI) analysis on intersection genes, and DAVID (https://david.ncifcrf.gov) to carry out gene annotation analysis. JASPAR website (http://jaspar.genereg.net) analyzed target genes.

Clinical specimens

From May 2019 to May 2020, 42 patients with steroid-induced femoral head necrosis were recruited in our hospital, including 18 males and 24 females, with an average age of 58.44 years old. At the same time, 20 patients with femur fractures caused by trauma were collected, 12 males and 8 females, with an average age of 56.21 years old.

Inclusion criteria: (1) Patients met the standards of the Association Research Circulation Osseous for steroid-induced femoral head necrosis. (2) Patients had normal test outcomes, except biopsy necrosis. (3) Patients had positive bone scan or/and magnetic resonance findings of steroid-induced femoral head necrosis [14]. Exclusion criteria: (1) Patients were

Table 1. Plasmid construction sequences

RELA	TTTGGCTATTATGCGCGCCTAAGAACACC
AKT1	GGTCTAAGAGACTCGCGCCAAAACCTTCGA
si-RELA	Sense-UCAAUUCCCGGUAUGAGCUCUCG
	Antosense-AAAUUUAAAGCGCGCUAUAUAACG

combined with chronic diseases such as hypertension, heart disease, diabetes. (2) Patients have recently used adrenal cortex hormones and lipid-lowering drugs. (3) Patients had alcoholism, recent use of hormonal drugs, or femoral head necrosis. There were no significant differences in age and gender between the two groups. During hip replacement surgery, the necrotic tissue of the fractured surface of the femoral head of patients in the two groups was collected and stored at -80°C. Each patient's written informed consent was obtained before specimen collection, and the study was approved by the local medical Ethics Committee.

Drug preparation

Dispensary of Chinese medicine in our hospital provided and decocted the medicinal herbs. Duhuo (Heracleum hemsleyanum Diels, 9 g), Sangjisheng (Herba Taxilli, 6 g), Duzhong (Eucommia ulmoides Oliv., 6 g), Niuxi (Achyranthes bidentatae), Xixin (Asarum sieboldi Mig, 6 g), Qinjiao (Gentiana macrophylla Pall, 6 g), Fuling (Poriae), Rouguixin (Cinnamomum cassia Presl, 6 g), Fangfeng (Saposhnikovia divaricata (Turcz.) Schischk, 6 g), Chuanxiong (Ligusticum striatum, 6 g), Renshen (Panax ginseng, 6 g), Gancao (Glycyrrhiza uralensis, 6 g), Danggui (Chinese angelica, 6 g), Shaoyao (Chinese herbaceous peony, 6 g), and Gandihuang (dried Radix Rehmanniae, 6 g) were soaked with 2,000 mL distilled water for 30 min and boild in high heat. After boiling, the decoction was allowed to simmer gently for 30 min. The medicine residue was boiled with 1,000 mL distilled water for 25 min. The filtrate collected from the two decoctions was mixed, concentrated to 1.0 g/mL, and stored at 4°C for later use.

ONFH animal model establishment

Sixty adult male Sprague Dawley rats (246 g-275 g) were obtained from Southern Medical University (Guangdong, China). Rats were bred for 1 week in an environment of 24±2°C, with free access to food and water, and a 12-hour cycle of light/darkness was maintained. Rats were injected with 50 mg/kg dexamethasone (D829854, Macklin Inc., Shanghai) into the gluteal muscles for 6 consecutive weeks, twice a week. Serum cholesterol and triacylglycerol tests were used to determine whether the modeling was successful. The increase of these indicators can lead to hyperlipidemia, causing fat embolism of small blood vessels around the femoral head and microcirculation disorders of the femoral head, which in turn leads to ONFH [15]. The 52 successfully modeled ONFH rats were divided into 6 groups (n=8 for each group) according to the random number table method. They were sham group (injection of saline), model group (ONFH group, injection of dexamethasone), DHJSD group (intragastrically administrated 3.4 g/kg DHJSD per day from 3 days before modeling to 12 days after modeling), DHJSD+RELA group (after intragastric administration of DHJSD, RELA overexpression plasmid was injected in the tail vein), DHJSD+AKT1 group (after intragastric administration of DHJSD, AKT1 overexpression plasmid was injected in the tail vein), DHJSD+AKT1+si-RELA group (after intragastric administration of DHJSD, AKT1 overexpression plasmid and si-RELA plasmid were injected in the tail vein), and the remaining 4 mice were set aside. The construction of the plasmid was provided by Shanghai Gema Gene Co., Ltd., Shanghai, China (**Table 1**). Rats in each group were euthanized by intraperitoneal injection of an overdose of sodium pentobarbital (100 mg/kg, Renfu Medical Co., Ltd.) after 2 weeks of treatment, and the femoral head tissues were taken out. The mRNA and protein expression levels of various factors in the femoral head tissue were analyzed by qRT-PCR and Western blot.

HE staining

HE Staining Kit (G1120, Soleibo, Beijing) was used for the experiment. The tissues were covered with the wax, and cut into slices of 6 μm section using a slicer, fixed on the glass slide. Drain xylene I/II was added to the tissue section successively and warmed for 10 min. The tissue section passed through an increasing concentration of alcohol baths (85%, 90%, 95%, 100%) and water for 5 min. After rinse with distilled water for 2 min, the tissue section was stained in hematoxylin for 10 min away from light. After the floating color was washed away with distilled water, the tissue section

Table 2. qRT-PCR primer sequences

Name of genes	qRT-PCR sequences	
	Forward	Reverse
RELA	GCCGGCATGGCATTTAGGGCTCT	TTCGGCGTATAAAGCTCAAAGC
AKT1	AATATCGGCTCTCTTCAGAAG	CAAATATCGCGCCGATTCCC
STAT1	CCTCTAGAGAGAGTCCGAAAT	TAGAGATCTCTTCTAGGCAA
PPARG	CTTTGTTAGAGTCTCATTTA	TGCAAATATGCCCGTCTTCTC
TNF	GTTATATGCGCTATGCGGTCTC	GGCTTAAGTTCGGTTAGAGA
ACP5	GTTTCGAGATACTGGGCTAAC	CCCCATTAAAGTTGGGGAG
CTSK	TAAGAGTAAGCTGCGCTTCG	AAAATCTGCGGCGCGGTAAG
RANK	CGGCATAGCGCGGCGATTA	TCGAGTAAAGACTGCGGTGCTC
GAPDH	TGGTGCGATTAGGAGCTTGCGC	CTTGAGATTCACCGCGTCCGA

was stained in Eosin for 2 min away from light. The section was rinsed in distilled tap water for 3 s before quick dehydration. Optical resin gum was used to seal the section, and the pathological changes in the tissues were observed under microscope (NE600, Shandong Micro instrument Co., Ltd.).

