ORIGINAL ARTICLE

Patellar Inward Pushing Method Relieves Knee Osteoarthritis via Regulating Cytokines

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ABSTRACT

Background and Objective: Knee osteoarthritis (KOA) is a chronic degenerative disease characterized by pain, morning stiffness and swelling in the knee joints. And KOA is common in the elderly and seriously affects the exercise function and physical health of patients. This study aimed to explore the curative effects of patellar inward pushing method (PIPM) on KOA.

Methods: In this study, we established rabbit animal models of KOA for the research by using the New Zealand white rabbits. A total of n=30 rabbits were divided into 5 groups by random number table method: blank group, model group, glucosamine hydrochloride (GH) group, PIPM group and PIPM combined with GH group. The rabbits were then modeled.

Results: After 9-weeks cultured in groups, 5 ml blood was collected from the heart, and cytokines were detected. The result suggested that iNOS, NO and TNF-α were the pathogenic inflammatory factor of KOA, and aggravated cartilage damage and degeneration. Besides, this study indicated that PIPM combined with GH treatment significantly reduced the activity of inflammatory cytokines in serum and joint fluid of KOA model in rabbits. In addition, PIPM combined with GH therapy exhibited the best therapeutic effect among these treatments, which was working on KOA better than PIPM treatment alone or GH treatment alone.

Conclusion: PIPM could effectively treat KOA via regulating cytokines, and the PIPM combined with GH therapy could be a novel therapeutic strategy for KOA.

KEYWORDS: Patellar inward pushing, Knee osteoarthritis, Orthopaedic therapy, Cytokines.

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INTRODUCTION

Knee osteoarthritis (KOA), also known as degenerative knee arthritis or deformable knee arthritis, is a chronic joint disease characterized by degeneration of articular cartilage and secondary hyperosteogeny, mainly manifestations are knee joint pain, swelling, stiffness, weakness and dysfunction.[1] KOA is a complete, multilevel, and varying degree of aseptic, chronic, and progressive disease of the bone, synovial membrane, joint capsule and other joints.[2] KOA is very common in clinic, especially in the middle and old age

population, which seriously affects the quality of life of patients.[3,4] According to China's epidemiological survey, the prevalence rate of KOA in China among people aged 60 – 75 years was 50%, and that of people aged over 75 years was as high as 80%.[5]5. With the aging of population in modern society, the incidence of KOA is increasing year by year.[6] KOA patients often suffer from disability due to pain and limitation of activities; the disability rate is 53%.[7] And because of long-term pain it is easy to produce negative emotions which seriously affects family harmony and therapeutic effect.[8] Therefore, it is necessary to apply effective treatment of KOA.

At present, the clinical treatment of KOA is still aimed at alleviating pain, delaying the disease and improving the daily activity ability of patients.[9,10] Drug treatment is a common treatment for KOA. In the drug treatment of KOA, anti-inflammatory painkillers, non-steroidal anti-inflammatories (NSAIDS) and glucocorticoids can alleviate symptoms, while intra-articular injection of sodium hyaluronate, D-glucosamine and statins can improve the condition.[11-14] Surgical treatments, include joint dissection, anterior tibial tuberosity displacement, and artificial knee replacement are also common KOA management strategies. Sun et al.[15] emphasized the acupuncture and moxibustion for the treatment of KOA. Manual therapy alone or combined with other therapeutic methods is also widely used in the treatment of KOA.[16,17] It has been found that manipulation and physical therapy had good clinical effects after a one-year study but the combination of the two methods did not improve the treatment effect.[18] Although the treatment of KOA is increasing, it is still in the stage of delaying the disease and existing treatment methods often bring various side effects. Hence, novel therapy for KOA is worthy to be developed.

Patellar inward pushing method (PIPM) is a kind of manual therapy on KOA. It is relatively non-invasive, safe and low priced. PIPM is easy to operate, grasp and popularize, too. It could be learned and operated conveniently for doctors and patients. Moreover, PIPM can be applied to KOA in all stages of operation with the effect of prevention, treatment and rehabilitation.

