Objectives—To investigate whether low-intensity pulsed ultrasound (US) has different protective effects on early and late rabbit osteoarthritis cartilage via the integrin/focal adhesion kinase (FAK)/mitogen-activated protein kinase (MAPK) signaling pathway.

Methods—Thirty-six New Zealand White rabbits were divided into early control, early osteoarthritis, early treatment, late control, late osteoarthritis, and late treatment groups. The early and late osteoarthritis and treatment groups underwent anterior cruciate ligament transection. The remaining groups underwent sham operations with knee joint exposure. The early and late treatment groups were exposed to low-intensity pulsed US 4 and 8 weeks after surgery. After 6 weeks of US exposure, pathologic changes on the articular surface of the femoral condyle were assessed by modified Mankin scores. Expression of type II collagen, matrix metalloproteinase, integrin \( \beta_1 \), phosphorylated FAK, and MAPKs (including extracellular signal-regulated kinase 1/2, MAPK 38, and c-Jun N-terminal kinase) was assessed by Western blot analysis.

Results—Cartilage damage was less severe in the early treatment group than the early osteoarthritis group. The Mankin score was significantly lower in the early treatment group than the early osteoarthritis group (\( P < .05 \)). There was no significant difference in cartilage damage or Mankin score between the late treatment and late osteoarthritis groups. There was a significant increase in type II collagen expression but a significant decrease in matrix metalloproteinase 13 expression in the early treatment group compared to the early osteoarthritis group, whereas no significant difference was found between the late treatment and late osteoarthritis groups. Integrin \( \beta_1 \) and phosphorylated FAK expression was significantly higher, and phosphorylated extracellular signal-regulated kinase 1/2 and phosphorylated MAPK 38 expression was significantly lower in the early treatment group than the early osteoarthritis group.

Conclusions—Our findings indicate that low-intensity pulsed US protects cartilage from damage in early-stage osteoarthritis via the integrin/FAK/MAPK pathway.

Key Words—focal adhesion kinase; integrin; low-intensity pulsed ultrasound; mitogen-activated protein kinase; musculoskeletal ultrasound; osteoarthritis

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Abbreviations
Akt, protein kinase B; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; p38, mitogen-activated protein kinase 38; PI3K, phosphatidylinositol 3-kinase; US, ultrasound


Osteoarthritis is a multifactor-related degenerate disease whose main feature is irreversible articular cartilage destruction.
Articular cartilage is composed of an extracellular matrix and a few chondrocytes. The extracellular matrix consists of affluent aggrecan and collagen, with type II collagen being the most abundant collagen and a typical marker of chondrocytes. Matrix metalloproteinase 13 (MMP-13), an important MMP, hydrolyzes the extracellular matrix, especially type II collagen. Extracellular matrix hydrolysis is a crucial step leading to irreversible loss of the chondrocyte extracellular matrix during cartilage degeneration. Therefore, anabolism of the extracellular matrix is of importance for maintenance of cartilage integrity.

Moderate mechanotransduction causes a series of changes in specific intracellular molecules and thereby their downstream signaling pathways by stimulation of mechanical loading, acceleration of chondrocyte differentiation and proliferation, and promotion of extracellular matrix anabolism to maintain articular cartilage integrity. Mechanical stress by low-intensity pulsed ultrasound (US) produces a certain kind of mechanical wave to activate osteoblasts and promote bone formation and strength. Low-intensity pulsed US promotes type II collagen synthesis for chondrocyte proliferation and cartilage formation.

Integrin, a heterodimeric glycoprotein composed of different α and β subunits, is one of the mechanical receptors on the cell surface, bridging signaling pathways from the extracellular matrix to cells. Of note, integrin α1β1 is the most common integrin on the adult chondrocyte cell membrane, and integrin β1, mainly expressed on the chondrocyte membrane, increases chondrocyte differentiation and maturation to promote cartilage formation and remodeling via regulation of extracellular matrix synthesis.

