



## The mechanism of Danzikang Knee Granule in regulating the chondrogenic differentiation of mesenchymal stem cells based on TGF- $\beta$ signaling pathway in cartilage repair in knee osteoarthritis

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### ABSTRACT

This study aimed to explore the mechanism of Danzikang Knee Joint Granules in regulating the differentiation of mesenchymal stem cells into cartilage to cartilage repair of knee osteoarthritis based on the TGF- $\beta$  signaling pathway. For this purpose, 60 SD rats were divided into four groups; the control group and treated groups with low, medium, and high concentrations of Danzikang. The histopathology of rats was analyzed and TGF- $\beta$  signaling pathway-related proteins were determined. Results showed that the average optical density in serum of the Danzikang Granule intervention group was significantly higher than the control group ( $P < 0.05$ ), and the average optical density increased with drug concentration increasing ( $P < 0.05$ ). Compared with the control group, Danzikang knee granule cell survival in the intervention group was elevated the serum and reduced cell apoptosis rate ( $P < 0.05$ ). Danzikang knee infusion concentrations were positively correlated with bone marrow mesenchymal stem cell survival rates ( $P < 0.05$ ), and negatively correlated with apoptosis rate ( $P < 0.05$ ). TGF- $\beta$ 1, BMP2, and BMP4 were significantly increased in the three concentrations of the Danzikang Granule serum intervention group ( $P < 0.05$ ). TGF- $\beta$ 1, BMP2 and BMP4 were significantly increased in the high concentration group, while TGF- $\beta$ 1, BMP2 and BMP4 were significantly decreased in the low concentration group ( $P < 0.05$ ). The Wakitani histological score of the control group was significantly lower than the other three groups ( $P < 0.05$ ). In general, Danzikang Knee Granule plays a role in cartilage repair in knee osteoarthritis by promoting mesenchymal stem cell proliferation and cartilage differentiation, and the specific mechanism may be related to TGF- $\beta$ 1/BMPs signaling pathway.

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### Introduction

Knee osteoarthritis is a degenerative disease, which is mainly caused by age increase, obesity, trauma, joint deformity and many other factors, as well as reactive hyperplasia of joint edge and subchondral bone. Clinically, the main manifestations are slowly developing joint pain, limited movement, stiffness, tenderness, joint swelling, etc., and the quality of life of serious patients (1). Currently, the treatment of knee joints can be divided into surgical treatment and non-surgical treatment. In non-surgical treatment, drugs and physical therapy can temporarily delay the clinical symptoms of the disease and relieve pain. However, knee cartilage is still difficult to repair (2). Surgical treatment includes high tibial osteotomy

and arthroscopic debridement, which can achieve a certain degree of efficacy, but still cannot solve the cartilage injury (3). Bone marrow mesenchymal stem cells are a kind of cells with self-renewal and multidirectional differentiation potential, which can differentiate into chondrocytes, osteoblasts, adipocytes, etc., through special induction factors (4, 5). In recent years, many scholars for the cells used in the treatment of osteoarthritis in extensively studied, between a number of research results confirm the bone marrow mesenchymal stem cells in certain conditions to the normal cartilage cell differentiation, animals can be used for osteoarthritis made modulus normal hyaline cartilage to repair cartilage defect, have normal biomechanical function (6-8). Danzikang

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Knee granule is a traditional Chinese medicine treatment of osteoarthritis recurrence, now there are research results have confirmed that the drug treatment of osteoarthritis effect is significant, and can improve the water content of cartilage, and protect the cartilage matrix proline, and prevent the degeneration of knee osteoarthritis. However, there is no research on the molecular mechanism of Danzikang Granule in the treatment of knee osteoarthritis. Therefore, 30 rat BMSCs and SD rats were selected in this study to explore the specific mechanism of Danzikang Knee granule in regulating the chondrogenic differentiation of mesenchymal stem cells and the repair effect of Danzikang Knee granule in knee osteoarthritis. The results are as follows.

