

## Original Article

# The mechanism of medial collateral ligament repair in knee osteoarthritis based on the TLR4/MyD88/NF- $\kappa$ B inflammatory signaling pathway

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## Abstract

**Objectives:** To explore the role of medial collateral ligament repair in knee osteoarthritis based on TLR4/MyD88/NF- $\kappa$ B inflammatory signaling pathway. **Methods:** The modified Hulth method was used to establish models, which were divided into a repair group, a model group, and a sham operation group. The repair group was treated with medial ligament repair technology. Synovium and cartilage morphological changes were evaluated by hematoxylin-eosin staining to determine the degree of reparation. The cartilage was evaluated by the Mankin's score, and inflammatory factors in cartilage tissues were determined by ELISA. The changes in TLR4, MyD88, and NF- $\kappa$ B levels were analyzed using the real-time quantitative PCR and Western blot assays. **Results:** The synovial and cartilage damages in the repair group and the sham operation group were significantly alleviated compared to the model group. The Mankin's score of the model group was significantly lower than the other two groups. The expression of inflammatory factors in the repair group and the sham operation group were significantly lower than in the model group. The expressions of those factors in the repair group and the model group were higher than those in the model group. **Conclusions:** Medial ligament repair can improve the cartilage morphology and delay the development and progression of knee osteoarthritis by inhibiting the TLR4/MyD88/NF- $\kappa$ B signaling pathway.

**Keywords:** Collateral Ligament, Inflammatory Factor, MyD88, NF- $\kappa$ B, TLR4

## Introduction

Knee osteoarthritis (KOA) is the most common form of arthritis that affects the elderly. Its clinical symptoms include joint swelling, pain, stiffness, and its pathological processes are mainly synovial inflammation, meniscus injury, and chondromalacia patella. When joints are injured or suffering from lesions, synovium will be hyperemic and edematous due to stimulation, resulting in hypersecretion of synovial fluid.

With a large amount of inflammatory factors, synovial fluid accelerates the progression of hyperosteoarthritis, meniscus injury, and patella softening, eventually aggravating KOA. Studies have revealed that a variety of inflammatory signaling pathways are involved in the development of KOA, and inhibition of inflammatory signaling pathways can slow down the development and progression of the disease<sup>1-4</sup>. The Toll-like receptor 4/human myeloid differentiation factor 88/nuclear factor kappa-B (TLR4/MyD88/NF- $\kappa$ B) inflammatory signaling pathway plays a crucial role in the development and progression of KOA<sup>5-7</sup>. Studies have revealed that in the KOA rat models of synovial inflammation, with the increase of TLR4 expression, intra-articular inflammation and synovial lesions are both aggravated<sup>8</sup>. In addition, one study has found that indexes related to the TLR4/MyD88 signaling pathway in the synovial fluid and synovial tissues of patients with KOA increased, indicating that TLR4/MyD88 signaling pathway may be related to synovial inflammation of KOA<sup>9</sup>. Medial collateral ligament (MCL) injury is a common knee

The authors have no conflict of interest.

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Edited by: G. Lyritis

Accepted 10 June 2020



joint injury, and poor ligament healing is easy to bring about secondary osteoarthritis. MCL is an essential structure for the stability of medial knee joint, and adjusting the ligament of knee joint can alleviate articular cartilage wear and thus relieve KOA. This study aimed to explore the effects of TLR4/MyD88/NF- $\kappa$ B inflammatory signaling pathway changes on KOA in MCL repair. Therefore, this study investigated the expression of TLR4/MyD88/NF- $\kappa$ B inflammatory signaling pathway after MCL repair and its mechanism of alleviating the degeneration or destruction of articular cartilage, promoting cartilage formation in the treatment of KOA.

## Materials and methods

### Experimental animals

Thirty 8-week-old clean-grade male Wistar rats were purchased from the Experimental Research Center, China Academy of Chinese Medical Sciences, and the study was approved by the Animal Ethics Committee of our hospital.

