Cell Biochem Biophys (2011) 61:427–434
DOI 10.1007/s12013-011-9206-4

TRANSLATIONAL BIOMEDICAL RESEARCH

# Effect of Low-Intensity Pulsed Ultrasound on MMP-13 and MAPKs Signaling Pathway in Rabbit Knee Osteoarthritis

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Published online: 13 May 2011


**Abstract** We evaluated the effect of low-intensity pulsed ultrasound (LIPUS) on MMP-13 and MAPKs expression in rabbit knee osteoarthritis (OA). For this purpose, 18 New Zealand white rabbits were randomly and equally divided into O + L, O – L, and SO groups. In O + L group, animals underwent right back leg ACLT operation and LIPUS radiation. In O − L group, animals underwent ACLT but no LIPUS treatment. In SO (control) group, animals underwent sham operation without LIPUS. After 6 weeks, we assessed the pathologic changes in the articular surface of femoral condyle and compared using Mankin scores. Also, expression of type-II collagen, MMP-13, ERK1/2, p38, and JNK was measured by Western blot. Compared with controls, Mankin scores were higher in O + L ($P < 0.05$)/O − L ($P < 0.01$) groups. Compared with O + L group, score was higher in O − L group ($P < 0.05$). Compared with controls, type-II collagen expression was less in O + L/O − L groups, with more significant decrease in O − L group ($P < 0.05$). Contrarily, expression of MMP-13, p-ERK1/2, and p-p38 was enhanced in O + L/O − L groups as compared with controls, with more significant increase in O − L group ($P < 0.01$). Compared with O + L group, expression was higher in O − L group ($P < 0.05$). We, therefore, concluded that LIPUS application promoted cartilage repair in OA through the down-regulation of MMP-13, ERK1/2, and p38.

**Keywords** Osteoarthritis · Articular cartilage · Rabbit · LIPUS · MMP-13 · MAPK · ACLT

## Introduction

Osteoarthritis (OA) is a common chronic degenerative joint disease. Joint pain, stiffness, dysfunction, and different degrees of deformity are the clinical manifestations observed in the late stage of the disease which adversely affect the quality of life of the patients [1]. In the United States, about 13% of the population aged 60 years or over lives with symptomatic knee OA and faces the risk of loss of function, i.e., walking or climbing stairs require assistance [2]. The risk of loss of function caused by OA is as high as caused by cardiovascular disease and is higher than other age-related diseases [3]. Although the targeted organs of OA are subject to the synovium, the loss of articular cartilage is the main feature of the disease [4]. The articular cartilage is composed of extracellular matrix and a small amount of cartilage cells. The extracellular matrix of cartilage is mainly composed of proteoglycan and collagen, whereas collagen type II accounts for 90% of collagen contents. The excessive protease hydrolysis of extracellular matrix of cartilage is the key step involved in destructive process caused by OA [5]. In this regard, early stage OA can cause loss of the collagen network in extracellular matrix of cartilage, leading to the increased destruction of collagen fibers [6].

Matrix metalloproteinases (MMPs) are the most important proteolytic system for the degradation of extracellular

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matrix [7]. MMP-13 (collagenase-3) can be produced by human cartilage cells and can degrade almost all components of the extracellular matrix, especially collagen type II [8, 9]. MMP-13 is the most important enzyme involved in the process of degradation of collagen in cartilage cells [10]. MMP-13 expression is regulated by mitogen-activated protein kinases (MAPKs) and nuclear factor-$\kappa$B (NF-$\kappa$B) signaling pathways. MAPKs including extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinase (JNK), and p38 are highly activated during the inflammation process [11–13]. A study in animal model showed that MMP-13 and ERK1/2 play an important role during the pathophysiologic process of OA [14].