In the current study, the white rabbit animal model of KOA was established, and the PIPM and PIPM combined with glucosamine hydrochloride (GH) were used to intervene the animal model. Then, the changes of serum and articular fluid cytokine levels were detected and the pathological changes were observed. Hence, the therapeutic effect of this method on KOA was discussed. This study further revealed the pathogenesis of KOA, and further elaborated the mechanism of the treatment ofKOA by the PIPM. Moreover, this study provided experimental data and theoretical support for the clinical application of the PIPM for the treatment of KOA.

METHODS

Reagents

Superoxide dismutase (SOD) assay kit (WST-1 method) and Malondialdehyde (MDA) assay kit (TBA method) was provided by Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). IL-1b Platinum Elisa kit was purchased from Thermo Co. (USA). Neutral formalin stationary solution, xylene, paraffin, hematoxylin staining solution, 1% eosin alcohol solution, neutral gum and glycerol protein adhesive tablets were obtained from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Glucosamine hydrochloride capsules were purchased fromBright Future Co. (Hongkong, China).

Animals

The animal study protocol was approved by the InstitutionalAnimal Care and Use Ethical Committee of PLA 371 Center Hospital, China. Thirty common grade healthy New Zealand white rabbits aged 6 months, regardless of gender and weight 3.0-3.5kg, purchased from Shanghai jiesjie experimental animals co., Ltd (Shanghai, China), and then 30 New Zealand white rabbits were raised in the same laboratory environment. The temperature, humidity and sunshine in the laboratory of the animal test center were all within the suitable living range of New Zealandwhite rabbits. The experimental living environment was clean and sanitary, and the molding was started after 1 weeks of routine adaptation.

Animals Grouping and Animal Model of KOA

Twenty-four rabbits were randomly divided into the model group, GH group, PIPM and PIPM combined GH group (6 rabbits per group) and the remaining 6 rabbits were as blank group, without modeling or treatment. The specific molding method was modified Hulth's method.[19] The rabbits were anesthetized along the rabbit ear margin vein (4% pentobarbital sodium, 0.8 ml/kg) under aseptic conditions. After anesthesia, the rabbits were fixed on the operating table in supine position. The hair of animal's knee was removed, and routine disinfection of the skin was carried out. A 2.5 cm longitudinal incision was performed on the medial collateral ligament of the rabbit right knee joint. The medial collateral ligament was cut off and the joint cavity was exposed. After determining no primary lesion, the anterior cruciate ligament was cut and the medial meniscus was excised. Articular cartilage was not damaged during the operation, the bleeding was completely stopped, and the incision was sutured by layer. The wound healing of the model rabbit was observed after operation; the drug was cleaned and changed regularly. Penicillin (50,000 u/kg/d) and streptomycin (100,000 u/kg/d) were injected into the muscle to prevent infection from the day of operation. The drug was discontinued after 3 consecutive days of antibiotic treatment.

PIPM Treatment on Rabbit KOA Models

After 1 week of animal modeling, the PIPM and PIPM combined with GH were treated on the PIPM rabbit group and PIPM combined with GH group, respectively. The operation steps of PIPM were as follows: After the experimental rabbits were fixed in lateral decubitus, the emotions were soothed and stabilized, then rabbit's knee joint was straightened, the operators touched the middle of the inner and outer edge of the patella with the thumb and forefinger, and exerted balanced force, pushed the patella in parallel to the inner side, and was pushed to the limit positionwhich was maintained for 5 seconds, and then released for 3 seconds to the natural restoration of the patella. The operation time in this method was 10 minutes, once daily treatment, 8 weeks continuous manipulation treatment.

GH Treatment on Rabbit KOA Models

A group of the rabbits KOA models were fed with GH capsules at the dose of 250 mg/kg, which was 10 times the maximum clinical dose (25 mg/kg) per day, and the volume of the dose was 5 ml/kg. The rabbits were fed 1 time a day with a continuous feeding for 8 weeks.