### Experimental reagents

Trizol (Sigma Company, USA); RNA reverse transcription kit (TOYOBO), SYBY®  $\kappa$ Green Real-time PCR Master Mix (TOYOBO), and 5 $\times$ Loading Buffer (Takara Company, USA); DNA Marker (Shanghai Sangon Biotech Co., Ltd.); paraffin, paraformaldehyde, and hematoxylin-eosin (HE) staining reagent (Shanghai Beyotime Institute of Biotechnology), protein extraction kit and sodium-dodecyl-sulfate-polyacrylamide gel-electrophoresis (SDS-PAGE) kit (Shanghai Beyotime Institute of Biotechnology); electrochemiluminescence (ECL) liquid (Applygen Technology Company); goat anti-rabbit immunoglobulin G (IgG)/horseradish peroxidase (HRP), goat anti-rabbit MyD88, TLR 4, and NF- $\kappa$ B (ab102890, ab16894, ab22048, and ab22146, Abcom Company, USA); TNF- $\alpha$ , IL-6, and IL- $\beta$  enzyme-linked immuno-sorbent assay (ELISA) kits (Shanghai Beyotime Institute of Biotechnology). Primers were designed by the Shanghai Sangon Biotech Co., Ltd.

### Experimental instruments

Microplate reader (Thermo, USA); FACS Calibur flow cytometer (BD company, USA), 4 low-temperature high-speed centrifuges (Thermo Scientific, USA); Sihuan freeze dryer (Sihuan Scientific Instrument Factory Co., Ltd., Beijing, China).

### Rat model preparation and sample collection

Thirty 8-week-old clean-grade male Wistar rats ((230 $\pm$ 10) g) were randomly assigned to a repair group, a model group, and a sham operation group (each n=10). All rats were fasted for solids and liquids for 12 h, and then injected with 2% pentobarbital (0.2 mL/100 g) via enterocoelia for anesthesia. Each rat in the sham operation group was incised from the medial side of joint to separate the muscle and ligament and expose the joint cavity, and then the incision was sutured.

Rats in the repair group and the model group were modeled into KOA rats by the modified Hulth method, and each rat was incised from the medial side of joint to separate the muscle and ligament and expose the joint cavity. Subsequently, the MCL was cut off, and the medial meniscus was removed. In addition, the anterior cruciate ligament was cut off, and the incision was sutured layer by layer. Afterwards, each rat was injected with penicillin for 3 continuous days after operation. Rats in the repair group were given medial ligament reconstruction and repair 4 weeks after operation, and they were injected with penicillin for 3 continuous days after modeling, and fed routinely. Rats in each group were dissected to take their femoral condyle and synovial tissues of knee joint 8 weeks later, which were labeled and stored in liquid nitrogen.

### Observation of cartilage histomorphology

The right knee joint of each rat was taken out, and the separated cartilage was adjusted to 0.5 cm  $\times$  0.4 cm  $\times$  0.3 cm. Subsequently, the cartilage tissues were embedded with paraffin, and cut into slices, stained through HE, and then analyzed under an optical microscope.

### Evaluation of degree of cartilage degeneration

The Mankin score was used to evaluate the articular cartilage of each rat. 0-2 points = normal cartilage, 3-5 points = cartilage surface fibrosis, 6-7 points = moderate cartilage damage, 8-10 points = severe cartilage damage, and scores more than 10 points = complete cartilage missing.

### Detection of inflammatory factors in tissues by ELISA

A part containing cartilage, subchondral bone, and synovial tissues was sampled from each rat, weighed, and placed in a 5 mL EP tube. Phosphate buffer saline (PBS) buffer (PH7.2-7.4) was added into the EP tube at the mass (g) and volume (ml) ratio of 1: 9 through a pipette gun, and the tube was also added with protease inhibitor, homogenized by a high-speed homogenizer at 3800 r/min, and centrifuged for 20 min to take the supernatant. Finally, the levels of inflammatory factors (TNF- $\alpha$ , IL-6, and IL- $\beta$ ) in rat tissues were determined.