How to delay the cartilage necrosis and promote cartilage regeneration in OA patients have been a research hot spot. Limited by the poor self-regeneration of articular cartilage during injury, the current treatments could not significantly improve the progress of OA such as medications, joint injection, physical therapy, and surgical treatment. Low-intensity pulsed ultrasound (LIPUS) is a newly approved physical therapy in the field of plastic surgery and is used to promote bone healing [15]. LIPUS was described to enhance endochondral bone formation by inducing the proliferation of chondrocytes [16]. The previous studies found that LIPUS could promote the morphological and histological features of interface formation in the damaged cartilage [17] and autologous chondrocyte transplantation [18]. LIPUS positively regulated proteoglycan synthesis [19] and promoted cartilage formation [20]. Studies also show that LIPUS probably functions through MAPKs, JNK, p38, and ERK [21] or phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling pathways [22]. Although studies reported that LIPUS reduces MMP-13 expression in chondrocytes [23], the effect of LIPUS on the expression of MMP-13 and the related signaling pathways remain poorly understood and require further investigation.

Herein, we analyzed the expression of MMP-13 and the activation of MAPKs signalling pathways including p38, ERK 1/2, and JNK as well as evaluated the role of LIPUS on the regeneration of articular cartilage using the rabbit knee OA model with the intent of finding whether LIPUS inhibits the expression of MMP-13 in articular cartilage chondrocytes and this effect is exerted through the inhibition of p38 and ERK1/2 activation.

## Materials and Methods

### Experimental Animals and Grouping

Eighteen New Zealand white healthy rabbits, male or female, weighing 2.5–3.0 kg, were purchased from the Experimental Animal Center of Jiangsu Provincial Academy of Agricultural Sciences. All animals were housed individually in cages under 12-h day and night cycle with free access to food and water. The experimental protocol was in accordance with the NIH guidelines of laboratory animals and approved by the Nanjing First Hospital affiliated to Nanjing Medical University Ethics Committee.

The rabbits were randomly and equally divided into three groups named operation plus LIPUS (O + L) group; operation minus LIPUS, i.e., LIPUS sham irradiation (O − L) group; and sham operation (SO) group. The rabbits in O + L group underwent right back leg anterior cruciate ligament transection (ACLT) and were subjected to LIPUS treatment from the 3rd postoperative day (once per day, 6 times per week for 6 weeks); the rabbits in O − L group underwent right back leg ACLT with sham LIPUS starting from the 3rd postoperative day; and the rabbits in SO group underwent sham surgery, i.e., only an incision was made into the right knee joint capsule and the anterior cruciate ligament was not transacted.

### Surgical Procedures

Our surgical procedure was modified from OA animal model ACLT method described previously [24]. Rabbits were anesthetized by 3% sodium pentobarbital administered intravenously at the arte of 1 ml/kg body weight. After shaving the knee joint, the skin was disinfected with iodine and a parapatellar skin incision was made on the medial side of the joint. An incision on the medial side of the patellar tendon provided access to the joint space after which the patella was dislocated laterally with the leg in extension. The anterior cruciate ligament was transected using eye scissors. A positive anterior drawer test ensured complete transaction of the ligament. After relocation of the patella, the wound was closed with vicryl 4/0 braided absorbable suture. The skin was closed with two staples, and penicillin and fentanyl were given to prevent bacterial infection and pain. Care was taken to keep the operation area moist with physiological saline and the procedures were performed using a surgical microscope. In the sham operation group, the anterior cruciate ligament was not transacted.

### Post-operative Intervention

In O + L group, the HT2009-1 low-intensity pulsed ultrasound (Ito Corporation, Japan) was applied as follows: FREE mode, on–off ratio of 20%, frequency of 3 MHz, irradiation intensity of 40 mW/cm$^2$, irradiation time of 20 min, and treatment frequency was 1 time/day, 6 days/week for 6 weeks. For sham LIPUS treatment of O − L group, the exposure intensity, time, and duration were the same as for O + L group but no ultrasound output was used.