## **Materials and methods**

### **Experimental animals**

A total of 105 healthy SPF male SD rats were studied with 8 weeks aged and  $210 \pm 20$ g body weight. They were kept in separate cages at room temperature (22°C) with 12h of light and night. All experimental animals in this study were provided by the Animal Experiment Center of the Hunan University of Chinese Medicine. The animal-related operations in the experiment were in accordance with the requirements of animal ethics.

### **Traditional Chinese medicine prescription**

In this study, an empirical Fontaine Zikang Knee granule was used. The specific formula was paeoniae alba (25 g), *Salvia miltiorrhiza* (15 g), Ziheche (15 g), *Rehmannia glutinosa* (15 g), Duhuo (12 g), *Caulis spatholobus* (12 g), Frankincense (10 g), myrrh (10 g) and licorice root (6 g).

### **Preparation of drug-containing serum of Danzikang Granule**

Twenty healthy SD rats were randomly divided into 4 groups (5 rats in each group) and converted according to the equivalent dose ratio of human and animal body surface area. The rats were given 0.8g/kg·d, 1.6g/kg·d and 3.2g/kg·d of Danzikang Granule, and the normal saline group was given an equal volume of intra-gastric administration. After 7 days of continuous administration, blood was collected from the abdominal aorta. Centrifuged at 3000r/min for 15min, the supernatant was taken, and

the mechanical energy of 0.22um microporous membrane was used for sterilization to obtain normal saline serum, low, medium and three concentrations of Danzikang granule containing serum, and stored in the refrigerator at -25°C for use.

### **Isolation, culture, identification and grouping of rat BMSCs**

Five healthy SD rats were anesthetized, killed by neck removal, and disinfected with 75% ethanol. The tibia and femur of rats were removed in a sterile operating table, the ends of the long bones were cut off, and the bone cavity was rinsed with DMEM culture solution. Then, BMSCs were cultured by full adherent method and sub-cultured at the same time, and the third generation BMSCs were identified by flow cytometry. After completion, BMSCs cells identified as blank group, low concentration group, medium concentration group and high concentration group were divided into groups. The blank group was given DEME medium containing 15% normal saline for BMSCs culture. BMSCs were cultured in low concentration group, medium concentration group and high concentration group with different concentrations of Danzikang Granule drug-containing serum DEME medium. BMSCs suspension was prepared after 7 days of culture and used in animal experiments.

### **Establishment of animal model**

Eighty healthy SD rats and 20 normal control rats were selected, and the rest rats were modeled by Hulth, namely, the skin was prepared on the right back stream of rats before surgery, and the abdominal cavity was anesthetized with chloral hydrate. After that, the rats were fixed in a supine position on the operating table, and a 2cm longitudinal incision was taken in the right knee joint of the rats under sterilized and sterile conditions, and the ACL was cut with the knee joint cavity exposed. The medial meniscus was completely resected and the articular cartilage surface was preserved. Then the surgical field was infiltrated and irrigated with complex iodine, normal saline and antibiotics to prevent intra-articular infection. Then the incisions were sutured layer by layer and the animals were placed in cages for free movement and feeding. Antibiotic injection and wound disinfection were given continuously for 3 days after the operation. After surgery, all the model animals

survived, and no redness, swelling, suppuration or other phenomena occurred in the incision. After 7 weeks of feeding, the rat model of knee osteoarthritis was obtained (9).

### **Grouping of animal models**

SD rats were divided into the control group, model group, normal saline group and Danzikang granule group. The blank control group consisted of 20 healthy SD rats without modeling, and the modeling group consisted of 20 rats with osteoarthritis. Rats in the normal saline group were injected into the joint cavity of BMSCs suspension with the intervention of saline-containing drug serum, while rats in the Danzikang granule group were injected into the joint cavity of BMSCs suspension with the intervention of Danzikang granule containing drug serum. At the same time, 0.5ml corresponding culture medium was injected into the joint cavity for continuous injection for 3 days. After injection, normal activities and free drinking water were observed.