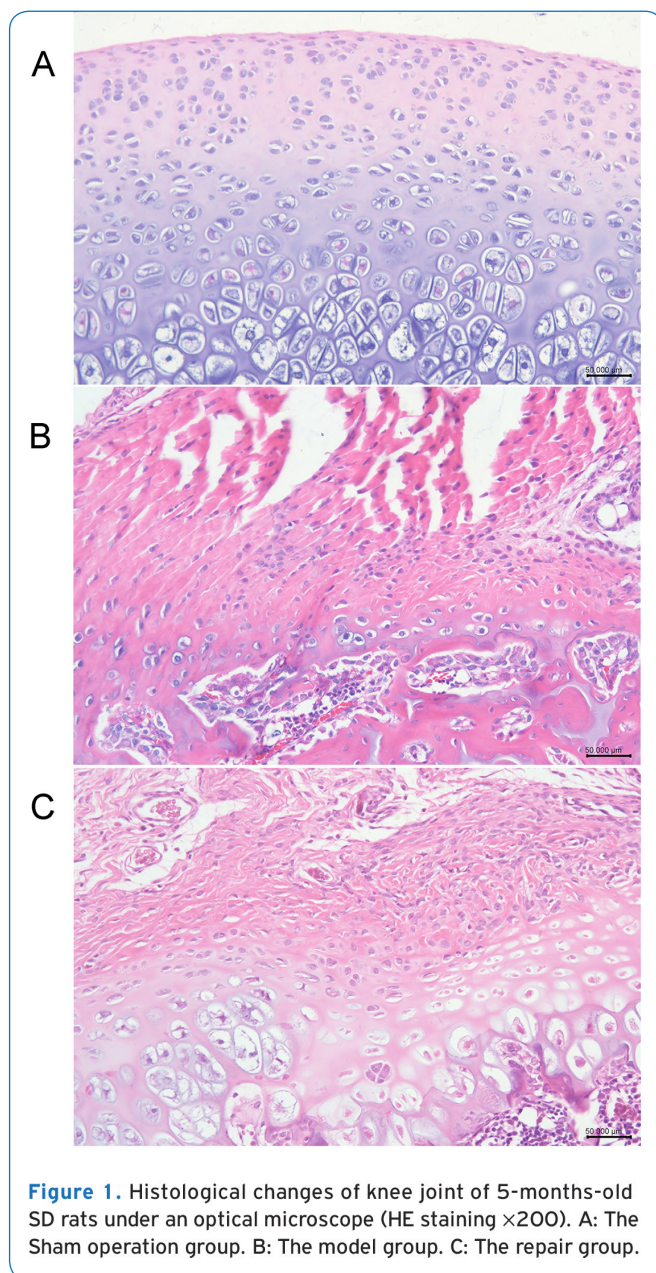
### Determination of mRNA expression changes of TLR4, MyD88 and NF- $\kappa$ B in each group of samples by the fluorescence quantitative PCR

Total RNA of synovial tissues of knee joint was extracted from each rat using the Trizol method, and then it was reversely transcribed into cDNA, and frozen and stored at -20°C. Designed Primer5.0: Upstream primer of TLR4: 5'-GATCTACTCACTTTACCATA-3'; downstream primer of TLR4: 5'-GCTAATCGAGGCTACGACT-3'; upstream primer of MyD88: 5'-GATGCCGATATGCCGATTCA-3'; downstream primer of MyD88: 5'-GCGACACGATGGGTCGCCAT-3'; upstream primer of MyD88NF- $\kappa$ B: 5'-CATCCGACGTATGACCATGA-3'; downstream primer of MyD88NF- $\kappa$ B:

**Table 1.** Mankin's score of articular cartilage of rats in each group ( $\bar{x} \pm S$ ) (n=10).

Groups	The model group	The repair group	Sham operation group
Score	3.27±1.05	8.29±1.23**	8.41±0.95**

\*\*P<0.01



5'-GCAACGTGAAGTGAAGTGA-3'. The total reaction system of qRT-PCR was 25.0  $\mu$ L, and the reaction condition of the factors was as follows: For TLR4: 95°C for 30 s, followed by 50 cycles of 58°C for 40 s and 72°C for 20 s; for MyD88: 95°C for 35 s, followed by 50 cycles of

55°C for 40 s and 72°C for 25 s; for NF-κB: 95°C for 30 s, followed by 50 cycles of 50°C for 40 s and 72°C for 30 s.