Integrin mediates the mechanotransduction pathway by activating a crucial adhesive molecule, focal adhesion kinase (FAK), which activates FAK downstream signaling pathways, such as mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) pathways. The MAPK signaling pathway, which includes extracellular signal-regulated kinase 1/2 (ERK1/2), mitogen-activated protein kinase 38 (p38), and c-Jun N-terminal kinase (JNK), plays important roles in regulating the secretion of MMPs in chondrocytes. Upregulation of this signaling pathway affects the normal extracellular matrix synthesis, causing cartilage damage. It was suggested in our previous studies that low-intensity pulsed US can promote osteoarthritis cartilage repair by downregulation of chondrocyte MMP-13 and p38 MAPK signaling pathways, and that low-intensity pulsed US protects osteoarthritic chondrocytes by downregulation of the integrin/Akt pathway.

Although low-intensity pulsed US is effective in protecting osteoarthritic chondrocytes from degeneration by downregulation of either the MAPK or PI3K/Akt pathway and healing bone fractures, using low-intensity pulsed US to repair cartilage damage is still limited clinically. Furthermore, osteoarthritis is a chronic and progressive disease; thus, choosing a correct stage for low-intensity pulsed US treatment is necessary. However, to our knowledge, there has been no study comparing the effect of low-intensity pulsed US on early and late osteoarthritis. Also, the effect of low-intensity pulsed US on osteoarthritic cartilage via the integrin/FAK/MAPK signaling pathway is not understood. The aim of this study was to investigate whether low-intensity pulsed US has different protective effects on early and late osteoarthritis via the integrin/FAK/MAPK signaling pathway in cartilage repair.

Materials and Methods

Reagents

Phosphate-buffered saline, a total protein extraction kit, and an enhanced chemiluminescence kit were purchased from KeyGEN (Nanjing, China). Mouse antirabbit monoclonal antibodies against type II collagen, MMP-13, integrin β1, FAK, phosphorylated FAK, ERK1/2, phosphorylated ERK1/2, p38, phosphorylated p38, JNK, phosphorylated JNK, and β-actin were purchased from Acris (Herford, Germany). Goat antimouse (Fab) secondary antibody was purchased from Santa Cruz Biotechnology (Dallas, TX).

Experimental Animals and Grouping

Thirty-six 2-month-old healthy male New Zealand white rabbits weighing 2.5 to 3.0 kg were purchased from the Qinglongshan Experimental Animal Center (Nanjing, China). All animals were housed individually in cages under a 12-hour day and 12-hour night cycle with free access to food and water. The experimental protocol was in accordance with the US National Institutes of Health guidelines for laboratory animals and approved by the Nanjing Medical University Ethics Committee of Nanjing Hospital.

The rabbits were randomly and equally divided into 6 groups, named early control, early osteoarthritis, early treatment, late control, late osteoarthritis, and late treatment. Rabbits in the early osteoarthritis, early treatment, late osteoarthritis, and late treatment groups underwent anterior cruciate ligament transection. The remaining groups underwent sham operations with knee joint exposure. The early and late treatment groups were exposed to low-intensity pulsed US 4 and 8 weeks after surgery.
Surgical Procedures
Anterior cruciate ligament transection of the rabbit osteoarthritis model was performed as described previously.19,20 Briefly, rabbits were intravenously anesthetized with 3% sodium pentobarbital (1 mL/kg). The knee joint skin was disinfected with iodine after the fur was shaved, and a parapatellar skin incision was made on the medial side of the joint. The anterior cruciate ligament was transected with eye scissors, and a positive anterior drawer test result was used to ensure complete transection of the ligament. The patella was relocated, and the wound was closed with 4-0 braided absorbable polyglactin 910 sutures. Penicillin and fentanyl were given to prevent bacterial infection and pain, respectively, after the incised skin was closed. Early and late osteoarthritis was defined according to the cartilage change and Mankin score as in a previous study, and early and late rabbit osteoarthritis models were established after 4 and 8 weeks of anterior cruciate ligament transection, respectively.20,21 The control groups underwent sham operations (the skin was cut and the joint capsule exposed, but the anterior cruciate ligament was not transected). The early and late control groups were established 4 and 8 weeks after sham surgery.

Postoperative Intervention
For the early and late treatment groups, low-intensity pulsed US was applied 4 and 8 weeks after anterior cruciate ligament transection, respectively. Low-intensity pulsed US (HT2009-1; Ito Corporation, Tokyo, Japan) was applied as follows: free mode, on-off ratio of 20%, frequency of 3 MHz, irradiation intensity of 40 mW/cm², irradiation time of 20 minutes, and treatment frequency of once per day at 6 d/wk for 6 consecutive weeks. For the sham early and late osteoarthritis groups, the exposure intensity, time, and duration were the same as for the early and late treatment groups, but without US output.