Cell culture and transfection

HEK293T cells (CL-0005, Wuhan Punosel, China) were used for molecular relationship verification. The cells were cutured in DMEM medium (PM150210, Wuhan Punosi, China) supplemented with 10% fetal bovine serum (164210, Wuhan Punosi, China) and 1% penicillin-streptomycin (PB180120, Wuhan Punosi, China). The cells were collected at logarithmic phase and transfected. The knocked down RELA, over-expressed RELA and negative control plasmids (Shanghai Gemma Gene, China) were transfected using Lipofectamine 3000 (L3000007, Thermo Fisher, USA) in accordance with the instructions. After transfection, cells were placed in an incubator at 37°C and 5% CO_2 for 48 hours before subsequent experiments.

qRT-PCR

The tissue was ground into homogenates. Trizol reagent (R0016, Beyotime Biotechnology, Shanghai) was used to extract total RNA. Hifair® III 1st Strand cDNA Synthesis SuperMix for qPCR (11141ES60, Yeasen, Shanghai) was adopted for reverse transcription. Hieff UNICON® qPCR SYBR Green Master Mix (11200ES25, Shanghai Yisheng) was used for quantitative PCR reactions. The reaction was completed on the ABI 7300 system (Applied Biosystems, Massachusetts, USA). The reaction system was 25 μL of Hieff UNICON® qPCR SYBR Green Master Mix, 1 μL of forward primer, 1 μL of reverse primer, DNA template, and pure water. The reaction conditions were 1 cycle for pre-denaturation at 95°C for 30 s, followed by denaturation at 95°C for 10 s, and 40 cycles for annealing/extension at 60°C for 30 s. GAPDH was used as the internal reference. Relative expression was calculated using the $2^{-\Delta\Delta Ct}$ method. The primer sequences provided by Sino Biological, Beijing are shown in **Table 2**.

Western blot

The tissue was ground thoroughly. RIPA cell lysates (Solarbio Science & Technology, Beijing) were added to extract total proteins. The protein concentration was determined by the bisindanedionic acid kit (Beyotime Biotechnology, Shanghai). A 10% sodium lauryl sulfate polyacrylamide gel was prepared, and the protein samples were boiled at 100°C for 5 min, ice bathed, and centrifuged. The protein samples were separated by gel electrophoresis, and transferred onto a nitrocellulose membrane (FFN08, Beyotime Biotechnology, Shanghai), sealed with 5% skimmed milk powder at 4°C. Primary antibodies RELA (1:2000, ab32536, abcam, UK), AKT1 (1:5000, ab81283, abcam, UK), ACP5 (1:1000, ab235448, abcam, UK), CTSK (1:1000, ab207086, abcam, UK), RANK (1:1000, ab182158, abcam, UK), and GAPDH (1:500, ab8245, abcam, UK) were added onto the membrane and incubated overnight. Horseradish peroxidase labeled IgG (1:2000, ab6721, abcam, UK) were added to incubate for 1 h at 37°C the next day. The membrane was soaked in ECL chemiluminescence solution (36208ES76, Shanghai Yisheng) and incubated at room temperature for 1 min. In a dark environment, after developvemt and fixation, the results were observed. Image Pro Plus 6.0 software (Media Cybernetics, USA) was used to analyze the Western blot images.

ChIP experiment

ChIP experiment was used to verify whether RELA could bind to the promoter region of AKT1

by ChIP Assay Kit (P2078, Beyotime Biotechnology, Shanghai). HEK293T cells were cultred in a petri dish, mixed with 1% formaldehyde, and incubated at 37°C for 10 min to cross-link the target protein and the corresponding genomic DNA. 1.1 mL Glycine Solution (10×) was added. The mixture was allowed to stand for 5 minutes. Ice-cooled PBS (10 mL) containing 1 mM PMSF was added to wash the cells and aspirate the liquid. The cells were collected in a new centrifuge tube, centrifuged at 1,000 g at 4°C for 2 min, and ice bathed for 10 min. The genomic DNA was sheared using a 2 mm ultrasonic head into a size of 400-800 bp, 10 s/time, 4 times in total. NaCl was added into 0.2 mL ultrasonically processed samples. Then the samples were heated at 65°C for 4 h. Tris balance phenol was added, vortex vigorously mixed and centrifuged for 5 min. The supernatant was collected. Then chloroform was added, vortex vigorously mixed and centrifuged for 5 min. The supernatant was collected. Agarose gel electrophoresis was performed. 0.2 mL of supernatant was took to ice bath. Protein A+G Agarose/Salmon Sperm DNA was added to the sample and mixed at 4°C for 30 min. Samples were centrifuged and the supernatant was collected. Primary antibodies IgG (1:1000, ab17-2730, abcam, UK), RELA (1:2000, ab32536, abcam, UK) was added overnight at 4°C. 60 microliters of Protein A+G Agarose/Salmon Sperm DNA was added and mixed at 4°C for 60 min. Samples were centrifuged at 1000 g for 1 min at 4°C. Then the samples were washed and analyzed by PCR amplification.

Statistical analysis

Statistical analysis was performed with SPSS 23.0 statistical software to analyze measurement data, which were expressed as mean ± standard deviation ($\overline{x}$±sd). Independent sample t test was used for the comparison between two groups. Differences among groups were assessed using one-way analysis of variance and Tukey post-hoc test. Differences with *P*-values <0.05 were considered statistically significant.