### Post-therapeutic Analyses

Rabbits were killed after 6 weeks of LIPUS exposure, the knee joint in each animal was immediately opened, and the improvement in femoral condyle articular surface was assessed using Mankin scores as described previously [23]. Further studies were made as below:

#### *Histopathology*

The femoral condyle articular cartilage from knee joint specimens was collected, placed in neutral formalin, and processed for histopathological examination. After slow decalcification in ethylene diamine tetraacetic acid (EDTA) for 3 weeks, samples were embedded in paraffin and microtomed into 4-μm thick sections for microscopic examination. All samples were processed simultaneously. The knee joint cartilage specimens were examined microscopically for pathologic changes including surface irregularities, decreased toluidine blue staining of articular cartilage, formation of cracks, etc. The modified Mankin score (Table 1) was used in evaluating fibrosis, matrix distribution, cartilage loss, and chondrocyte colonization.

**Table 1** Modified Mankin scoring scale

| |
|---|
| Subgroup 1: fibrillation |
| 1. Even surface |
| 2. Uneven surface |
| 3. Fibrillated and fissured within superficial zone only |
| 4. Fissures and erosions extending below the surface zone, without extending beyond the radial zone |
| 5. Fissures and erosions extending into the deeper zone |
| Subgroup 2: matrix distribution |
| 1. Normal staining |
| 2. Moderate loss in staining |
| 3. Severe loss in staining |
| 4. No staining |
| Subgroup 3: chondrocyte loss |
| 1. Loss extending into superficial zone |
| 2. Loss extending into mid-zone |
| 3. Loss extending into radial zone |
| Subgroup 4: chondrocyte cloning |
| 1. No clusters |
| 2. Chondrocyte clusters in superficial zone |
| 3. Chondrocyte clusters in superficial to mid-zone (less than four cells) |
| 4. Chondrocyte clusters of more than four cells located in superficial to mid-zone, or chondrocyte clusters in deeper zone |

Grading was performed separately for the medial femoral condyle, lateral femoral condyle, medial tibial plateau, and lateral tibial plateau. The minimum total score is 4 and the maximum total score is 16

The microscopic evaluations were performed independently by two double-blinded experts.

#### *Western Blot*

The expression of MMP-13, type-II collagen, p38, ERK1/2, and JNK proteins was determined by Western blot. For this purpose, the rabbits were killed by overdose injections of sodium pentobarbital. Under sterile conditions, the femoral condyle articular cartilage in the knee, weighing about 50 g, was pulverized into powder in liquid nitrogen, then the lysis buffer (500 μl) was added and centrifuged at $16{,}060\times g$ for 10 min at 4°C. Cell lysates (40 μg each) were used for sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE). The gel-separated proteins were electroblotted onto nitrocellulose membranes. After blocking with skim milk for 2 h, the membranes were incubated with primary antibodies (goat anti-rabbit monoclonal antibodies; 1:500 dilution; Thermo scientific, USA) against type-II collagen, MMP-13, p-ERK1/2, p-JNK, and p-p38 for overnight at 4°C. Next, the membranes were incubated with peroxidase-conjugated secondary antibody (1:5000 dilution; KeyGEN, China) for 2 h at 37°C and bands were developed through specific chromogenic reaction following the manufacturer's instructions.

### Statistical Analysis

All data were expressed as mean ± standard deviation (SD) values and analyzed using SAS9.1 software. The differences between groups were compared using single-factor analysis of variance (ANOVA). Modified Mankin score was analyzed using Wilcoxin signed rank test. All $P$ values $<0.05$ were considered as statistically significant.

## Results

### Histopathological Observations

After 6 weeks of irradiation, the femoral condylar cartilage samples in three groups were stained with toluidine blue and microscopically examined at ×40 magnification. The histopathological observations are shown in Fig. 1. In O + L group, we observed minor surface cracks in the articular cartilage with moderate loss of staining. In O − L group, we found the apparent articular cartilage fissures, extending from the surface to the depth with significant loss of staining. In SO group, we found that the articular cartilage surface was smooth and evenly stained. At the ×100 magnification, in O + L group, chondrocytes showed slightly abnormal arrangement with decreased superficial cells and formation of cluster. In O − L group,