### **BMSCs identification in rats**

The third-generation cells were collected, the medium was discarded, digested with 0.25% trypsin, centrifuged at 1000r/min for 10min, cell precipitate was collected and prepared as a cell suspension, which were divided into two groups. Mouse anti-human mab CD34, CD14, CD44, CD45 and CD105 were added into one group, and mouse IgG1FITC and IGG1-PE were added into the other group as the control group and incubated for 30min before routine PBS washing and centrifugation. The expression of CD44, CD105, CD34 and CD45 antibodies was determined by flow cytometry.

### **Chondrocyte identification**

The chondrocyte identification is mainly determined by toluidine blue staining (10), and the proportion of chondrocytes is determined by SBAC immunohistochemistry. Toluidine blue staining : (1) cell plates were washed with conventional PBS solution and fixed with 4% paraformaldehyde for 60min; (2) Wash with running water for 15min; (3) wash with distilled water for 5min; (4) 1% toluidine blue was added for 2h; (5) removal and dyeing; (6) Light microscope observation. SBAC immunohistochemistry method: (1) The cell plates

were washed with conventional PBS solution, (2) fixed with 4% paraformaldehyde, incubated with conventional 3% H<sub>2</sub>O<sub>2</sub> solution for 10min, and then citrate buffer was used for antigen repair. (3) The cells were heated in a water bath for 45min, then cooled, and the rabbit anti-collagen II primary antibody was dropped and incubated for 2h. After rinsing with PBS solution, biotin-labeled secondary antibody working solution was dropped and incubated at 37°C for 30min. (4) after washing with PBS solution, DAB color reagent was dropped and incubated for 30min at 37°C. After the incubation, tap water was used for washing, hematoxylin was dyed, alcohol was dehydrated to transparent, neutral gum was used for sealing, and films were taken under an inverted fluorescence microscope.

The average optical density value was measured (11), and an Eclipse CI-L photo microscope was used to select the target area for 200-fold imaging. Try to fill the field of vision with tissue so that the background light is consistent in each image. After the imaging was completed, Image-Pro Plus 6.0 analysis software was used to measure the positive cumulative optical density values of the three fields in each section with the unified pixel area as the standard unit, denoted as A. And the corresponding tissue pixel area was denoted as B, and the average optical DENSITY = A/B was calculated as C. Note: positive cytoplasm was brownish yellow, cell and purple.

### **Cell proliferation and apoptosis were measured by the MTT method**

Cells at the logarithmic growth stage were selected to be inoculated in a 96-well plate with 5×10<sup>3</sup> cells/well. After cells were adherent to the wall, the above groups were performed. Namely, the normal saline intervention group, low concentration group, medium concentration group and high concentration group were cultured for 8h, and 20ulMTT was added to each well, and the culture was continued for 4h in the incubator. After that, the medium was discarded, dimethyl sulfoxide was added to each well, and the mixture was shaken. The enzyme plate was used to measure the absorbance value (A value) at 490nm, and the cell survival rate was calculated. Cell apoptosis rate was determined by conventional 96-well plate cell culture, and the cell suspension was performed with 400ul 1×Binding Buffer. Annexin V-

FITC (5 $\mu$ L) and 5 ULPI were added to the stain and incubated for 5min. The apoptosis rate was determined by up-flow cytometry.

### **Grading evaluation of knee cartilage defects in rats**

Four weeks later, the rats were sacrificed under uratan anesthesia, and the joint capsule of the rats was opened to observe the repair of the defect area or the degeneration of cartilage and the photos were taken. Then, Wakitani histological score of cartilage repair was given (12) to evaluate the cell morphology, surface regularity, matrix staining, cartilage thickness and the degree of adhesion with normal cartilage.

### **Histopathological observation**

The cartilage tissues of rats in the four groups were conventionally fixed with methanol, dehydrated, paraffin-embedded sections, and then stained with HE. The specific steps were (i) routine section dewaxing; (ii) Gradient dehydration of alcohol and injection of distilled water into dyeing solution; (iii) Place in hematoxylin staining solution for 5~15min; (iv) Remove excess dye; (v) Place in lithium carbonate saturated solution for alkalization; (vi) Dyeing with 0.1~0.5% eosin solution for 1~5min; (vii) Gradient alcohol dehydration, xylene transparent; (viii) Drop an appropriate amount of neutral gum, seal the sheet with a cover glass, and observe it under a microscope.