PIPM Combined with GH Treatment on Rabbit KOA Models

According to the above methods, a group of the rabbit KOA models were treated by PIPM combined with GH. These rabbits were treated by PIPM and fed with GH at the same time for 8 weeks.

Detection of Cytokine Content in Serum and Synovial Fluid

At the end of 9 weeks after molding, 5 ml blood and synovial fluid were collected from the rabbits' heart. Cytokines were detected according to the operation recommended instructions from the manufacturer. Inducible nitric oxide synthase (iNOS) was detected by superoxide dismutase (SOD) assay kit. Nitric oxide (NO) was tested by lipid peroxidation (MDA) assay kit and tumour necrosis factor (TNF-α) was examined by IL-1b platinum Elisa kit.

General Observation of Intra-articular Region

After 9 weeks of molding and the detection of cytokine were finished, the experimental animals were killed by air embolism. The experimental joints were cut opened layer by layer and the intra-articular region was exposed. Cartilage smoothness, color, ulcer, erosion, fibrous hyperplasia and osteophyte formation of articular surface were mainly observed. Overall observation of rabbit knee cartilage was conducted according to the standard of Pelletier's et al.[20] report (Table-1).

Table-1: Evaluation criteria of articular cartilage.

Items	*Grade (Point)*
The cartilage surface is smooth and light blue or colorless and translucent	0
The cartilage surface is soft but smooth	1
Cartilage thinning, small fibrous fascicular change forms	2
Cartilaginous fascicular change	3

Abrasion of fibrous fascicular degeneration with subchondral bone exposure and bone sclerosis.	4

Light Microscopic Observation of Articular Cartilage

The sample was taken from the femoral condyle joint of the rabbit by a scalpel with a diameter of 3 mm and the depth reaching the subchondral bone. At the same time, the synovial tissue near the joint gap was excised with a sharp knife slice, the size was about 3 mm x 3 mm, and was fixed with 10% neutral formaldehyde solution. After sampling and fixation, the preparation of paraffin sections was completed step by step: the cartilage specimens were successively washed, dehydrated, decalcified, and embedded with paraffin, which were then perpendicular to the cartilage surface. Then, staining procedures such as dewaxing, hydration, staining, dehydration, transparency and sealing were carried out. Finally, Hematoxylin and Eosin (HE) staining and histochemistry (toluidine blue staining) were performed for the specimens. Surface structure of cartilage, number of cells and integrity of the tide line of articular cartilage were mainly observed by HE staining, while cartilage matrix staining were mainly observed by toluidine blue staining. The degree of regression was graded according to Mankin's rating standard (Table-2).[21]

Observation and scoring of synovial tissue

The preparation of synovial tissue paraffin sections and HE staining was basically the same as that of articular cartilage, and the pathological changes of synovial tissue were observed by light microscopy. As previous report synovial cell hyperplasia, interstitial hyperemia edema and inflammatory cell infiltration were the main evaluation criteria (Table-3).[22]

Table-2: Mankin's scoring criteria of Joint cartilage.

The structure of Articular Cartilage	*Grade (Point)*	*Number of Cells*	*Grade (Point)*	*Toluidine Blue Staining*	*Grade (Point)*	*Integrity of Tide-Line*	*Grade (Point)*
Normal	0	Normal	0	Normal	0	Integrated	0
Disordered, but levels are distinguishable	1	Mild hyperplasia	1	Moderately reduced	1	Multilevel	1
Obviously irregular and the level is disordered	2	Moderate hyperplasia	2	Modestly reduced	2	Fuzzy	2
Serious disordered	3	Obvious hyperplasia	3	Serious reduced	3	Blood vessels pass through	3
				Non-staining	4		

Table-3: Synovial tissue grading standards.