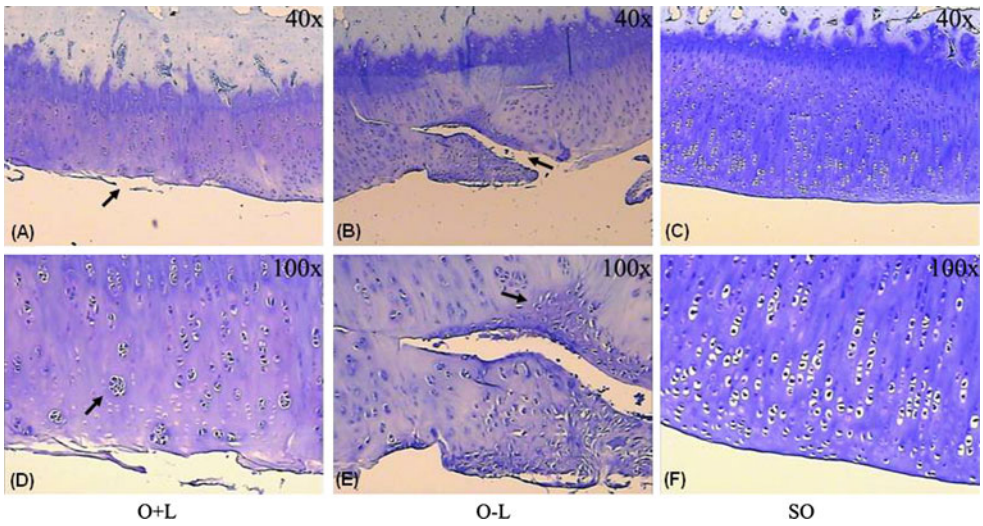


**Fig. 1** Comparison of the femoral condylar cartilage stained with toluidine blue in three groups. The animals in O + L group were subjected to the anterior cruciate ligament transection (ACLT) operation with postoperative low-intensity pulsed ultrasound (LIPUS) irradiation; the animals in O − L group were subjected to ACLT with sham LIPUS; and the animals in SO group were subjected to sham surgery as described in "Materials and methods". **a** The formation of articular cartilage surface cracks (*arrow*) with moderate loss of staining is shown in O + L group; **b** apparent articular cartilage fissures (*arrow*) extending from the surface to deep below as well as significant loss of staining are shown in O − L group; **c** the articular cartilage surface is smooth and evenly stained in SO group; **d** the chondrocytes show slightly abnormal arrangement (*arrow*) with decreased superficial cells and the formation of cluster set in O + L group; **e** cartilage cells are disorganized (*arrow*) and each layer of chondrocytes is reduced and clustered in O − L group; **f** chondrocytes are arranged in SO group

cells were disorganized with reduced number of chondrocytes in each layer and cluster; while in SO group, chondrocytes were arranged in normal order.

## Mankin Score Profile

The Mankin scores after 6 weeks of LIPUS irradiation are summarized in Table 2. As compared with SO group, the scores were significantly increased in O + L ($P < 0.05$) and O − L ($P < 0.01$) groups. The score in O − L group was significantly higher ($P < 0.05$) than that in O + L group. In subgroups 2 and 4, Mankin scores were significantly increased in O + L ($P < 0.05$) and O − L ($P < 0.01$) groups as compared with SO group. The score in O − L group was significantly higher ($P < 0.05$) than that in O + L group. In subgroups 1 and 3, Mankin score was significantly increased ($P < 0.05$) in O − L group as compared with SO group.

## Protein Expression

The expression of select proteins after 6 weeks of irradiation is shown in Fig. 2. Type-II collagen expression was reduced in O + L and O − L groups as compared with SO group; the decrease was significant in O − L group ($P < 0.05$). As though type-II collagen expression was relatively higher in O + L group than in O − L group, the increase was found to be non-significant ($P > 0.05$). MMP-13 expression was increased in O + L and O − L groups as compared with SO group; the increase was significant in