### **Western blotting**

The protein expressions of TGF- $\beta$ , BMP2 and BMP4 in rat mesenchymal stem cells and cartilage tissues were determined by the western-bolt method. The specific steps were as follows: the cells in the above-related groups were collected, washed with PBS buffer solution 3 times, centrifuged at 10000r/min for 10min to precipitate the cells, and the supernatant was discarded. Add cell lysates, or take tissue samples for full grinding, and add cell lysates containing protease inhibitors in a ratio of 1:9. Then it was shaken evenly with a low-temperature operating homogenizer and fully cracked for 30min. After that, it was transferred to a centrifugal tube with a pipette and centrifuged at 12000r/min at 4°C for 5min. Then the protein concentration of supernatant samples was detected by BCA protein concentration. Then adjust

the protein concentration to the same, after boiling for 10min, take 50ug total protein in sodium dodecyl sulfate-polyacrylamide gel for electrophoresis. The classified proteins were transferred to PVDF membranes using a wet membrane transfer device, and then the membranes were sealed overnight with 5% skim milk powder solution at 4°C. Then the membrane was washed with TNST buffer solution for 2mi and the primary antibodies, namely TGF- $\beta$ , BMP2 and BMP4 antibodies, were added respectively, sealed at room temperature for 2h, and cleaned with TBST solution for 3 times. The second antibody was added and incubated for 1h, protected from light, and then TBST solution was applied 3 times, 10min each time. After that, GAPDH protein was used as the internal reference protein, and the absorbance value of the bands was analyzed by gel imaging system. The relative protein expression is the ratio of the absorbance value of the target band to that of the reference protein.

### **Statistical methods**

SPSS20.0 statistical arrest was used to process the data. The measurement data were expressed as mean  $\pm$  standard deviation, the paired sample T-test was used to compare the data among the groups, the counting data were expressed as N or percentage, and the Chi-square test was used. The difference between P<0.05 and P<0.05 was considered statistically significant.

## **Results and discussion**

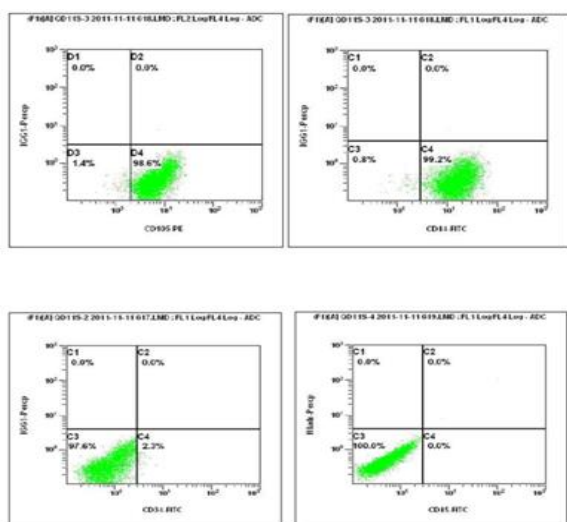
### **Identification of bone marrow mesenchymal stem cells**

The results showed that the expression of THE third-generation BMSCsCD44 and CD105 was positive, while the expression of CD34 and CD45 was negative, which was consistent with the immune-phenotype of MSCS and the general characteristics of stem cells. BMSCS were identified as mesenchymal stem cells, as shown in Figure 1 and Table 1.

### **Identification of chondrocytes**

Toluidine blue staining showed no staining and cell death in the blank group, and toluidine blue heterostaining was found in the other three groups. The high dose group had the highest heterochromatism, while the low dose group had the lowest heterochromatism, and chondrocytes were oval

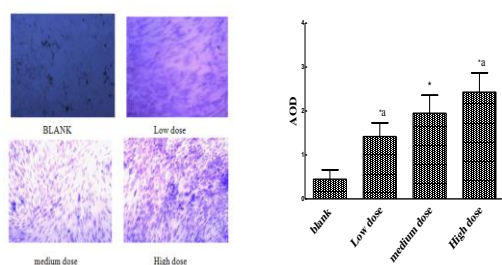
or round. The immunohistochemical method was used to measure the average optical density of the cells in the four groups, and the results showed that the average optical density of the serum intervention group was significantly higher than that of the blank group, and the difference was statistically significant ( $P < 0.05$ ). In the serum intervention group of Danzikang Granule, the average optical density value increased with the increase of drug concentration, and the difference was statistically significant ( $P < 0.05$ ), as shown in Figure 2.



