EFFECTS OF ASTRAGALOSIDE ON CHONDROCYTE PROLIFERATION BY REGULATING THE WNT/B-CATENIN SIGNALING PATHWAY

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ABSTRACT

Objective: *To investigate the effect mechanism of astragaloside on chondrocyte proliferation by regulating the Wnt/β-catenin signaling pathway, and to further elaborate the molecular mechanism of astragaloside in the treatment of osteoarthritis from the molecular biological level.*

Methods: *A rabbit OA model was constructed, and chondrocytes were cultured. According to different treatment methods, the cells were divided into a normal control group, an OA model group, 12h low-, medium-, and high-dose astragaloside groups, 24h low-, medium-, and high-dose astragaloside groups, and 48h low-, medium-, and high-dose astragaloside groups. The protein expression of chondrocytes was detected by the western blot assay. The mRNA expression of chondrocytes was measured by the PCR method. The expression of MMP-7, MMP-13, type II collagen carboxy-terminal peptide (CTX-II), Runx2, ADAMTS-4, ADAMTS-5, Wnt2, and β-catenin in the supernatant of human chondrocytes was detected by an ELISA assay. The expression changes of related factors were analyzed to further study the mechanism of astragaloside on chondrocyte proliferation.*

Results: *Western blotting showed that the β-catenin protein was not expressed in a normal control group. The expression of β-catenin protein in the chondrocyte nucleus of an OA model group was significantly higher than that of the normal control group (P<0.05). After the intervention of astragaloside, the expression of the β-catenin protein in the chondrocyte nucleus of the 12h low-, medium-, and high-dose astragaloside groups had no significant changes compared with that of the pathway activation group. While the expression of β-catenin protein in 12h, 24h and 48h low-, medium-, and high-dose astragaloside groups was lower than that in the pathway activation group, and it was related to the intervention time (P<0.01). The protein expression of β-catenin in the 48h high-dose astragaloside group was dramatically lower than that in the 48h low-dose astragaloside group. The ELISA assay showed that the expression of MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, and Wnt2 protein in the supernatant of chondrocytes in the pathway activation group was higher than that in the normal control group (P<0.05) and the astragaloside group (P<0.05).*

Conclusion: *Astragaloside can inhibit the Wnt/β-catenin signaling pathway of chondrocytes, reduce the expression of β-catenin, and downregulate the expression of MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5 and Wnt2. It reveals that the mechanism of astragaloside in the treatment of osteoarthritis may be achieved through inhibiting the Wnt/β-catenin signaling pathway of OA to prevent the degradation of cartilage matrix, inhibit the apoptosis of chondrocytes, and promote the recovery of cartilage function.*

Keywords: *Astragaloside, Wnt/β-catenin signaling pathway, chondrocyte proliferation.*

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Introduction

Osteoarthritis (OA) is a chronic joint disease caused by joint degenerative diseases and destruction of articular cartilage, which commonly occurs in the elderly. In the process of osteoarticular development, chondrocytes are mainly affected by apoptosis and necrosis, and the Wnt/β-catenin signaling pathway has a greater influence on the apoptosis and necrosis of chondrocytes in osteoarticulation[(1)]. Some researchers[(2)] have found that the activation of the Wnt/β-catenin signaling pathway inhibits growth of chondrocytes. Astragaloside can decrease the expression of β-catenin protein and thus promote osteoblast differentiation by upregulating the Wnt/β-catenin signaling pathway. Moreover, in vitro and in vivo studies have also demonstrated that the Wnt/β-catenin signaling pathway is closely related

to cartilage activity[3]. Traditional Chinese medicine therapy can promote the proliferation of chondrocytes, inhibit apoptosis and necrosis, improve the joint internal environment, and repair articular cartilage, which has become a hot topic in OA research this year. Astragaloside has the effect of promoting blood circulation and replenishing Qi. Studies have shown[4] that Bushen Huoxue prescription can inhibit the Wnt/β-catenin signaling pathway in chondrocytes and significantly downregulate metalloproteinase-7 (MMP7) expression.

Furthermore, through fluorescence quantitative PCR detection, Li Chenrui et al. found that astragaloside could increase the levels of GSK-3β and Runx2, thereby promoting osteogenic differentiation of rat bone marrow mesenchymal stem cells (BMSCs)[5]. Therefore, this study mainly explores the effect mechanism of astragaloside regulating the Wnt/β-catenin signaling pathway on chondrocyte proliferation, and further elaborates the molecular mechanism of astragaloside in the treatment of osteoarthritis from the molecular biological level.

Materials and methods

Culture method of chondrocytes

The rabbit OA model was established by using the modified stretch fixation method. After the modeling, 2 New Zealand white rabbits of the same age in weeks were killed, and the cartilage of both hip joints, knee joints, shoulder joints, and elbow joints was collected in a sterile environment.