**Table 2** Mankin scores in three groups

| | O + L group Right: operation side | O − L group Right: operation side | SO group Right: operation side |
|---|---|---|---|
| Mankin scores (total scores) | 6.67 ± 1.21*,# | 10.33 ± 2.66** | 4.83 ± 0.75 |
| Subgroup 1 | 2.0 ± 1.26 | 3.17 ± 1.17* | 1.67 ± 0.52 |
| Subgroup 2 | 1.67 ± 0.52*,# | 2.33 ± 0.52** | 1 ± 0 |
| Subgroup 3 | 1.5 ± 0.55 | 1.83 ± 0.41* | 1.17 ± 0.41 |
| Subgroup 4 | 1.5 ± 0.55*,# | 3 ± 1.10** | 1 ± 0 |

* $P$ value of <0.05 (compared with SO group)

** $P$ value of <0.01 (compared with SO group)

# $P$ value of <0.05 (compared with O − L group)

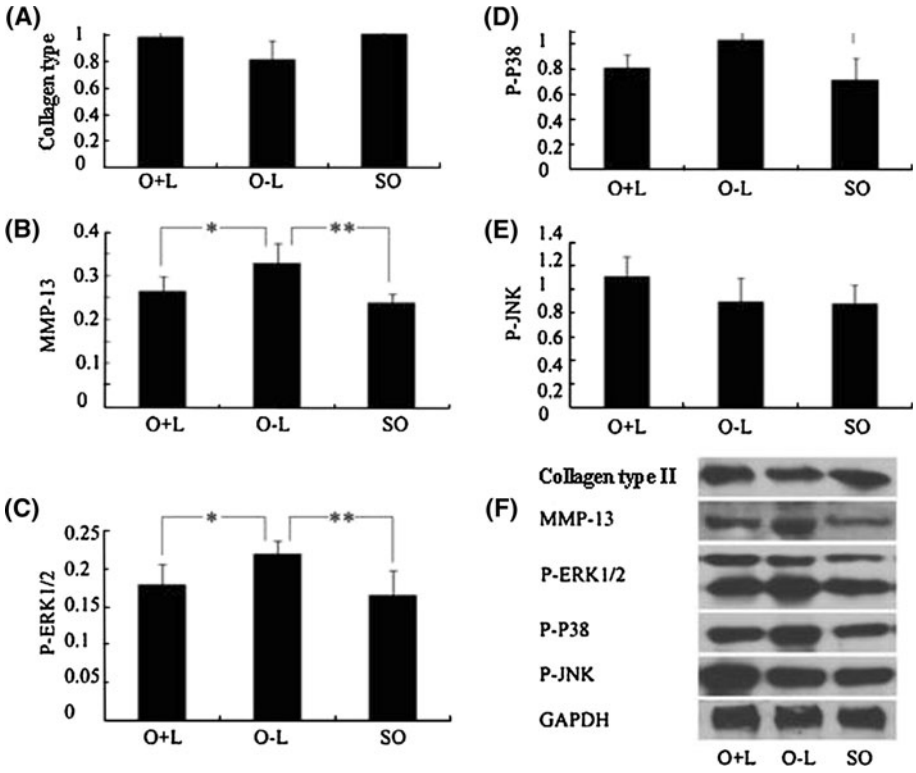


**Fig. 2** Comparative expression of type-II collagen, MMP-13, and MAPK family proteins in three groups. O + L group underwent surgery followed by LIPUS irradiation; O − L group underwent surgery followed by sham irradiation; and SO group underwent sham operation as described in "Materials and methods". **a** Compared with SO group, type-II collagen expression was reduced in both O + L and O − L groups, and the decrease was significant in O − L group ($P < 0.05$) while there was no significant difference between O + L and O − L groups; **b** compared with SO group, MMP-13 expression was enhanced in both O + L and O − L groups, and the increase was significant in O − L group ($P < 0.01$). However, MMP-13 expression in O + L group was significantly reduced as compared with O − L group ($P < 0.05$); **c** compared with SO group, p-ERK1/2 expression was enhanced in both O + L and O − L groups, and the increase was significant in O − L group ($P < 0.01$). However, p-ERK1/2 expression in O + L group was significantly reduced as compared with O − L group ($P < 0.05$); **d** compared with SO group, p-p38 expression was enhanced in both O + L and O − L groups, and the increase was significant in O − L group ($P < 0.01$). However, p-p38 expression in O + L group was significantly reduced as compared with O − L group ($P < 0.05$); **e** there was no significant difference in p-JNK expression between O + L, O – L, and SO groups; **f** The representative Western blot from three independent determinations is shown. Cell lysates from O + L, O – L, and SO groups were analyzed by SDS–PAGE for the expression of collagen type-II, MMP-13, ERK1/2, p-38, and JNK whereas GAPDH expression was used as internal control for sample loading. The ratio between target and housekeeping gene was used to normalize the data for comparison