**Figure 1.** Identification results of CD105, CD44, CD34 and CD45 by flow cytometry

**Table 1.** Flow cytometry identification results

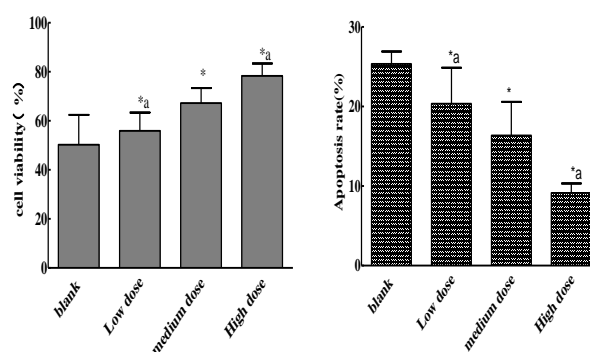
Phenotypic antibody detection	Positive rate
CD44	99.27%
CD105	97.69%
CD34	2.14%
CD45	0.00%



**Figure 2.** Identification and optical density analysis of chondrocytes

### Effects of Danzikang Granule at different concentrations on proliferation and apoptosis of bone marrow mesenchymal stem cells

Compared with the blank group, the cell survival rate of danzikang granule serum intervention group was significantly increased, and the cell apoptosis rate was significantly decreased, with statistical significance ( $P < 0.05$ ). In the serum intervention group of Danzikang Knee Granule, the serum drug concentration of Danzikang Knee granule was positively correlated with the survival rate of BMSCS ( $P < 0.05$ ), and negatively correlated with the apoptosis rate ( $P < 0.05$ ), as shown in Table 2 and Figure 3.



**Figure 3.** Effects of Danzikang Granule at different concentrations on proliferation and apoptosis of bone marrow mesenchymal stem cells

**Table 2.** Effects of Danzikang Granule at different concentrations on proliferation and apoptosis of bone marrow mesenchymal stem cells

Group	Blank group	Low concentration group	Middle concentration group	High concentration group
Cell viability	50.25 ± 12.19	55.89 ± 7.45 <sup>a</sup>	67.23 ± 6.14 <sup>*</sup>	78.25 ± 5.12 <sup>a</sup>
Apoptosis rate	25.34 ± 1.58	20.35 ± 4.53 <sup>a</sup>	16.37 ± 4.23 <sup>*</sup>	9.14 ± 1.19 <sup>a</sup>

Note: Compared with blank group,  $*P < 0.05$ ; Compared with the medium-dose group,  $aP < 0.05$

### Effect of Different concentrations of Danzikang Granule on BMSCs protein expression

Compared with the blank group, TGF- $\beta$ 1, BMP2 and BMP4 were significantly increased in the three concentrations of Danzikang Granule serum intervention group, with statistical significance ( $P < 0.05$ ). Compared with the medium concentration

group, TGF- $\beta$ 1, BMP2 and BMP4 were significantly increased in the high concentration group, while TGF- $\beta$ 1, BMP2 and BMP4 were significantly decreased in the low concentration group ( $P < 0.05$ ), as shown in Table 3.