Synovial Cell Proliferation	*Grade (Point)*	*Interstitial Hyperemia and Edema*	*Grade (Point)*	*Inflammatory cell Infiltration (Lymphocytes, Plasma Cells and Monocytes)*	*Grade (Point)*
The surface is smooth, the cells are arranged in one to four layers, and the shape and size are normal, adipose subintimal integrity	0	Non	0	Non	0
Low villous process on the surface, loose arrangement of 1-4 layers of cells, slight enlargement of nucleus, and the subintimal layer was mostly replaced by proliferative fibrous tissue.	1	Mild	1	Little	1
The surface cells were lost and damaged, the cells were 4∼5 layers, the nucleus slightly increased, and the subintimal layer was mostly replaced by proliferative fibrous tissue.	2	Moderate	2	High	2
The surface is rough and uneven, the cell layer is more than 5 layers, the nucleus is enlarged, and the subintimal layer is replaced by proliferative fibrous tissue.	3	Serious	3	Large	3

STATISTICAL ANALYSIS

Data were presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) following Turkey's text was used to compare the variables among the different treatment groups. Statistical analysis was performed using Statistical Package for Social Sciences ((SPSS, 20.0 software, Inc., Chicago, IL, USA) and $P < 0.05$ was considered to indicate a statistically significant difference.

RESULTS

PIPM Combined with GH Significantly Inhibited Inflammatory Factors

Inflammatory factors such as iNOS, NO and TNF-α in blood and synovial fluid were detected by SOD assay kit, MDA assay kit and IL-1b Platinum Elisa kit, respectively. As shown in Figure: 1, the activity of iNOS, NO and TNF-α in the model group was significantly higher than that in the blank group ($P<0.01$), which indicated that iNOS, NO and TNF-α were all inflammatory factors in KOA. Figures 1A and 1B exhibited the influence of PIPM on KOA models was similar to GH treatment, both of PIPM and GH significantly reduced iNOS activity in blood (Fig.1A) and synovial fluid (Fig. 1B). However, PIPM combined with GH treatment has the most significant inhibition ability to iNOS when compared with other groups ($P < 0.01$). Similar results were found in the detection of NO and TNF. Although both PIPM and GH significantly inhibited the content of NO (Fig. 1C and 1D) and TNF-α (Fig. 1E, $P < 0.01$), PIPM combined with GH treatment showed the highest inhibitory capacity for inflammatory factor ($P < 0.01$).

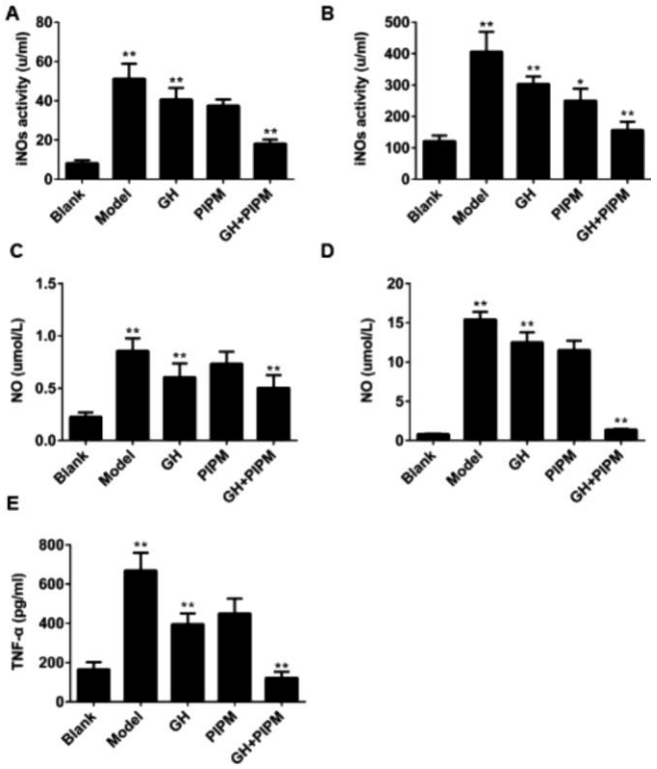

Fig. 1: Effect of PIPM on cytokines in blood and joint fluid. (A-B) Whierebbits were divided into 5 groups: blank, model, PIPM, GH, PIPM combined with GH, PIPM combined with GH treatment significantly reduced the iNOS activity both in blood and joint fluid. (C-D) PIPM combined with GH treatment significantly decreased the NO content both in blood and joint fluid. (E) PIPM combined with GH treatment significantly inhibited the TNF-α in blood.
**Represents $P < 0.01$ versus the control group.