#### Determination of protein expression changes of TLR4, MyD88, and NF-κB in each group of samples by the Western blot assay

Articular tissues were sampled from each rat, homogenized, and added with 40  $\mu$ l lysis solution (890  $\mu$ l NP40 cell lysis buffer). They were placed on ice for 30 min, then lysates were collected and clarified by centrifugation at 13,000 rpm for 10 minutes, and then stored at -80°C. The protein content was determined using a Bicinchoninic acid (BCA) kit. Protein samples (10  $\mu$ g) were separated on 13% preformed gel, electrophoresed, and then transferred to a membrane, and cultured overnight at 4°C. The membrane was sealed with 3% bovine serum albumin (BSA), and incubated overnight at 4°C with one of the following antibodies: Mouse monoclonal antibody anti- $\beta$ -actin (working dilution of 1: 25000), rabbit polyclonal anti-rat TLR4 (dilution of 1: 300), rabbit polyclonal anti-rat MyD88 (dilution of 1: 200), rabbit polyclonal anti-rat NF-κB (dilution of 1: 300). Subsequently, the membrane was washed, and then cultured with a suitable secondary antibody (goat anti-rabbit IgG horseradish peroxidase conjugated or goat anti-mouse IgG horseradish peroxidase conjugated) at a dilution of 1: 4000 for 1 hour. Imprinting was developed using an enhanced chemiluminescent substrate and immunoreactivity was developed using the VersaDoc imaging system. Finally, the protein expression was normalized to  $\beta$ -actin for band density quantification.

#### Statistical analysis

Data in this study were statistically analyzed using SPSS17.0. Measurement data were expressed as the mean  $\pm$  standard deviation ( $\bar{x} \pm S$ ). Normal distributed variables between groups were compared using the non-paired student t test, and those within groups were compared using the one-way ANOVA. Differences between groups were compared using the Fisher's least significant difference (LSD).  $P < 0.05$  indicates a significant difference.

## Results

### HE staining and histological grading of cartilage

The morphology results of cartilage were as follows: The cartilage tissues of rats in the sham operation group showed unobvious fissures, clear stratification, and no interrupted tidal line; the cartilage tissues of rats in the model group

showed deepened and widened fissures, large-scale defects on the surface that were covered by thin layers of scar fiber tissue, unclearly stratificated chondrocytes, and disordered tidal line; Cartilage tissues of rats in the repair group showed deeper fissures, more cells, higher density, clearer stratification, and more interruptions of tidal line than those in the sham operation group, and also showed a more significant alleviation in lesion than those in the model group (Figure 1).

#### Comparison of morphological changes of articular cartilage between different groups

The Mankin's score of the model group was significantly lower than that of the sham operation group ( $P < 0.01$ ), and the Mankin's score of the repair group was significantly higher than that of the model group ( $P < 0.01$ ), indicating that arthritis of rats was ameliorated by medial ligament repair (Table 1).

#### Detection of inflammatory factors in synovial tissues by ELISA

Compared with the sham operation group, the model group showed significant increased levels of inflammatory factors (TNF- $\alpha$ , IL-6, and IL- $\beta$ ) (all  $P < 0.01$ ), while compared with the model group, the repair group showed significantly decreased levels of them (all  $P < 0.05$ ), implying that MCL repair relatively ameliorated inflammatory factors (Figure 2).

#### Determination of mRNA expression changes of TLR4, MyD88, and NF- $\kappa$ B by the real-time fluorescent quantitative PCR

Synovial tissues of keen joint were sampled from each rat 8 weeks after operation, and the mRNA expression of TLR4, MyD88, and NF- $\kappa$ B in the tissues was determined using real-time fluorescent quantitative PCR. It was found that the mRNA expression of TLR4, MyD88, and NF- $\kappa$ B in the model group was significantly higher than that in the sham operation group (all  $P < 0.01$ ), and the mRNA expression of them in the repair group was significantly lower than that in the model group ( $P < 0.05$ ). In addition, there was no significant difference between the repair group and the sham operation group in the mRNA expression of them (Figure 3).

#### Determination of protein expression changes of TLR4, MyD88, and NF- $\kappa$ B by Western blot assay

Synovial tissues of keen joint were sampled from each rat 8 weeks after operation, and the protein expression of TLR4, MyD88, and NF- $\kappa$ B in the tissues was determined by Western blot assay. It was found that the protein expression of TLR4, MyD88, and NF- $\kappa$ B in the model group was significantly higher than that in the sham operation group (all  $P < 0.01$ ), and the protein expression of them in the repair group was significantly lower than that in the model group (all  $P < 0.05$ ). In addition, there was no significant difference between the repair group and the sham operation group in the protein expression of them (Figure 4). Those results indicated that