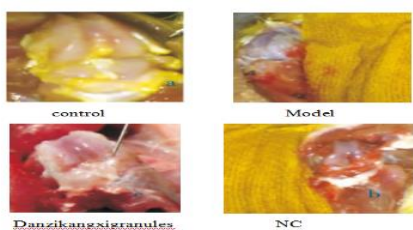
**Table 3.** Effects of Danzikang Granule with different concentrations on BMSCs protein expression

Group	Blank group	Low concentration group	Middle concentration group	High concentration group
TGF- $\beta$ 1	0.82 $\pm$ 0.15	1.35 $\pm$ 0.68 <sup>a</sup>	1.71 $\pm$ 0.42 <sup>*</sup>	2.53 $\pm$ 0.88 <sup>a</sup>
BMP2	0.37 $\pm$ 0.22	0.54 $\pm$ 0.16 <sup>a</sup>	0.93 $\pm$ 0.27 <sup>*</sup>	1.55 $\pm$ 0.55 <sup>a</sup>
BMP4	0.70 $\pm$ 0.36	0.93 $\pm$ 0.41 <sup>a</sup>	1.05 $\pm$ 0.53 <sup>*</sup>	1.72 $\pm$ 0.49 <sup>a</sup>

Note: Compared with blank group, <sup>\*</sup> $P < 0.05$ ; Compared with the medium-dose group, <sup>a</sup> $P < 0.05$

### Gross observation of knee cartilage in rats

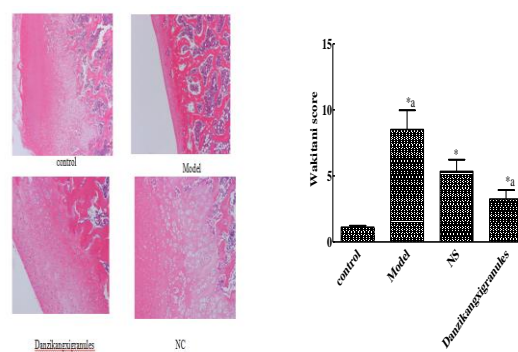
After 4 weeks, the joint morphology of the control group was normal, indicating smooth and no damage and the articular synovial fluid was clear, colorless and transparent. In the model group, joint swelling was severe, bone hypertrophy was observed in the tibial plateau and femoral condyle, and synovial fluid increased significantly, showing turbidness yellow. The intrasynovial surface is yellow and the articular surface is fuzzy and dark red. Articular cartilage was markedly damaged, with cartilage shedding and subchondral bone exposed in some areas. In the saline group, partial cartilage repair was found, and the tissue surface was more smooth than that in the modeling group, and the cartilage fissure was significantly reduced. Compared with the sham operation group, the joint of danzikang Knee granule group was slightly swollen, but there was no bony hypertrophy, the synovial fluid was colorless and transparent, the articular surface and synovial inner surface were smooth, the color was not bright, and the articular cartilage was intact. There were repair marks on the articular surface. The joint edges were regular and neat, without osteophyte formation, and the synovium was smooth and pale red, as shown in Figure 4.



**Figure 4.** Gross observation of knee cartilage in rats

### Comparison of histopathological observation and Wakitani histological score in rats

In the control group, the cell-matrix was uniformly colored, and each cell level showed emotion, arranged in orderly order and uniform size. In the model group, there were large inflammatory cell infiltration, synovial tissue and vascular hyperplasia, and some obvious osteophytes. In the normal saline group, matrix staining was not uniform, cell cluster aggregation was common, and a large number of chondrocyte necrosis areas could be seen, and part of synovial tissue inflammatory infiltration could be seen. In the group of Danzikang granules, rough articular surface, uneven staining of cell-matrix, no aggregation of cell clusters and a small amount of chondrocyte necrosis were observed. Synovial tissue is normal without obvious infiltration and proliferation. The Wakitani histological score of the control group was significantly lower than that of the other three groups. Compared with the normal saline group, the Wakitani histological score of the modeling group was significantly increased, while the Wakitani histological score of the Danzikang Granule group was significantly decreased, with statistical significance ( $P < 0.05$ ), as shown in Figure 5.



**Figure 5.** Comparison of histopathological observation and Wakitani histological score in rats

### Protein expression in rat cartilage tissue

The protein expressions of TGF- $\beta$ 1, BMP2 and BMP4 in Danzikang granule group were significantly higher than those in the normal saline group, Dan control group and modeling group, and those in the normal saline group were significantly higher than those in the control group and modeling group. The control group was significantly higher than the

modeling group, and the differences were statistically significant ( $P < 0.05$ ), as shown in Table 4.