General Observation of Knee Cartilage in Different Treatment Groups

To evaluate the effect of PIPM on the cartilage of the knee joint, external features such as smoothness, color, ulceration or erosion, fibrous hyperplasia and osteophyte formation were selected as evaluation criteria. The external features of different treatments were as follows: the cartilage surface of blank group was light blue and translucent, with smooth edges and no obvious osteophytes formed. The cartilage surface of the model group was rough and uneven and lost the normal luster, it also showed a clear fiber bundle change as well as cartilage defected and subchondral bone exposure. In the KOA model group, obvious osteophytes formed on the edge, and the texture was mainly cartilage. GH group: cartilage surface was slightly yellow, s normal luster, less smoothness, and cartilage wear degree was lighter, less osteophyte formation formed at the edge, the texture was mainly cartilage. In the PIPM group, the cartilage surface was slightly yellow, with normal luster and small fiber bundle changes. The cartilage weared lightly and there were less osteophytes at the edges, and the texture was mainly cartilage. Finally, in the PIPM combined with GH group, the cartilage surface was smooth, with normal luster, it presented small fiber bundle shape change, cartilage wear degree was lighter,

marginal osteophyte formation was very small, the texture was mainly cartilage (Fig.2). Overall observation of rabbit knee cartilage was showed as (Table-4).

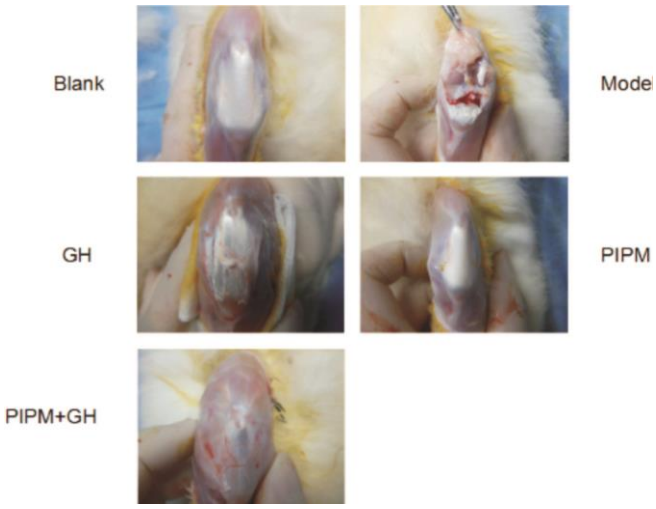

Fig.2: General observation of knee cartilage in different treatment groups. Rabbit models were respectively treated by GH, PIPM and PIPM combined with GH for 8 weeks. Cartilage smoothness, color, ulcer, erosion, fibrous hyperplasia and osteophyte formation of articular surface were mainly observed.

Microscopic Pathological Findings of Articular Cartilage of the KOA Models

To obtain the microscopic pathological findings of articular cartilage of the KOA models, HE staining and histochemistry (toluidine blue staining) were performed. In the blank group, the cartilage surface was smooth and orderly, with clear stratification, orderly cell arrangement and regular structure. In the model group, there were defects on the cartilage surface; cracks were inserted underneath; the stratification was not clear. The cells showed focal and diffuse hyperplasia with irregular structure, and the calcified layer was obviously thickened. The structures of each layer in GH group were gradually better than that in model group. The structures of all layers in PIPM group were almost as same as that in GH group. In the PIPM combined with GH group: the cartilage surface was smooth and orderly, stratified clearly; cells are arranged orderly, and the structure is regular (Fig.3). Besides, the Markin's grade of articular cartilage is shown in (Table-5) where PIPM combined with GH obtained the best grade in comparison with other treatments.