Results

Effective ingredients of DHJSD

DHJSD consists of Duhuo (Heracleum hemsleyanum Diels), Sangjisheng (Herba Taxilli), Duzhong (Eucommia ulmoides Oliv.), Niuxi (Achyranthes bidentata), Xixin (Asarum sieboldi Mig), Qinjiao (Gentiana macrophylla Pall), Fuling (Poria), Rouguixin (Cinnamomum cassia Presl), Fangfeng (Saposhnikovia divaricata (Turcz.) Schischk), Chuanxiong (Ligusticum striatum), Renshen (Panax ginseng), Gancao (Glycyrrhiza uralensis), Danggui (Chinese angelica), Shaoyao (Chinese herbaceous peony), and Gandihuang (dried Radix Rehmanniae). The effective ingredients and targets of each Chinese herbal medicine were searched in the TCMSP database, the names were standardized on the Uniprot database, and a total of 231 effective genes were obtained. See **Figure 1** and Supplementary Table 1.

PPI analysis for core gene screening

The risk genes were retrieved in the DisGeNET database using the keyword “osteoclasts in osteonecrosis of the femoral head” (Supplementary Table 2). The risk genes were intersected with the target genes of DHJSD, obtaining 20 potential target genes, which may play a strong role in ONFH and DHJSD (**Figure 2A**). PPI analysis in the STRING revealed that AKT1 had the strongest interaction with other genes (**Figure 2B**).

KEGG enrichment analysis for core gene screening

In the KEGG enrichment analysis, there was an osteoclast differentiation pathway closely associated with ONFH (hsa04380: Osteoclast differentiation, P.3.12E-04, FDR-0.003112595), which was rich in STAT1, AKT1 (a key gene obtained from protein interoperability analysis), PPARG, TNF, RELA (**Figure 3A**). The qRT-PCR experiment found that compared with normal femoral head specimens, STAT1 expression was decreased in femoral head necrosis specimens, and the expression of the other four genes were increased to varying degrees (**Figure 3B-F**, all P<0.05), among which, AKT1 had the highest level.

RELA activated AKT1 transcription

The JASPAR indicated that RELA bound to the promoter region of AKT1 (**Figure 4A**), which was proved by the ChIP experiment. The experiment showed that the promoter region of AKT1 had binding motifs (**Figure 4B**, P<0.05). A plas-

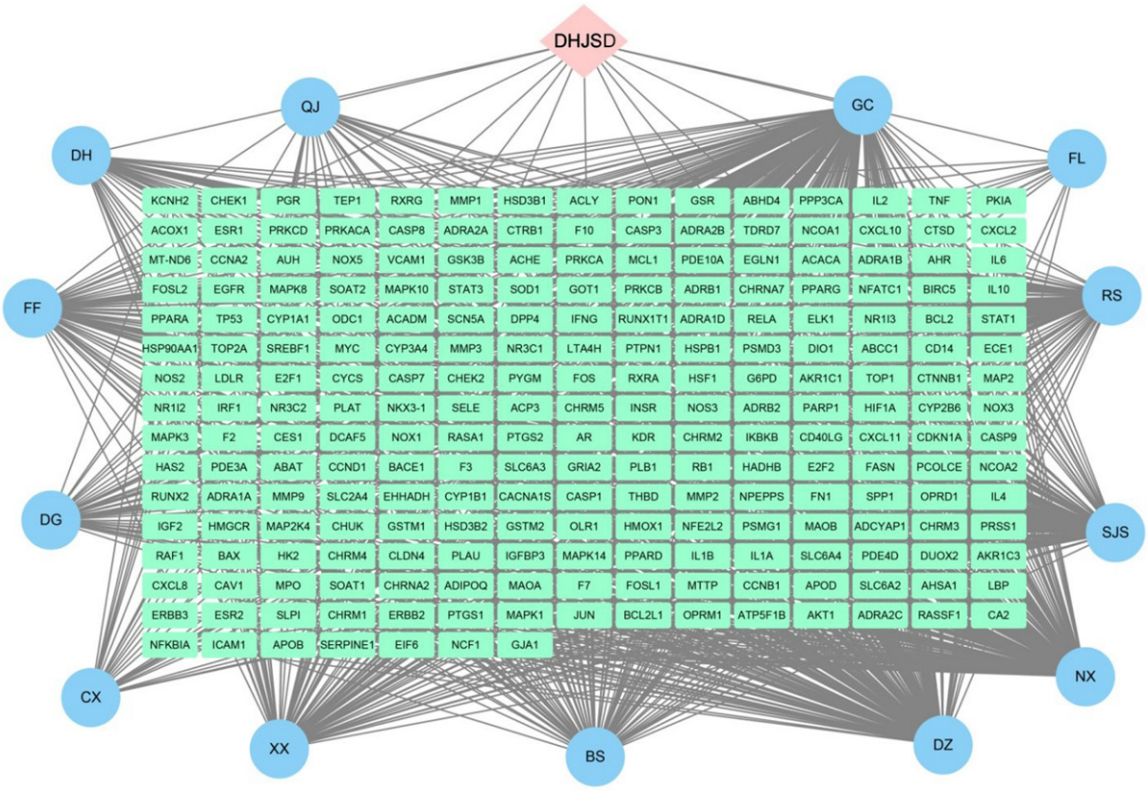

Figure 1. The target gene regulatory network of DHJSD in the treatment of osteonecrosis of the femoral head. The abbreviations of DHJSD and Chinese herbal medicine were used in the network diagram. DHJSD: Duhuo Jisheng Decoction; DH: Duhuo (Heracleum hemsleyanum Diels); SJS: Sangjisheng (Herba Taxilli); DZ: Duzhong (Eucommia ulmoides Oliv.); NX: Niuxi (Achyranthes bidentata); XX: Xixin (Asarum sieboldi Mig); QJ: Qinjiao (Gentiana macrophylla Pall); FL: Fuling (Poria); RGX: Rouguixin (Cinnamomum cassia Presl); FF: Fangfeng (Saposhnikovia divaricata (Turcz.) Schischk); CX: Chuanxiong (Ligusticum striatum); RS: Renshen (Panax ginseng); GC: Gancao (Glycyrrhiza uralensis); DG: Danggui (Chinese angelica); SY: Shaoyao (Chinese herbaceous peony); GDH: Gandihuang (dried Radix Rehmanniae).