Post-Therapeutic Analysis
Rabbits were euthanized after US exposure for 6 weeks, and the knee joint in each animal was immediately opened. Improvement in the femoral condyle articular surface was assessed by Mankin scores as described previously.22

Histopathologic Analysis
The femoral condyle articular cartilage collected from the knee joints was fixed in neutral formalin, decalcified in EDTA for 3 weeks, embedded in paraffin, and divided into 4-μm-thick sections with a microtome. All samples were processed simultaneously. The knee joint cartilage specimens were examined under a microscope for pathologic changes, including surface irregularities, decreased hematoxylin-eosin staining of articular cartilage, and formation of cracks. Fibrosis, matrix distribution, cartilage loss, and chondrocyte colonization were evaluated in a double-blind fashion by 2 independent experts using the Mankin score system (Table 1).

Western Blot Analysis
Expression of type II collagen, MMP-13, integrin β₁, FAK, phosphorylated FAK, p38, phosphorylated p38, ERK1/2, phosphorylated ERK1/2, JNK, phosphorylated JNK, and β-actin proteins was determined by Western blot analysis. Briefly, the rabbits were euthanized by overdose injections of sodium pentobarbital. Under sterile conditions, the femoral condyle articular cartilage in the knee (≈50 g), was pulverized into powder in liquid nitrogen; then the lysis buffer in the protein extraction kit (500 μL) was added, and samples were centrifuged at 16,000g and 4°C for 10 minutes. The cell lysates (40 μg each) were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the separated proteins were electroblotted onto nitrocellulose membranes. After blocking with skim milk for 2 hours, the membranes were incubated with primary antibodies against type II collagen (1:500), MMP-13 (1:500), integrin β₁ (1:500), FAK (1:500), phosphorylated FAK (1:500), ERK1/2 (1:1000), phosphorylated ERK1/2

<table>
<thead>
<tr>
<th>Table 1. Mankin Scoring Scale</th>
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<tbody>
<tr>
<td>Subgroup 1: fibrillation</td>
</tr>
<tr>
<td>1. Even surface</td>
</tr>
<tr>
<td>2. Uneven surface</td>
</tr>
<tr>
<td>3. Fibrillated and fissured within superficial zone only</td>
</tr>
<tr>
<td>4. Fissures and erosions extending below surface zone, without extending beyond radial zone</td>
</tr>
<tr>
<td>5. Fissures and erosions extending into deeper zone</td>
</tr>
<tr>
<td>Subgroup 2: matrix distribution</td>
</tr>
<tr>
<td>1. Normal staining</td>
</tr>
<tr>
<td>2. Moderate loss in staining</td>
</tr>
<tr>
<td>3. Severe loss in staining</td>
</tr>
<tr>
<td>4. No staining</td>
</tr>
<tr>
<td>Subgroup 3: chondrocyte loss</td>
</tr>
<tr>
<td>1. Loss extending into superficial zone</td>
</tr>
<tr>
<td>2. Loss extending into mid zone</td>
</tr>
<tr>
<td>3. Loss extending into radial zone</td>
</tr>
<tr>
<td>Subgroup 4: chondrocyte cloning</td>
</tr>
<tr>
<td>1. No clusters</td>
</tr>
<tr>
<td>2. Chondrocyte clusters in superficial zone</td>
</tr>
<tr>
<td>3. Chondrocyte clusters in superficial to mid zone (&lt;4 cells)</td>
</tr>
<tr>
<td>4. Chondrocyte clusters of &gt;4 cells located in superficial to mid zone or chondrocyte clusters in deeper zone</td>
</tr>
</tbody>
</table>

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

Pathologic Changes in Early and Late Osteoarthritis Cartilage Before and After Low-Intensity Pulsed US Exposure

After 6 weeks of low-intensity pulsed US exposure, osteoarthritis cartilage was harvested for hematoxylin-eosin staining and then observed under a microscope to compare the morphologic differences and Mankin scores among the different groups. The results showed that the cartilage surface in the early and late control groups was regular and even, with no obvious cartilage fibrosis and a well-stained extracellular matrix. Chondrocytes were evenly arrayed and well organized within the extracellular matrix. In the early osteoarthritis group, the surface was slightly uneven and irregular; cartilage fibrosis was slight; staining was slightly to moderately lost; chondrocytes were disarranged; and the number of the chondrocytes was reduced compared to the early control group. In the late osteoarthritis group, there were some obvious disruptions on the surface of the cartilage, a severe loss of staining, severe cartilage fibrosis, and decreased chondrocytes compared to the early osteoarthritis group. After low-intensity pulsed US exposure, a slightly uneven cartilage surface and chondrocyte proliferation in the early treatment group were observed, but in the late treatment group, cartilage damage was almost the same as that in the late osteoarthritis group (Figure 1).