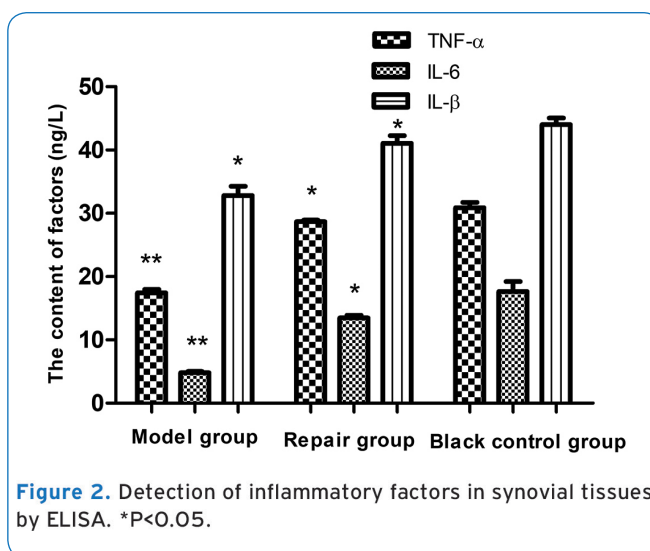


Figure 2. Detection of inflammatory factors in synovial tissues by ELISA. \* $P < 0.05$ .

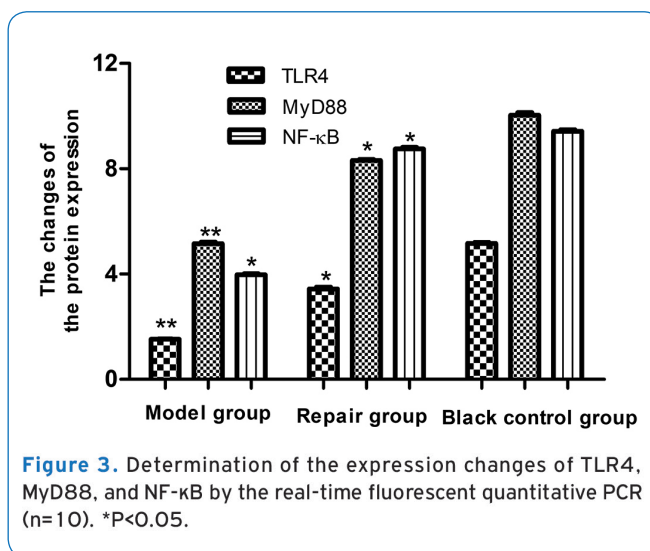
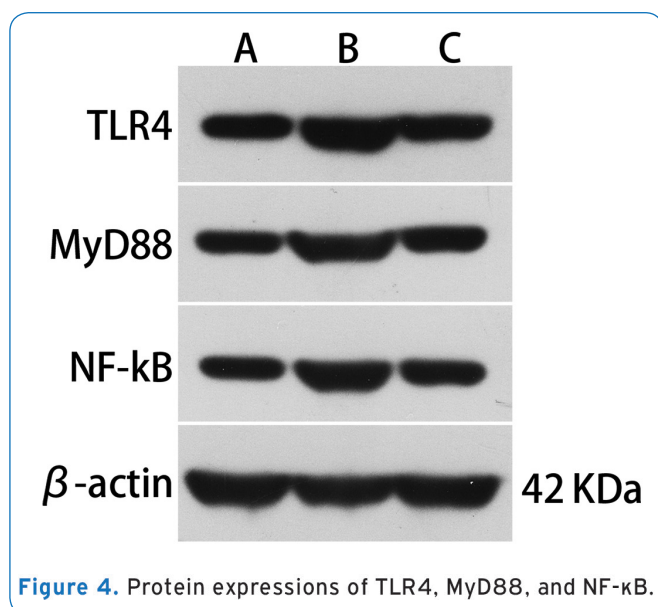


Figure 3. Determination of the expression changes of TLR4, MyD88, and NF- $\kappa$ B by the real-time fluorescent quantitative PCR ( $n = 10$ ). \* $P < 0.05$ .

MCL repair had a certain regulatory effect on inflammatory signaling pathways.