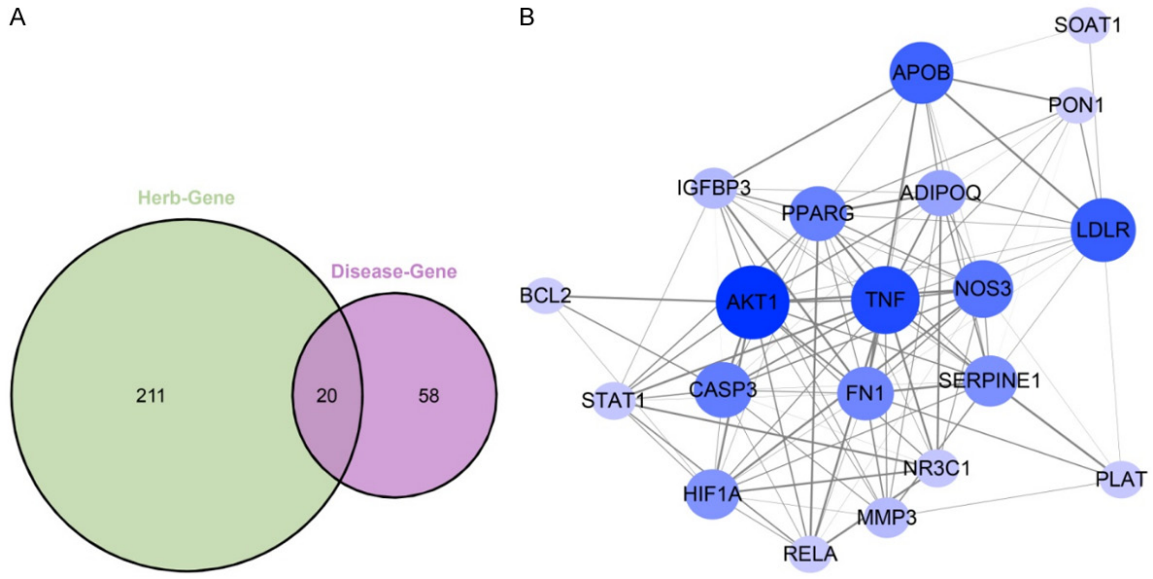

Figure 2. Protein interaction analysis for core gene screening. A: The intersection analysis results of the target genes of DHJSD and the pathogenesis genes of ONFH; B: The protein interaction results of the intersection genes (the darker color and the rounder icon indicated the stronger interaction).