The cartilage was rinsed with phosphate buffer solution (PBS) buffer and digested using 0.25% trypsin in water bath at 37 °C for 30 min. Chondrocytes were isolated and then inoculated in 4 culture dishes with 10% FBS and 1% double-antibody LG-DMEM, respectively. The chondrocytes were completely cultured in a sterile incubator at 37 °C with 5% CO_2.

When the cell adherent growth in the medium reached more than 90%, the culture medium was poured out and rinsed by the PBS buffer 3 to 4 times. Then the PBS buffer was poured out, and the residual liquid was removed with a pipetting device.

Digestion and passage were carried out with 0.125% trypsin, and the cell density of the passage cells was adjusted to 105/L. Six EP tubes (2 mL) were prepared and loaded with 2 mL 10% FBS and 1% double-antibody LG-DMEM, respectively, for a complete culture.

Grouping and processing methods

After passage, cells were inoculated in 12-well culture plates until their growth density was 80% to 90%. According to different treatment methods, the cells were divided into a normal control group, an OA model group, 12h low-, medium-, and high-dose astragaloside groups, 24h low-, medium-, and high-dose astragaloside groups, and 48h low-, medium-, and high-dose astragaloside groups.

Treatment methods:

• Preparation of standard substance: The standard substance was diluted into 7 concentrations (10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 ng/mL).

• Sample loading: the protein wells of sample to be tested, 7 standard protein wells and 2 blank control wells were set on the ELISA plate. 100 μL corresponding liquid was added to each well, and 3 duplicate wells were set for each sample, incubated at 37 °C for 2 h, and the residual liquid was discarded;

• 100 μL of primary antibody working fluid for test solution was added to each well;

• 100 μL of secondary antibody detection working fluid was added to each well;

• Color development;

• Detection and calculation.

The control group was treated with serum-free LG-DMEM. The 12h low-, medium-, and high-dose astragaloside groups, the 24h low-, medium-, and high-dose astragaloside groups, and the 48h low-, medium-, and high-dose astragaloside groups were treated with serum-free LG-DMEM containing 5%, 10% and 15% astragaloside at 12 h, 24h and 48h, respectively.

Total protein extraction from chondrocytes after astragaloside treatment

Preparation of the lysis buffer: 5 mL RIPA+1% protease inhibitor; PBS buffer rinsing, 5 times; cell protein lysis; scraping the adherent cells; cell disruption; centrifugation; loading the tube.

Instruments and reagents

Fluorescent inverted microscope (TKO Optical Instrument Co., Ltd., Japan); Cell CO2 incubator (Hangzhou Leiqi Experimental Equipment Co., Ltd.); Micropipette (Guangzhou Leide Biotechnology Co., Ltd.); Electrophoresis apparatus, electronic balance, low temperature refrigerator (Shanghai Tianneng Technology Co., Ltd.); Low-temperature high-speed centrifuge (Shanghai Aiyan Biotechnology Co., Ltd.); Constant temperature blast drying oven

(Shanghai Jinghong Co., Ltd.); Automatic microplate reader (Bio-Rad Company, USA); Ultrapure water system (Germany/Think-Lab); Magnetic stirrer (Jiangsu Haimen Qilinbeier Instrument Manufacturing Co., Ltd.); Electric thermostatic water bath (Shanghai Fuma Experimental Equipment Co., Ltd.); Astragaloside standard substance (Shanghai Guangrui Biotechnology Co., Ltd.); Chondrocyte culture medium (Scien Cell, USA); Fetal bovine serum (ExCell); Dimethylsulfoxide (Phytotech, USA); Cytoplasmic protein and nuclear protein extraction kit, BCA protein concentration detection kit (enhanced), SDS-PAGE electrophoresis solution (Beyotime Biotechnology Company); SDS-PAGE gel preparation kit, SDS-PAGE protein loading buffer (Shanghai Yiji); Protein electrophoresis molecular weight standard substance (Pierce); β-catenin antibody (CST); Goat anti-mouse HRP secondary antibody (Abcam); HRP-ECL Luminescence detection kit (Pierce); MMP-7 ELISA kit, COMP ELISA kit, CTX-II ELISA kit (Cloud-clone); ALP ELISA kit, Run2 ELISA kit, OC ELISA kit, Wnt2 ELISA kit, and GSK-3β ELISA kit were all purchased from Shanghai Chunshi Biotechnology Co., Ltd.

Observation indicators

The protein expression of chondrocytes was detected by the western blot assay; the mRNA expression of chondrocytes was measured by PCR method; the expression of MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, Wnt2, and β-catenin in the supernatant of human chondrocytes was detected by ELISA assay.

The normal control group was given normal saline, and the astragaloside group was given 5%, 10% and 15% astragaloside suspension. The human chondrocytes activated by the pathway were interfered for 12h, 24h and 48h, and the culture supernatant of human chondrocytes was collected.