The Mankin score in the early treatment group was much higher than that in the early control group ($P < .05$) but was obviously lower than that in the early osteoarthritis group ($P < .05$). There was no difference in Mankin scores between the late treatment and late osteoarthritis groups ($P > .05$), but both scores were significantly higher than that in the late control group ($P < .05$; Figure 1).

Type II Collagen, MMP-13, and Integrin β1 Expression and Phosphorylation Level of FAK Before and After Low-Intensity Pulsed US Exposure in Early and Late Osteoarthritis Cartilage

Compared to the early osteoarthritis group, type II collagen expression in the early treatment group was significantly increased ($P < .05$), but MMP-13 expression was significantly decreased ($P < .05$). No significant difference in the expression of the two proteins was found between the late treatment and late osteoarthritis groups ($P > .05$). The integrin β1 and phosphorylated FAK levels were significantly higher in the early treatment group than the early osteoarthritis and early control groups ($P < .05$), whereas the integrin β1 and phosphorylated FAK levels in the late treatment and late osteoarthritis groups were significantly decreased ($P < .05$). No significant difference in integrin β1 or phosphorylated FAK expression was found between the late treatment and late osteoarthritis groups ($P > .05$; Figure 2).

Phosphorylated ERK1/2, JNK, and p38 Expression Before and After Low-Intensity Pulsed US Exposure in Early and Late Osteoarthritis Cartilage

Phosphorylated ERK1/2 and p38 expression in the early treatment group was significantly lower compared to the early osteoarthritis group ($P < .05$). Compared to the late control group, phosphorylated ERK1/2 and p38 expression in the late osteoarthritis and late treatment groups was significantly increased ($P < .05$), but no significant difference in the expression of the two phosphorylated proteins was found between the late osteoarthritis and late treatment groups ($P > .05$). There was no significant difference in phosphorylated JNK expression between early and late osteoarthritis cartilage before and after US exposure ($P > .05$; Figure 3).

Discussion

It is important to better understand the signaling pathway whereby a correct low-intensity pulsed US intervention time for osteoarthritis therapy can be implemented; however, to our knowledge, there has been no study of the integrin/FAK/MAPK signaling pathway by which low-intensity pulsed US acts on both early and late osteoarthritis. In this study, different protective effects of low-intensity pulsed US on early and late osteoarthritis via the integrin/FAK/MAPK signaling pathway by which low-intensity pulsed US acts on both early and late osteoarthritis models by anterior cruciate ligament transection. Early low-intensity pulsed US treatment was
found to prevent cartilage damage, especially at the early stage of osteoarthritis, by increasing type II collagen and decreasing MMP-13 levels and upregulating integrin β1 and phosphorylated FAK and downregulating phosphorylated ERK1/2 and p38 in the signaling pathway.

Our findings demonstrate that the low-intensity pulsed US/integrin/FAK/MAPK mechanotransduction pathway plays an important role during the pathologic process of osteoarthritis by regulating extracellular matrix expression in early and late osteoarthritis cartilage. Type II

**Figure 1.** Morphologic changes and Mankin scores for early- and late-stage osteoarthritis cartilage in each group. Morphologic changes were revealed by hematoxylin-eosin staining and observed under a microscope: EC indicates early control group; EO, early osteoarthritis group; ET, early treatment group; LC, late control group; LO, late osteoarthritis group; and LT, late treatment group (n = 6 per group). *P < .05.
collagen expression was found to be significantly increased, but MMP-13 expression was significantly decreased after application of low-intensity pulsed US in early osteoarthritis cartilage. However, in late osteoarthritis cartilage, US application had no effects. Type II collagen is an important extracellular matrix component and maintains cartilage integrity; MMP-13 is an important inflammatory factor in the MMP family that degrades the type II collagen components, which destroys joint cartilage, causing osteoarthritis.