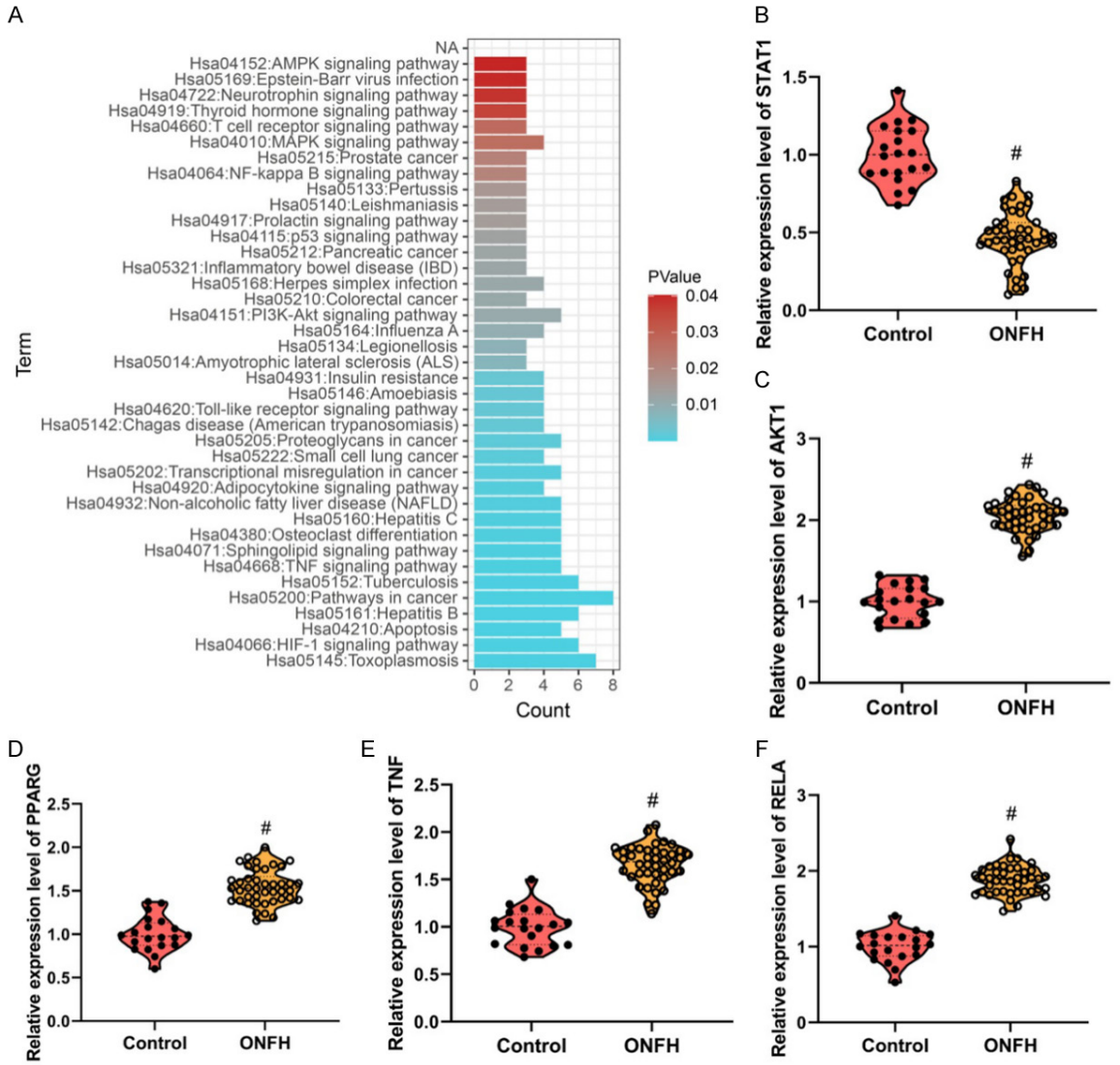

Figure 3. KEGG enrichment analysis for core gene screening. A: KEGG enrichment analysis; B-F: qRT-PCR was used to detect the level of expression of STAT1, AKT1, PPARG, TNF, AND RELA in clinical tissue specimens. Compared to the control group, #P<0.05. Control group, n=20; ONFH group, n=42. ONFH: osteonecrosis of the femoral head.

mid with knocked down or overexpressed RELA was transfected into HEK293T cells. qRT-PCR showed that knockdown or overexpression of RELA had no significant effect on the mRNA expression of AKT1, but had a significant inhibitory or promotion effect on protein expression (**Figure 4C**, both P<0.05).

DHJSD inhibited osteoclast differentiation in ONFH rats

ONFH rats were treated with intragastric administration of DHJSD. HE staining revealed that there was normal bone trabeculae, no fracture, normal vascular tissue in the bone marrow cavity and a large number of red blood cells in the sham group; irregular trabecular fracture, light fat accumulation and emerging inflammatory cells in the ONFH group; no trabecular fracture, light fat accumulation and a small number of inflammatory cells in the DHJSD group (**Figure 5A**).

Compared with the ONFH group, the expression levels of RELA and AKT1 in the bone tissue of the rats were reduced after treatment (**Figure 5B**, both P<0.05). The expression of osteoclast-related factors ACP5, CTSK and RANK decreased (**Figure 5C**, all P<0.05). It suggested that DHJSD improved pathological damage to the tissues from ONFH rats, inhibited the levels of RELA and AKT1 in ONFH

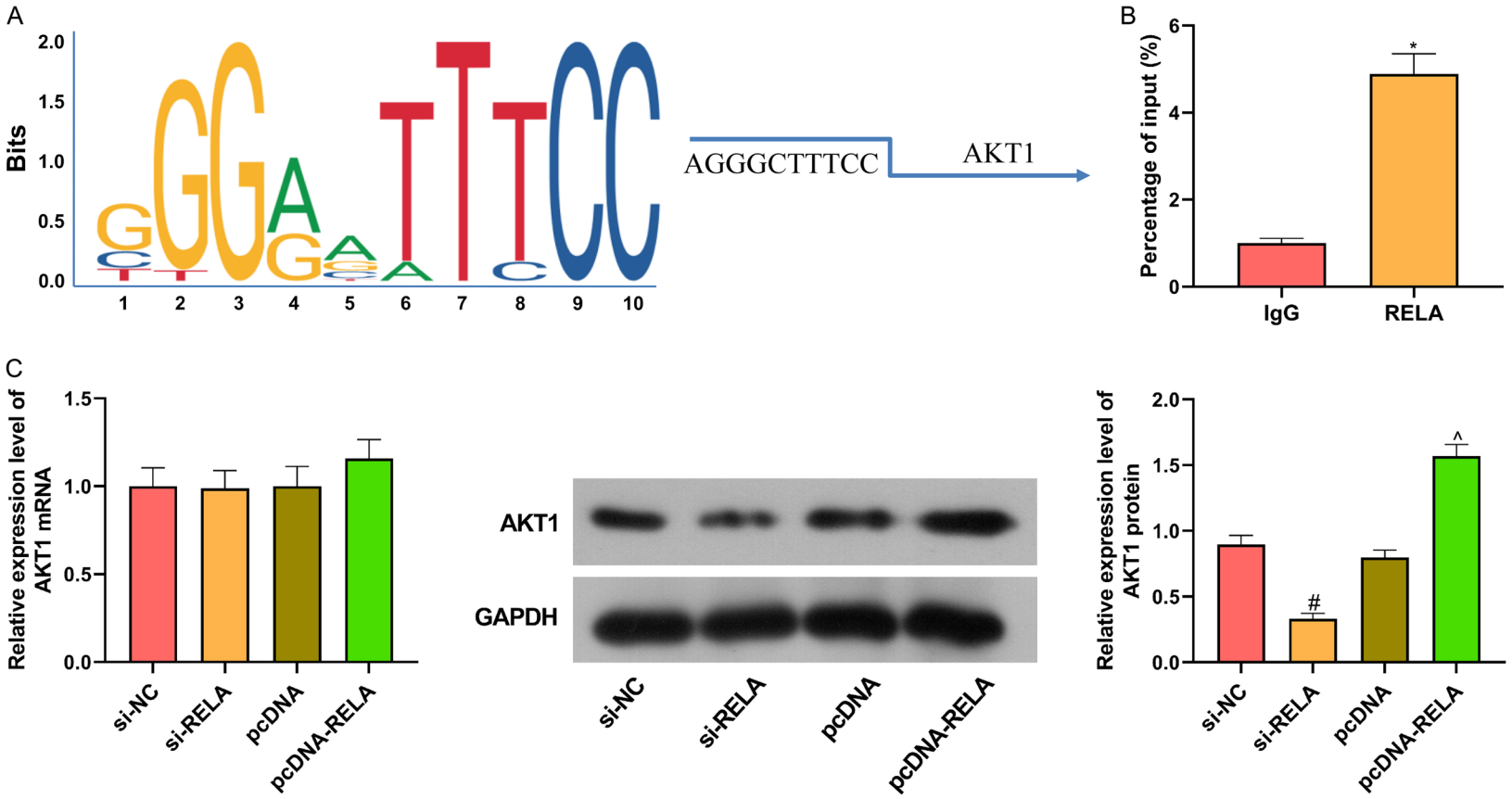

Figure 4. RELA activated AKT1 transcription. A: The binding site of RELA and AKT1 promoter predicted by JASPAR; B: Results from ChIP experiment; C: qRT-PCR and Western blot were used to detect the effect of RELA on AKT1 expression. Compared with IgG group, *P<0.05; Compared with si-NC group, #P<0.05; Compared with pcDNA group, ^P<0.05.