Statistical treatment

SPSS 20.0 software was used for statistical analysis. Variance analysis of repeated measurement data was used for analyzing the difference between groups. If symmetry was not satisfied, ε-corrected results were used.

Results

Construction of OA model

The normal control group had a smooth surface, and the chondrocytes were located in the cartilage lacuna, with regular distribution, clear structure, and complete tidal line. The surface of the OA model group was rough, with cracks forming.

The number of chondrocytes decreased with some pyknosis, which were to one side, and there was obvious inflammatory cell exudation, indicating that the OA model was successfully established. The results are shown in Figure 1.

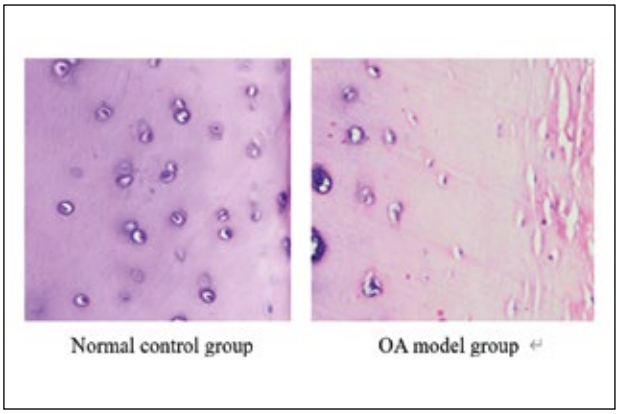

Figure 1: Cell exudation.

Effects of different concentrations of astragaloside suspension on β-catenin in the nucleus and cytoplasm of chondrocytes in the OA model group after intervention at different times

The β-catenin protein was not expressed in the nucleus of normal chondrocytes.

The expression of β-catenin protein in the chondrocyte nucleus of the 12h low-, medium-, and high-dose astragaloside groups had no significant changes compared with that of the pathway activation group. While the expression of β-catenin protein in 12h, 24h and 48h low-, medium-, and high dose astragaloside groups was lower than that in the pathway activation group, and it was related to the intervention time ($P<0.01$).

The protein expression of β-catenin in the 48h high-dose astragaloside group was dramatically lower than that in the 48h low-dose astragaloside group. The results are shown in Table 1.

Effects of astragaloside on MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, and Wnt2 expression in supernatant of chondrocytes

The expression of MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, and the Wnt2 protein in the supernatant of chondrocytes in the pathway activation group was higher than that in the normal control group ($P<0.05$) as well as in the astragaloside group ($P<0.05$). The results are shown in Table 2 and Table 3.

Groups	n	β-catenin gray value	Multiples
Normal control group	3	207.68±15.73	0.11±0.0056
OA model group	3	1918.57±81.39#	1.00±0.0321
12h low-dose group	3	2126.76±139.84	1.11±0.0613
12h medium-dose group	3	1816.17±102.14	0.95±0.0416
12h high-dose group	3	1697.83±60.85	0.88±0.0201
24h low-dose group	3	1792.3±178.63	0.93±0.0815
24h medium-dose group	3	1410.10±48.44&	0.74±0.0136
24h high-dose group	3	1336.22±78.98&	0.70±0.0306
48h low-dose group	3	2073.14±253.62*	1.08±0.1205
48h medium-dose group	3	822.94±76.27*ω	0.43±0.0281
48h high-dose group	3	894.88±62.46*ω	0.47±0.0210

Table 1: Effect of astragaloside on β-catenin expression in the nucleus of human chondrocyte.
*#Compared with the normal control group, P<0.05, with statistical significance. *Compared with the pathway activation group, P<0.05, with statistical significance. &Compared with the pathway activation group, P<0.05, with statistical significance. ωCompared with 48h low-dose group, P<0.05, with statistical significance.*

Groups	n	MMP-7	MMP-13	CTX-II
Normal control group	3	0.1337±0.0235*	25.0831±3.0237*	1353.30±142.44*
OA model group	3	0.3371±0.0174	58.6557±0.9279	4064.82±70.00
Astragaloside group	3	0.1237±0.0250*	33.5555±2.3193*	1876.49±43.676*

Table 2: Expression of MMP-7, MMP-13 and CTX-II in supernatant of human chondrocytes.
**Compared with the pathway activation group, P<0.05, with statistical significance.*

Groups	Runx2	ADAMTS-4	Wnt2	ADAMTS-5
Normal control group	0.76±0.09*	1.08±0.14*	0.062±0.002*	0.298±0.0125*
OA model group	1.045±0.12	1.35±0.24	0.071±0.003	0.424±0.0323
Astragaloside group	0.81±0.16*	1.01±0.11*	0.064±0.006*	0.344±0.0231*