O − L group ($P < 0.01$). As well, MMP-13 expression was significantly higher in O − L group as compared with O + L group ($P < 0.05$). Similarly, the expression of ERK1/2 and p38 proteins was increased in both O + L and O − L groups as compared with SO group; the increase was significant in O − L group ($P < 0.01$). While the expression of p-ERK1/2 and p-p38 in O − L group was significantly higher than that in O + L group ($P < 0.05$). Interestingly, p-JNK expression was found to be unchanged among O + L, O – L, and SO groups.

## Discussion

The use of animal models in the study of pathogenesis, progression, and intervention strategies of knee OA has proved effective and essential. The commonly used animal models include mice, dogs, rabbits, and guinea pigs. Among them, guinea pigs [25, 26] and transgenic mice [27, 28] were mainly used to study the natural course of knee OA while rabbits, dogs, and rats were mainly used in studies of induced OA through drugs or surgical methods such as papain injections in rats [29], canine meniscectomy [30], transection of the anterior cruciate ligament [31, 32], rabbit meniscectomy [33], or estrogen (17$\beta$-estradiol) injections in rats [34]. In ACLT-induced rabbit OA model, the damage of articular cartilage is relatively mild, similar to the early and middle stages of OA. This model is particularly good for studies of pathogenesis and drug treatment of OA. The compression factor (confined compression modulus) and glycosaminoglycan (GAG) density are decreased significantly in the ACLT rabbit knee cartilage model.

Besides, changes in the physical properties of articular cartilage are similar to those observed in human OA. Moreover, in the 6–24 weeks post-surgical follow-up, the changes such as fibrosis of articular cartilage, loss of full-thickness articular cartilage and osteophyte formation can be observed in this rabbit ACLT model [35]. The imaging changes in ACLT-treated side articular cartilage or meniscus can be detected by ultrasound [36], microtomography (Micro-CT), or magnetic resonance (MR) [37]. In this study, we used ACLT method to establish the animal model of OA. Consequently, we observed the surface irregularities, loss of staining, and formation of surface cracks in the operated side of femoral condyle which indicated that the intermediate stage of knee OA was established. Besides, Mankin score changes also confirmed the OA-associated pathology of femoral condyle in our rabbit model.

Type-II collagen is the main component of the fiber network structure of articular cartilage. Its functions include the maintenance of spatial structure of proteoglycan and anti-tensile shear strength. Proteoglycans are glycoproteins that are richly glycosylated. The basic unit of proteoglycan is composed of a core protein having one or more covalently attached GAG chains. Proteoglycans are involved in binding cations (like $Na^+$, $K^+$, and $Ca^{++}$) and water, and also regulate molecular movement through the extracellular matrix. Proteoglycan is resistant to the extrusion force, and combined with water, it results in swelling pressure [1]. It was shown that [38] injuries to the ligament and meniscus could cause load changes inside the joints, leading to the rapid destruction of cartilage matrix. This type of damage includes the degeneration of proteoglycan and type-II collagen which induces loss of fiber network structure of articular cartilage. These morbid changes can occur during the early stages of OA [39]. In the pathologic process of OA, the degeneration of proteoglycan is mainly related to MMP-3 and aggrecanase-1/a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADMSTS 4) while the degeneration of type-II collagen is mainly affected by MMP-13 [10, 11, 40]. Previously, it was shown [41] that in the ACLT-induced rabbit knee OA model, the expression of MMP-13 was significantly increased in the knee cartilage even in the early stage of operation. Whereas another study [23] demonstrated that in the early stage of OA in the guinea pig model, LIPUS irradiation could decrease the expression of MMP-13 but not that of MMP-3. In our study, we started to apply LIPUS radiation from the third day of ACLT operation. After 6 weeks of irradiation therapy, the results showed that compared with SO group, MMP-13 expression was significantly increased in O − L group, while the increase in O + L group was not as high as observed in O − L group. Compared with O − L group, the degree of proteoglycan staining loss was significantly low in O + L group. However, type-II collagen expression increased only slightly and there was no significant difference between O + L and O − L groups, indicating that the proteoglycan metabolism might be affected by LIPUS during the initial stage of rabbit knee OA.