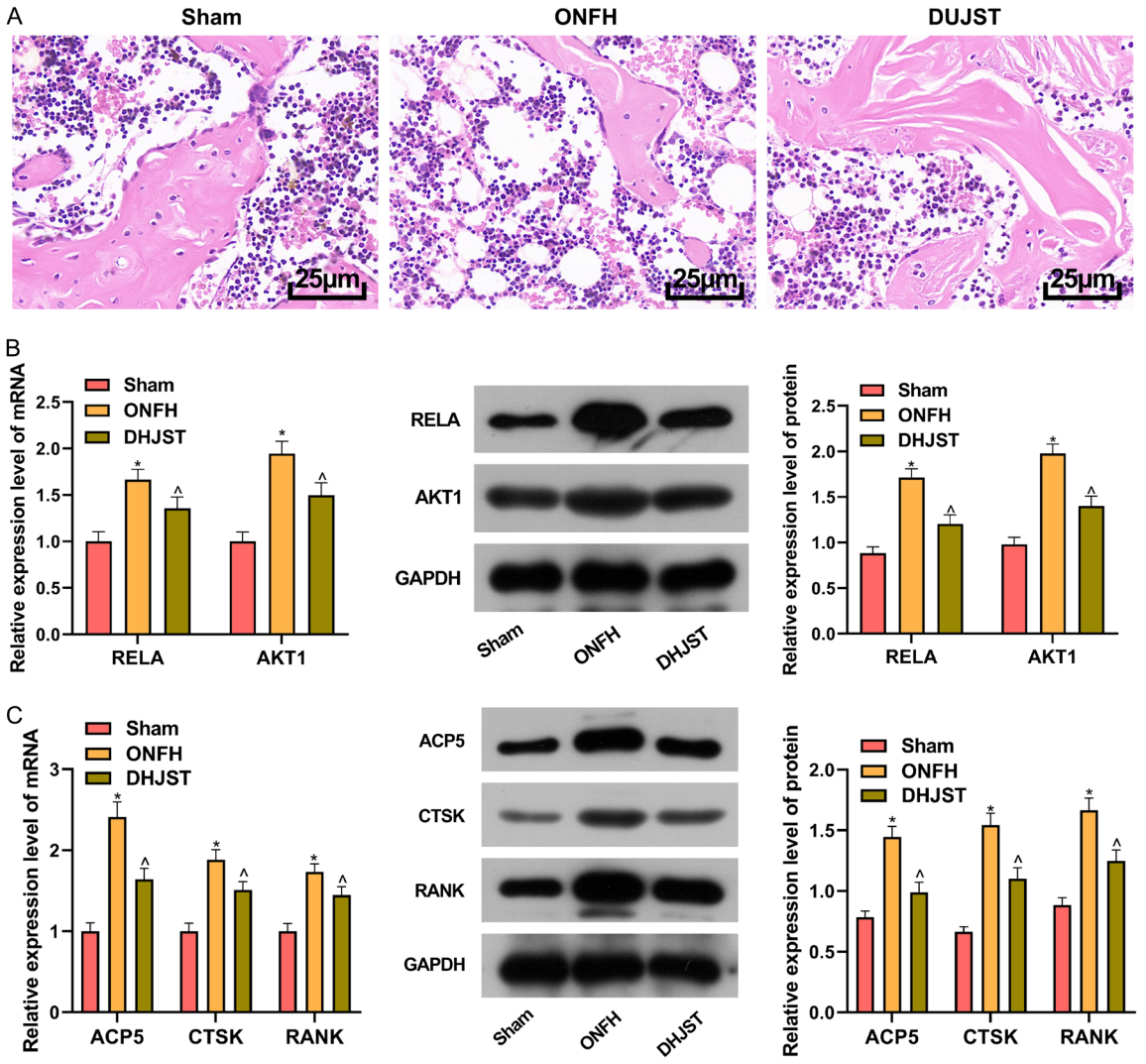

Figure 5. DHJSD inhibited osteoclast differentiation in ONFH rats. A: Pathology of rat tissues from HE staining; B: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of RELA and AKT1; C: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of ACP5, CTSK and RANK. Compared with sham group, *P<0.05; Compared with ONFH group, ^P<0.05. DHJSD: Duhuo Jisheng Decoction; ONFH: osteonecrosis of the femoral head.

rats and inhibited the differentiation of osteoclasts.

Overexpression of RELA or AKT1 partially attenuated the effect of DHJSD

When DHJSD was used to treat ONFH rats, the plasmids that overexpressed RELA or AKT1 were transfected in the rats, which increased the expression of RELA or AKT1 in the rats (**Figure 6A**, both P<0.05), and reversed the effect of DHJSD on the expression of osteoclast-related factors ACP5, CTSK, and RANK (**Figure 6B**, all P<0.05). It indicated that overexpression of RELA or AKT1 can partially offset the inhibitory effect of DHJSD on osteoclast differentiation.