Western blot results showed higher expression of MMP-13, integrin β1, phosphorylated FAK, ERK1/2, JNK, and p38 in the early osteoarthritis group compared to the early control group. In the late osteoarthritis group, integrin β1 and phosphorylated FAK expression was at a lower level, and phosphorylated ERK1/2, JNK, and p38 expression was at a higher level compared to the late control group. These results demonstrate that the variable expression levels of proteins in the integrin/FAK/MAPK signaling...
pathway are closely related to pathologic changes in early and late osteoarthritic cartilage. In fact, integrin \( \beta_1 \), which is the major subunit of integrin in chondrocyte membranes,\(^{26}\) plays an important role in transmitting mechanical and chemical signals through signaling pathways in cartilage.\(^{11}\) Integrin \( \beta_1 \) is involved in the process that regulates proliferation, differentiation, extension, and migration of chondrocytes, thus providing cartilage-protective effects.\(^{27,28}\) Focal adhesion kinase is a vital mediator in the integrin/FAK/MAPK mechanotransduction pathway.\(^{29}\) Phosphorylated FAK regulates proliferation and differentiation of chondrocytes and type II collagen expression.\(^{30}\) Furthermore, phosphorylated FAK inhibits MAPK signaling pathway proteins, including ERK1/2, JNK, and p38.\(^{31}\)

Mitogen-activated protein kinases are associated with extracellular matrix synthesis and cartilage stabilization,\(^{32,33}\) and upregulation of the MAPK signaling pathway leads to cartilage dysfunction, accelerating the disease development process.\(^{34}\) Extracellular signal-regulated kinase 1/2, JNK, and p38 are related to chondrosteosis and play an important role in hypertrophy, calcification, and apoptosis of chondrocytes.\(^{14,28}\) Phosphorylated ERK1/2, JNK, and p38 have a negative impact on cartilage extracellular matrix regulation and are extensively involved in signal transduction and cartilage degeneration.\(^{35}\)

We found that exposure to early low-intensity pulsed US treatment promoted integrin \( \beta_1 \) and phosphorylated FAK expression and downregulation of phosphorylated ERK1/2, JNK, and p38.\(^{31}\) The data supports the hypothesis that low-intensity pulsed US treatment can be an effective therapeutic approach for early osteoarthritis.

**Figure 3.** Phosphorylated (p) ERK1/2, p38, and JNK expression before and after low-intensity pulsed US exposure in early- and late-stage osteoarthritis cartilage. A. Western blot analysis of total and phosphorylated ERK1/2, p38, and JNK. B. Quantitative analysis of phosphorylated ERK1/2 with total ERK1/2 as a loading control. C. Quantitative analysis of phosphorylated p38 with total p38 as a loading control. D. Quantitative analysis of phosphorylated JNK with total JNK as a loading control. Abbreviations are as in Figure 1. *\( P < .05 \).
ERK1/2 and p38 expression, leading to substantial protective effects against cartilage damage. In contrast, late low-intensity pulsed US treatment downregulated integrin β1 and upregulated phosphorylated FAK, ERK1/2, and p38 expression slightly. These changes had no statistical significance but suggested that late low-intensity pulsed US treatment did not provide remarkable protective effects against cartilage damage and may have had negative effects on late-stage osteoarthritis. These findings are in line with previous studies in which low-intensity pulsed US was found to increase integrin β1 expression, induce phosphorylated FAK, trigger ERK1/2 and p38 signaling pathways, and promote type II collagen synthesis.6,36,37

In conclusion, cartilage-protective effects from early low-intensity pulsed US treatment of osteoarthritis via the integrin/FAK/MAPK signaling pathway is time sensitive, and optimal intervention timing may be important for protecting or even repairing osteoarthritis cartilage. As low-intensity pulsed US is effective in protecting osteoarthritis chondrocytes from degeneration by upregulation of the PI3K/Akt pathway,17 the question of whether low-intensity pulsed US functions by upregulation of the PI3K/Akt pathway and downregulation of the MAPK pathway at the same time should be investigated in the future. Currently, low-intensity pulsed US is still not well accepted for treating osteoarthritis in clinical practice. Our findings may provide further evidence for the application of low-intensity pulsed US to clinical therapy, especially at an early stage of osteoarthritis.

References