Table 3: Expression of Runx2, ADAMTS-4, ADAMTS-5 and Wnt2 in supernatant of human chondrocytes.
**Compared with the pathway activation group, P<0.05, with statistical significance.*

Discussion

OA is a complex and independent disease related to inflammation, obesity, age, genetics and other factors, and its main clinical manifestations are joint pain, swelling and function limitation[6]. Although the pathogenesis is not clear, it is closely related to the process of articular cartilage change. Chondrocytes are the only cell type in mature cartilage tissue that can maintain the internal environment stability during cartilage injury and remodeling. The Wnt/β-catenin signaling pathway is one of the important signal transduction pathways of chondrocyte apoptosis[7]. It can also induce osteoblast differentiation[8] and promote chondrocyte proliferation by interfering with OA cell metabolism. The Wnt/β-catenin signaling pathway mainly affects the formation and decomposition of articular cartilage matrix in osteoarthropathy. Wnt is a secreted protein, which is homologous to the embryonic development gene wingless (WG) of drosophila. For that reason, it is named the Wnt gene, which includes a variety of Wnt molecules. The Wnt molecule is the upstream gene molecule of Wnt/β-catenin signaling pathway, and the Wnt2 protein is the initiation factor of the Wnt/β-catenin signaling pathway, inducing a series of signal transductions in osteocytes[9].

Studies have found that Wnt3A, Wnt4, Wnt5b, Wnt5a, Wnt7a, Wnt1, and Wnt14 were differentially expressed in interstitial polymerization and chondrogenic differentiation. β-catenin acts only as a cytoskeletal protein in normal somatic cells. When the extracellular Wnt signaling molecules combine with the Frizzled protein of the specific receptor on the cell membrane, β-catenin accumulates gradually in the cytoplasm. When it reaches a certain concentration level, it will be transferred to the nucleus and combine with the transcription factor family to activate the transcription of downstream genes[10] to promote the maturation of bone cells. MMP-7 and MMP-13 are downstream target genes of the Wnt/β-catenin signaling pathway, which are stromate-degrading enzymes secreted during inflammation and act as the messengers for regulating the environmental stability in the articular cavity. MMP-13 plays an important role in structural bone destruction. CTX-II is a bone matrix decomposition and can be used as markers of cartilage degradation[2].

Runx2 is a transcription factor that promotes differentiation of bone marrow stromal stem cells[11], which can induce osteoblastic differentiation and increase the number of immature bone cells during

bone development. Studies have shown that Runx2 is the intersection that integrates various signals affecting osteoblast differentiation. ADAMTS-4 and ADAMTS-5 are factors closely related to cartilage matrix catabolism and have the function of decomposing proteoglycan[12]. High levels of β-catenin were found in degenerated cartilage in osteoarthritis, suggesting that either inhibition or weakening of Wnt signaling could lead to loss of bone components. This experiment, based on the analysis and observation of the effects of astragaloside on active molecules expression through Wnt/β-catenin signaling pathway in chondrocytes, showed that the expression of MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, and Wnt2 protein in the supernatant of chondrocytes in the OA model group was higher than that in the normal control group ($P<0.01$) and astragaloside group ($P<0.01$). It may be due to astragaloside inhibiting the Wnt/β-catenin signaling pathway, alleviating inflammation, reducing cartilage matrix degradation, and improving endochondral environment, thus promoting chondrocyte proliferation.

The study also found that the β-catenin protein was not expressed in the nucleus of normal chondrocytes. The expression of the β-catenin protein in the chondrocyte nucleus of the OA model group was significantly higher than that of the normal control group ($P<0.01$). The expression of the β-catenin protein in the chondrocyte nucleus of the 12h low-, medium-, and high-dose astragaloside groups had no significant changes compared with that of the pathway activation group. While the expression of β-catenin protein in 12h, 24h and 48h low-, medium-, and high-dose astragaloside groups was lower than that in the pathway activation group, and it was related to the intervention time ($P<0.01$).

The protein expression of β-catenin in the 48h high-dose astragaloside group was dramatically lower than that in the 48h low-dose astragaloside group. These results indicated that astragaloside could decrease the expression of β-catenin protein in the chondrocyte nucleus, and the effect was related to the concentration of astragaloside and the intervention time, which was consistent with the results of many studies[13-14].

In conclusion, astragaloside could inhibit the Wnt/β-catenin signaling pathway, reduce the MMP-7, MMP-13, CTX-II, Runx2, ADAMTS-4, ADAMTS-5, Wnt2 and β-catenin protein expression. This may be an important mechanism for astragaloside to alleviate inflammation, reduce cartilage matrix degradation, promote chondrocyte recovery, differentiation, and treatment of OA. However, the Wnt/β-catenin signaling pathway has many involved and related factors; hence, it needs to be studied further.

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