## Discussion

KOA is a bone disease mediated by inflammatory factors and related signaling pathways<sup>11</sup>. The expression of inflammatory factors, including IL-6, IL-1 $\beta$ , and TNF- $\alpha$  in synovial fluid, are correlated with the pathological changes of KOA<sup>12</sup>. In addition, the expression of TLR4 in chondrocytes of patients with KOA was higher than that in healthy individuals<sup>13</sup>, and inhibiting the TLR4 signaling pathway can inhibit chondrocyte apoptosis<sup>14</sup>. Curcumin can alleviate joint inflammation in rats with KOA rats by inhibiting the TLR4/MyD88/NF- $\kappa$ B inflammatory signaling pathway<sup>15</sup>.



Toll-like receptor can induce the production of various inflammatory factors by sensing damage-associated molecular pattern molecules (DAMPs) and identifying pathogen-associated molecular patterns (PAMPs)<sup>16</sup>. TLRs activation signal can activate the NF- $\kappa$ B pathway and stress kinase pathway, and stimulate the production of inflammatory factor (IL-10), tumor necrosis factor (TNF), proteoglycan enzyme, transforming growth factor, and matrix metalloproteinase (MMPs)<sup>17</sup>, thus inducing inflammation. TLR4 is a transmembrane protein involved in mediating the release of inflammatory mediators. In recent years, TLRs-mediated innate immune response of chondrocytes has been verified to be a key link in promoting KOA<sup>18,19</sup>.

The increase in TLR4 expression could activate the RAF6/NF- $\kappa$ B signal transduction via MyD88. NF- $\kappa$ B, a key transcription factor of inflammatory cascade reaction, initiates the transcription of pro-inflammatory cytokines including TNF- $\alpha$ , IL-1 $\beta$  and IL-6. Studies have showed that TLR4-mediated signaling pathway activates NF- $\kappa$ B and induces the secretion of downstream cytokines, which plays a key role in the development and progression of KOA<sup>20</sup>.

In this study, we used the modified Hulth method to construct rats with KOA, and analyzed the morphological changes of cartilage tissues using the HE staining method. The cartilage tissues of rats in the sham operation group showed neatly arranged chondrocytes, clear stratification, and no interrupted tidal line; the cartilage tissues of rats in the model group showed deepened and widened fissures, integrity compromise, unclear stratification of chondrocytes, visible fibrosis, cartilage defect, disordered or disappeared tidal line; cartilage tissues of rats in the repair group showed deeper fissures, more cells, higher density, clearer stratification, and more interruptions of tidal line than those in the sham operation group, and also showed a more

significant alleviation in lesion than those in the model group. The cartilage structure in the repair group was similar to that in the sham operation group. In addition, in this study, the Mankin's score of the model group was significantly higher than that of the sham operation group, and the score of the repair group was significantly higher than that of the model group. The levels of TNF- $\alpha$ , IL-6, and IL- $\beta$  in synovium was detected by ELISA, and compared with the sham operation group, the model group showed significantly increased levels of TNF- $\alpha$ , IL-6, and IL- $\beta$ , while the model group showed significantly decreased levels of the three indicators. Furthermore, real-time fluorescent quantitative PCR and Western blot assays revealed that the expression of TLR4, MyD88, and NF- $\kappa$ B in the model group was significantly lower than that in the repair group and sham operation group, and the expressions in the repair group and sham operation group were similar, which suggested that MCL repair can inhibit the expression of TLR4, MyD88, and NF- $\kappa$ B. Therefore, it is speculated that MCL repair and TLR4/MyD88/NF- $\kappa$ B pathway play important roles in the development of KOA syndrome. MCL repair can prevent and treat KOA by regulating the TLR4/MyD88/NF- $\kappa$ B signaling pathway and then knee joint inflammatory reaction, inhibiting chondrocyte proliferation and inflammatory reaction, slowing down cartilage degeneration, and improving articular cartilage morphology.

#### Acknowledgements - Authors' contributions

ZL and ST conceived and designed the study, and drafted the manuscript. ZL, YZ, DF, WZ, HG and NG collected, analyzed and interpreted the experimental data. YZ revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

#### Funding

This work was supported by Experimental Study of 5-fluorouracil in the treatment of rabbit knee osteoarthritis (SFZD-2016109) and Clinical Study on the repair of medial collateral ligament in the treatment of elbow joint (SFZD-2019138).

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