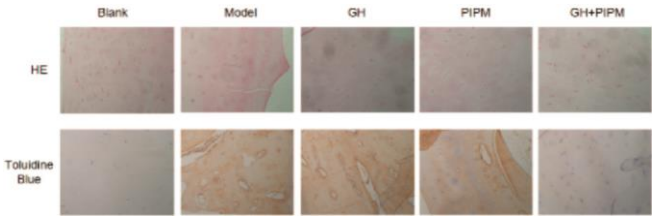

Fig.3: H&E staining and histochemical staining of articular cartilage. Rabbit models were respectively treated by GH, PIPM and PIPM combined with GH for 8 weeks.

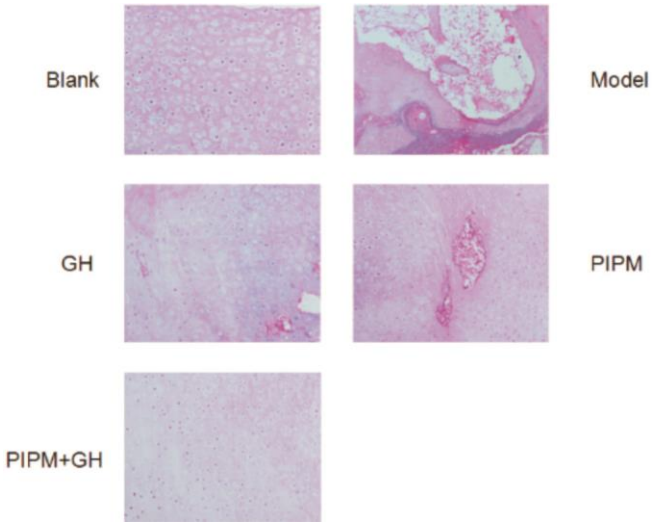

Fig.4: H&E staining of synovial tissue.Rabbit models were respectively treated by GH, PIPM and PIPM combined with GH for 8 weeks. The paraffin sections of synovial tissue were observed by light microscopy.

Table-4: Markin's grade of articular cartilage.

Group	*Tissue Structure (Point)*		*Number of Cells (Point)*	
Blank	0	0	0	0
Model	3	3	3	3
PIPM	3	2	2	3
GH	3	2	3	2
PIPM+GH	2	1	1	2

Observation of Knee Joint Synovium in KOA Models under Light Microscope

To exhibit the effects of different treatments on the knee joint synovium in KOA models, HE staining was applied in current study. As shown in Fig. 4, in blank group, the synovial surface was smooth with

clear cellular layer, and the adipose submucosa was intact. For model group, the surface of the synovial membrane is rough, with cell proliferation, the subintimal layer is replaced by fibrous tissue, and the interstitium was mildly hyperemic with edema and a large number of inflammatory cells infiltrated. In the GH group, the synovial cells were loosely arranged, and the under-layer of the inner membrane was replaced by proliferative fibrous tissue, and the interstitium was mildly hyperemic with edema and a few numbers of inflammatory cells infiltrated. The PIPM group showed almost same changes as of GH group. In the PIPM combined with GH group: the synovial membrane had a smooth surface, a clear cell level, and a small number of proliferating fibrous tissue substituted for the subintima, and there was no obvious congestion and edema in the interstitium with a little inflammatory cell infiltration. The grades of different treatments are shown in (Table-5).

Table-5: Grade of synovial tissue.

Group	*Synovial Cell Proliferation (Point)*		*Interstitial Hyperemia and Edema (Point)*		*Inflammatory Cell Infiltration (Point)*	
Blank	0	0	0	0	0	0
Model	3	3	3	3	3	3
PIPM	2	3	2	3	2	3
GH	3	2	3	2	3	2
PIPM+GH	2	1	1	2	1	1