Knockdown of RELA partially offset the attenuating effect of AKT1 on DHJSD

After the treatment of ONFH rats by DHJSD, overexpression of AKT1 partially offset the effect of DHJSD. On this basis, RELA was knocked down, and it was found that the expression level of AKT1 was inhibited (**Figure 7A**, all P<0.05). Similarly, the levels of osteoclast-related factors ACP5, CTSK, and RANK also decreased partially (**Figure 7B**, all P< 0.05). It showed that knocking down RELA

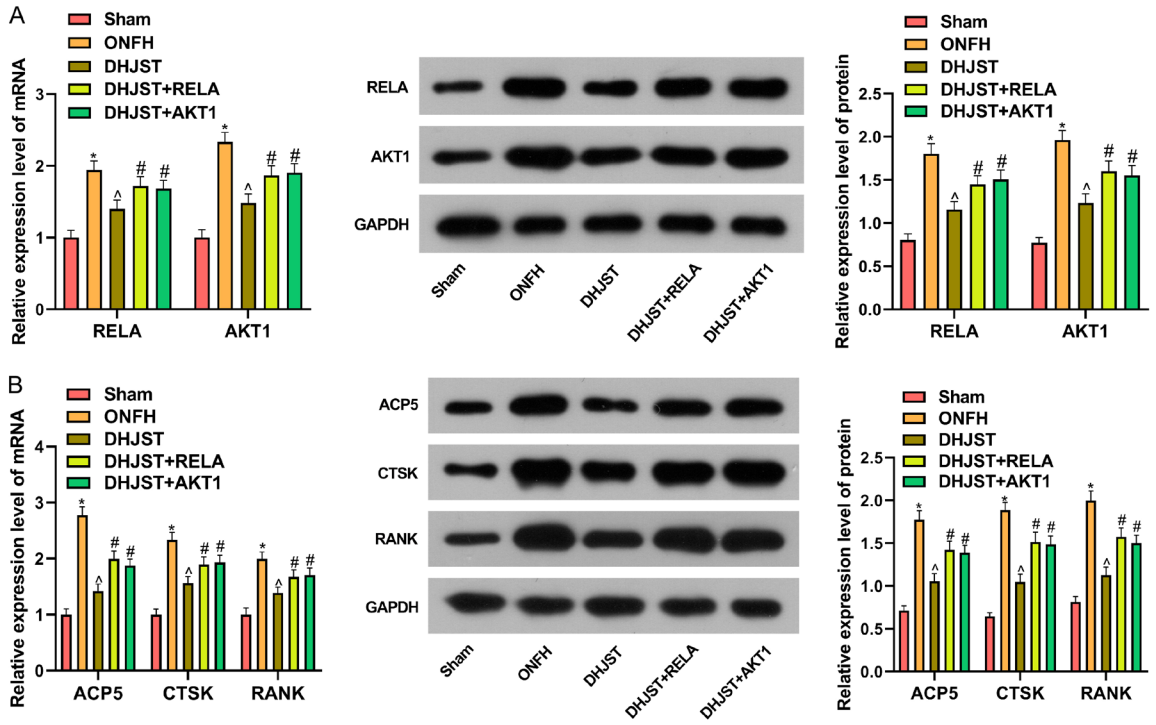

Figure 6. Overexpression of RELA or AKT1 partially attenuated the effect of DHJSD. A: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of RELA and AKT1; B: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of ACP5, CTSK and RANK (n=8 for each group). Compared with sham group, *P<0.05; Compared with ONFH group, ^P<0.05; Compared with DHJSD group, #P<0.05. DHJSD: Duhuo Jisheng Decoction; ONFH: osteonecrosis of the femoral head.

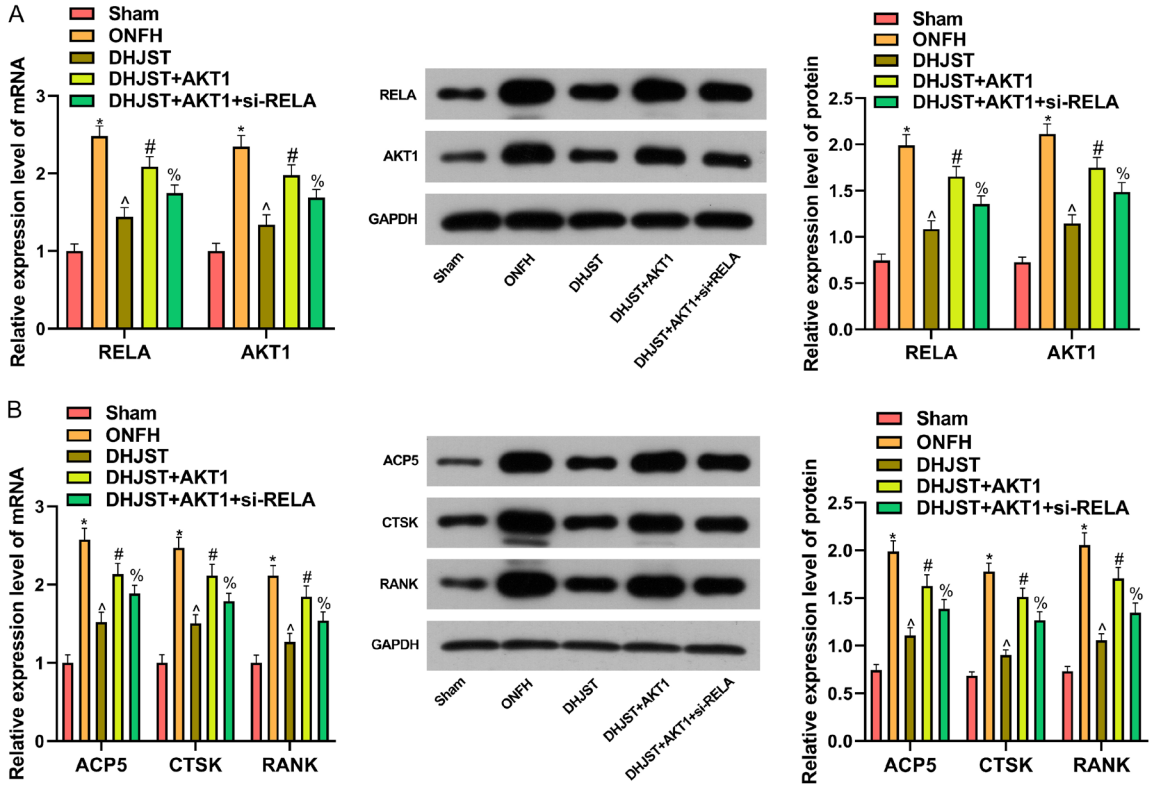

Figure 7. Knockdown of RELA partially offseted the attenuating effect of AKT1 on DHJSD. A: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of RELA and AKT1; B: qRT-PCR and Western blot were used to detect the effect of DHJSD on the levels of ACP5, CTSK and RANK (n=8 for each group). Compared with sham group, *P<0.05; Compared with ONFH group, ^P<0.05; Compared with DHJSD group, #P<0.05; Compared with DHJSD+AKT1 group, %P<0.05. DHJSD: Duhuo Jisheng Decoction; ONFH: osteonecrosis of the femoral head.