The expression of MMP-13 is regulated by MAPKs, NF-$\kappa$B, and Smad2/3 signaling pathways [42, 43]. Among them, ERK1/2, JNK, and p38 [11–13] are mainly involved in signaling transduction during the inflammatory process. It was reported that [43] in rat endplate chondrocytes, PI3K pathway was involved in the expression of type-II collagen while ERK pathway was involved in the expression of MMP-13. The pomegranate fruit extract (PFE) was reported [44] to inhibit the expression of MMP-13 in the OA cartilage via NF-$\kappa$B and JNK/p38. In IL-1beta-induced human OA chondrocytes, four inhibitors of p38-MAPK called Birb 796, pamapimod, SB203580, and CBS-3868 reduced the gene expression of MMP-13 [45]. Using ACLT + MMT-induced rabbit OA model, it was shown that [15] MMP-13 and ERK levels were significantly increased even in early stage. In regard to the effect of LIPUS treatment, it was demonstrated that [46] in C-28/I2 human chondrocyte cell line, LIPUS increased the expression of type-II collagen and proteoglycan via the activation of JNK and ERK signaling pathways. However, the effect of LIPUS on the expression of MMP-13 in OA cartilage is unclear. In our study, we observed the effect of LIUPS on MAPK signaling pathway in the rabbit OA model. Our data show that the expression of ERK and p38 was less in O + L group than in O − L group; however, the expression in both former groups was higher than that in SO group. Importantly, these changes were consistent with the modulations in MMP-13, type-II collagen and proteoglycan levels. Of note, we could not detect any change in the JNK expression. These results suggest that in the early stage of OA, the application of LIPUS can delay the degeneration of articular cartilage by decreasing the degradation of type-II collagen and proteoglycan which can be related, at least in part, to the reduced expression of MMP-13, ERK1/2, and p38. However, our results show that LIPUS does not affect the expression of JNK which is discordant with the previous studies. We believe that this disparity may be due to the reason that most of these studies measured MMPs expression in vitro whereas we determined their expression in vivo and, most likely, differences exist with regard to chondrocytes microenvironments between the two experimental systems.

In conclusion, we demonstrated that in ACLT-induced rabbit knee OA model, the early application of LIPUS could delay the degeneration of articular cartilage. This effect was related to the decreased expression of MMP-13 and suppression of ERK1/2, p38 signaling. However, due to the difficulty in applying the relevant signal transduction

pathway inhibitors, we could not directly show that the decreased MMP-13 expression by LIPUS was via the ERK1/2 and p38 signaling pathways. Moreover, since the clinical application of LIPUS in knee OA has not yet started, our follow-up part of this study will further elucidate the underlying mechanism of LIPUS therapy in OA.

**Acknowledgments** We thank Dr. Huang Wenbin and Mr. Wang Jinsong from Department of Pathology, Nanjing First Hospital affiliated to Nanjing Medical University for generous help with tissue production and scoring. We also thank: (1) National Key Program for Developing Basic Research (Grant # 2007CB936104); (2) National High Technology Research and Development Program of China (Grant # 2007AA021905); and (3) Science & Technology Support Project of Jiangsu Province (Grant # BE2010697) for financial support.

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