DISCUSSION

The therapies of KOA are mainly divided into surgical treatment and non-surgical treatment.[23,24] Surgical treatment mainly includes arthroscopic surgery, joint replacement and other operations, while non-surgical treatment includes traditional Chinese medicine treatment, western medicine treatment, integrated Chinese and western medicine treatment and other emerging treatment methods.[25-27] Among them, traditional Chinese medicine (TCM) treatment mainly includes internal and external treatment, acupuncture treatment, manipulation massage, physical therapy and functional exercise. Western medicine mainly includes articular cavity injection of glucocorticoid, sodium hyaluronate, ozone, and oral drugs.[28] At present, there are many reports on physical rehabilitation therapy for KOA, all of which can achieve certain curative effect but the treatment method is relatively complex, with a long learning curve, which is not easy for doctors and patients to master.[29,30] In this study, PIPM was used to treat KOA models. The method is simple and easy to be learned and mastered by both doctors and patients. It is relatively non-invasive and inexpensive. It can effectively reduce the cost of treatment, and PIPM could be applied to the prevention, treatment and rehabilitation of KOA.

From the perspective of biology, PIPM effectively regulates the changes of cytokines in the body, enhances the activity of SOD in the body, and eliminates the damage of oxygen free radical to knee cartilage. This method also reduces the MDA poisonous to the cells of the body of membrane system, as well as reducing the IL-1 beta of cartilage matrix degradation and destruction of articular cartilage. Besides, PIPM inhibits the formation and the release of inflammatory mediators which can protect the articular cartilage, delay the degeneration and apoptosis of cells and promote the repair and proliferation of articular cartilage. Eventually, the disease progress is delayed and the KOA is remitted.

From the perspective of mechanical mechanics, PIPM maintains the mechanical balance of periknee bone and soft tissue, thus promoting the dynamic and static stability of knee joint mechanics. PIPM effectively improves the movement track of patellar trochlear and facilitated the benign alignment of patellofemoral joint, thus greatly reducing the impact and wear of bone tissue, avoiding cartilage damage and obviously reducing the pain caused by malalignment. Besides, PIPM can increase the mobility of patellar and knee joints, promote the release of adhesion folds, and increase the space volume of knee joint space, thereby reducing the pressure in knee joint and avoiding local abnormal stress. Moreover, PIPM can promote the knee tendon ligament and muscle strength exercise balance, especially being helpful to the muscle strength growth of the vastus medialis oblique (VMO) and medial collateral ligament. Thus, it can promote the balance of the inner and outer muscle force and soft tissue of the knee joint, help the patella and the knee joint to stabilize, make the patella correct the wear and pain caused by external dislocation.[31] Finally, the degree of OA symptoms and function limitation can be

significantly improved.

Take the perspective of traditional Chinese medicine, PIPM can play a role in relaxing tendons, easing collateral pain, warming meridians, activating blood circulation, and nourishing joints. It is helpful to strengthen the body's surface defense ability and resist the cold and damp three evils outside the knee joint. PIPM improves blood circulation around the knee joint and the joint capsule by patching the vital energy and meridians. Moreover, the treatment can enhance the nutrient supply and absorption of articular bone, cartilage and soft tissue, and improve the efficiency of cartilage metabolites. Thereby, the degeneration of articular cartilage tissue can be delayed.

CONCLUSION

PIPM promote the repair of articular cartilage and synovial membrane and delay the degeneration of articular cartilage via inhibiting the release of inflammatory cytokines. This may be one of the effective mechanisms of PIPM on KOA. In addition, the curative effects of GH, PIPM and PIPM combined with GH were all detected in the current study, both of PIPM and GH treatment had good therapeutic effects on KOA, and there was no significant difference between them, while the PIPM combined with GH showed the best curative effect on KOA.

LIMITATIONS OF THE STUDY

Despite the above-mentioned findings are promising though further studies focusing on the mechanism of PIPM on the repair and degeneration of articular cartilage via inflammatory cytokines with more advanced techniques need to be conducted.

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CONFLICT OF INTEREST

None to declare.

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Author's Contribution

ZM: Conception and design of study.

XZ, TW, FS: Analysis and data interpretation.

FS, LL, DL: Conception and design of study, drafting of manuscript.

ALL AUTHORS: Approval of the final version of the manuscript to be published.