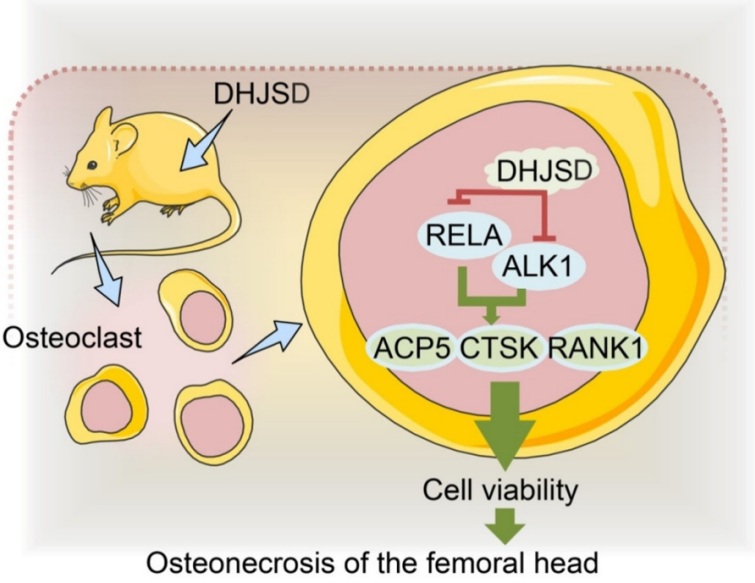

Figure 8. The mechanism diagram of Duhuo Jisheng Decoction inhibiting the activity of osteoclasts in osteonecrosis of the femoral head by regulating the RELA/AKT1 axis.

can partially offset the effect of AKT1 on the weakening of the treatment effect of DHJSD. The mechanism diagram of this research is shown in **Figure 8**.

Discussion

This study explored the active ingredients and target genes of 15 traditional Chinese herbal medicines that make up DHJSD through network pharmacology. A series of biological information tools were applied to analyze the target points, which screened out that RELA and AKT1 may play an important role in the treatment of ONFH by DHJSD. In this study, a binding site of RELA and promoter regions of AKT1 was found in the JASPAR website, and RELA may activate the AKT1 gene as a transcription factor, and ChIP experiments further verified the results. RELA belongs to the NF-κB protein family, David Baltimore first discovered the NF-κB protein, which selectively bound to the B cell κ-light chain enhancer to regulate the expression of a variety of genes [16]. NF-κB has been confirmed to exist in most animal cells, and can regulate cellular immune response, inflammatory response and other processes [17]. For example, RELA/p65 induced LncRNA00162 overexpression promotes the growth of pancreatic catheter adenocarcinoma [18].

The AKT gene family covers AKT1, AKT2, and AKT3 [19]. AKT1 encodes a specific protein kinase that mediates PI3K signaling and participates in the regulation of cell viability and apoptosis [20]. Mutations in the AKT1 gene activate kinase activity, stimulate downstream signal transduction, and trigger a variety of diseases [21]. Several studies have shown that inhibiting AKT1 inhibits the formation of osteoclasts [22, 23]. In this study, high activations of RELA and AKT1 were detected in the ONFH rat models, which was preliminaryly consistent with previous studies. Significantly, we found that DHJSD inhibited RELA and AKT1 expression in the ONFH rat models, and speculated that DHJSD may have a therapeutic effect on ONFH through the RELA/AKT1 axis.

The ONFH rat model was established. RELA and AKT1 were highly activated in the model. The expression of RELA and AKT1 in the femoral head tissue of rats treated with DHJSD was inhibited. The treatment of tartrate-resistant acid phosphatase (ACP5) through DHJSD is related to the osteoclast differentiation pathway [24]. Cathepsin K (CTSK) is mainly expressed in osteoclasts, as a lysosomal cysteine protease that can participate in bone remodeling and resorption [25]. As one of the members of the TNF-receptor family, the osteoclast differentiation factor receptor RANK is an essential mediator for the development of osteoclasts and lymph nodes [26]. The above three proteins are all landmark proteins for osteo-

clast differentiation. In the ONFH rat model, ACP5, CTSK, and RANK were all highly expressed, indicating that osteoclasts are highly differentiated, and the expression of ACP5, CTSK, and RANK was inhibited after treatment with DHJSD. This suggested that when rats were treated with DHJSD, injection of RELA or AKT1 overexpression vector in the rat inhibited the inhibitory effect of DHJSD on osteoclast differentiation, and further knockdown of RELA can weaken the inhibitory effect of AKT1. Therefore, we believe that RELA can activate the transcription of AKT1. The expression of AKT1 was inhibited after knocking down RELA, and DHJSD inhibited the osteoclasts differentiation by inhibiting the RELA/AKT1 pathway, so the injection of RELA or AKT1 overexpression vector in rats made the effect of DHJSD weakened.

However, our research still has some limitations. First, only marker proteins were detected for osteoclast differentiation, while tissue staining was not performed, and there is no more intuitive observation of osteoclast differentiation. Secondly, other pathways that DHJSD may act on in the KEGG enrichment analysis, such as the AMPK pathway, have been found, but we have not yet explored them. Since AKT1 can also influence the expression of RELA, the interaction between them may not only be achieved by regulating AKT1 transcription through RELA.

To sum up, DHJSD inhibits ONFH rat osteoclast differentiation by inhibiting the activation of AKT1 by RELA, which provides a theoretical basis for the development of DHJSD combined drugs in the treatment of ONFH.

Disclosure of conflict of interest

None.

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