**Table 4.** Protein expression in rat cartilage tissue

Group	Control group (n=20)	Model group (n=20)	Normal saline group (n=20)	Danzikang granule group (n=20)
TGF- $\beta$ 1	43.05 $\pm$ 4.12	37.12 $\pm$ 3.15 <sup>a</sup>	46.17 $\pm$ 6.52 <sup>*</sup>	52.12 $\pm$ 13.19 <sup>*a</sup>
BMP2	19.16 $\pm$ 2.11	10.19 $\pm$ 1.16 <sup>a</sup>	24.18 $\pm$ 4.18 <sup>*</sup>	32.18 $\pm$ 7.13 <sup>*a</sup>
BMP4	22.18 $\pm$ 3.15	15.15 $\pm$ 5.16 <sup>a</sup>	30.18 $\pm$ 6.48 <sup>*</sup>	39.27 $\pm$ 6.52 <sup>*a</sup>

Note: Compared with control group, \* $P < 0.05$ ; Compared with normal saline group, a $P < 0.05$

The main pathological features of knee arthritis are pathological degeneration of articular cartilage and subchondral bone. Since articular cartilage is mainly composed of cartilage matrix and chondrocytes, degeneration of articular cartilage can be specifically manifested as degradation of cartilage matrix and reduction of chondrocytes (13, 14). In the treatment of knee arthritis, it is feasible to promote cartilage repair by increasing chondrocytes. BMSCs are pluripotent stem cells with multidirectional differentiation potential, which can participate in the regulation of immunity, self-replication and other functions (15, 16). A large number of studies have confirmed that BMSCs can differentiate into chondrocytes in vivo and rely on inducers for chondrocyte differentiation in vitro (17-19). Danzikang knee granule is experienced for the treatment of knee osteoarthritis, the liver and kidney deficiency type of knee osteoarthritis with obvious therapeutic effect, side of dried human placenta essence of lean and raising blood yiqi, salvia miltiorrhiza promoting blood circulation to remove blood stasis, effectively for medicine, achyranthes kidney, rehmannia glutinosa lean body, mulberry parasitism kidney, (three fungicides have liver and kidney Yin and Yang double repair work. Blood exhaustion blood circulation and stasis, frankincense blood circulation and pain relief, caulis spatholobi line blood and blood, several drugs combined can strengthen danshen blood circulation and collaterals effect. Alone dispels wind and dampness, clears collaterals and eases pain, and Paeonia lactiflora relaxes the liver, collects Yin, nourishes blood and clears collaterals. The combined use of various medicines dispelling blood stasis, tonifying blood and qi, dispelling cold and dampness, stretching tendons and relieving pain.

In cell experiments, the present study obtained drug serum-containing normal saline, low, medium and high concentration drug serum-containing Danzikang granule by continuous drug intragastric administration of rats for 7 days, and explored the effect on BMSCs cell proliferation and differentiation in vitro. The results showed that compared with the blank group, the serum-containing Danzikang granule could promote the proliferation of mesenchymal stem cells and differentiation into chondrocytes. With the increase of drug concentration, cell proliferation and differentiation intensity also increased. As far as the current research results are concerned, danzikang Knee granule cannot prove its effect on the proliferation of mesenchymal stem cells and the promotion of chondrocyte differentiation because danzikang Knee granule is a traditional Chinese medicine experience prescription for the treatment of osteoarthritis. However, some studies have proved the effectiveness of some medicinal ingredients in the drug formula in promoting chondrocyte proliferation and the therapeutic effect on knee osteoarthritis. The drug formulae in this study were Paeonia lactiflora, Salvia miltiorrhiza, Ziheche, Rehmannia glutinosa, Duhuo, Achyranthes bidentata, Mulberry Jizi, Subtilis spatholobus, Sanguinae sanguinae, frankincense, myrrh and glycyrrhiza, among which, Wang et al. (20) showed that tanshinone I significantly inhibited IL-1 $\beta$ -induced apoptosis of CHon-001 cells. In osteoarthritis, IL-1 $\beta$ -induced collagen II, proteascan degradation, SOX11 downregulation and upregulation of MMP-13 and P-NF- $\kappa$ B in CHON-001 cells were reversed by tanshinone I treatment. Meanwhile, tanshinone I alleviated cartilage destruction and synovitis in OA mouse model, and decreased OARSI score and subchondral bone thickness. Weng et al. (21) showed that ACHYrantha polysaccharides promoted chondrocyte proliferation by activating the Wnt/ $\beta$ -catenin signaling pathway.

In animal experiments, the model rats of OSTEO arthritis were selected for intra articular injection of mesenchymal stem cells for four weeks in this study. The results showed that the symptoms of the model rats were significantly improved after injection of BMSCs suspension-cultured in danzikang granule drug-containing serum and normal saline drug-containing serum. The Wakitani histological score was significantly higher than that of the model group

and the normal saline group, but it still could not return to the normal bone and joint state. At present, it is difficult to demonstrate the efficacy of the Wudan Zikang Knee granule in animal experiments and clinical studies abroad. However, Li et al. (22) showed that total glucosides of *Paeonia lactiflora* can not only improve symptoms but also inhibit bone destruction. The therapeutic effect of total glucosides of *Paeonia lactiflora* on arthritis is mainly achieved by inhibiting the differentiation of spleen Tfh cells and GC formation through the STAT3 signaling pathway. Xu et al. (23) (23) showed that *ACHYranthes bidentata* inhibited IL-1 $\beta$  -induced apoptosis and proliferative nuclear antigen by inhibiting caspase-3 activation, and alleviated IL-1 $\beta$  -induced inflammation and matrix degradation by down-regulating the expression of matrix metalloproteinases 3 and 9 and cycidase 2. In addition, il-1 $\beta$ -induced nuclear factor  $\kappa$ B activation in rat cartilage was inhibited. Jia et al. (24) showed that tanshinone IIA inhibited articular cartilage degradation by inhibiting cell apoptosis and inflammatory cytokine expression levels, providing potential for the treatment of OA. The above results proved the effectiveness of Danzikang Knee granule in the treatment of knee osteoarthritis.

In terms of protein expression of mesenchymal stem cells and cartilage tissue, cell experiments and animal experiments in this study have confirmed that danzikangjin granule applied to mesenchymal stem cells can promote the protein expression of TGF- $\beta$ 1, BMP2 and BMP4. Therefore, it is speculated that the specific mechanism of DANzikang Granule promoting the proliferation of mesenchymal stem cells and differentiation into chondrocytes for cartilage repair in knee osteoarthritis may be related to TGF- $\beta$ /BMPs signaling pathway. TGF- $\beta$ 1 has been confirmed to promote BMSCs cells to express aggregative proteoglycan and type II collagen through a series of effects to present chondrocyte morphology. Fang et al. (25) showed that autologous platelet-rich plasma promoted gene and protein expression of cell proliferation and chondrogenesis markers through TGF- $\beta$ /SMAD signaling pathway. Zhou et al. (26) showed that BMP2 induced chondrogenesis and osteogenesis in vitro through the expression of Sox9, Runx2 and their downstream markers. According to the results of subcutaneous stem cell implantation

studies, BMP2 not only induces chondrogenesis but also promotes endochondral ossification during ectopic bone/chondrogenesis. The results of this study are consistent with those of the above studies, and all of them are chondrogenic differentiation of mesenchymal stem cells through the TGF- $\beta$  signaling pathway. In terms of specific reasons, Mei et al. (27) showed that *Salvia miltiorrhiza* injection can improve periodontal microcirculation, thus promoting the expression of TGF- $\beta$ 1 protein.

In conclusion, Danzikang Knee Granule plays a role in cartilage repair in knee osteoarthritis by promoting mesenchymal stem cell proliferation and chondrogenic differentiation, and the specific mechanism may be related to the TGF- $\beta$ /BMPs signaling pathway.

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### Interest conflict

The authors declare no conflict of interest.

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