Mechanical loading mitigates osteoarthritis symptoms by regulating the inflammatory microenvironment

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Abbreviations: AKT, Protein Kinase B; CC/TAC, ratio of calcified cartilage to total articular cartilage; IL-1β, interleukin-1 beta; IFN-γ, interferon-γ; LPS, lipopolysaccharides; MMPs, metalloproteinases; M-CSF, macrophage-colony stimulating factor; NF-κB, nuclear factor Kappa-B; PI3K, phosphatidylinositol-3-kinase; OARSI, osteoarthritis research society international. TNF-α, tumor necrosis factor-α.

Abstract: Osteoarthritis (OA) is one of the most common chronic diseases, in which inflammatory responses in the articular cavity induce chondrocyte apoptosis and cartilage degeneration. While mechanical loading is reported to mitigate synovial inflammation, the mechanism and pathways for the loading-driven improvement of OA symptoms remain unclear. In this research, we evaluated the loading effects on the M1/M2 polarization of synovial macrophages via performing molecular, cytology, and histology analyses. In the OA groups, the cell layer of the synovial lining was enlarged with an increase in cell density. Also, M1 macrophages were polarized and pro-inflammatory cytokines were increased. In contrast, in the OA group with mechanical loading cartilage degradation was reduced and synovial inflammation was alleviated. Notably, the polarization of M1 macrophages was diminished by mechanical loading, while that of M2 macrophages was increased. Furthermore, mechanical loading decreased the levels of pro-inflammatory cytokines such as IL-1β and TNF-α and suppressed PI3K/AKT/NF-κB signaling. Consistently, NF-κB inhibited decreased the polarization of M1 macrophages in RAW264.7 macrophages. Taken together, this study demonstrates that mechanical loading changes the ratio of M1 and M2 macrophage polarization via regulating PI3K/AKT/NF-κB signaling and provides chondroprotective effects in the mouse OA model.

Key words: Osteoarthritis, Inflammatory microenvironment, Macrophage polarization, PI3K/AKT/NF-κB signaling, Mechanical loading

1. Introduction

Osteoarthritis (OA) is a whole joint disease that induces structural alterations of the articular cartilage, subchondral bone, synovial membrane and ligaments [1, 2]. It is one of the most common chronic diseases and about 250 million people are currently affected [3]. Its main pathological characteristic is degeneration of the articular cartilage and its repair is particularly important to reduce OA symptoms [4]. The self-repair ability of chondrocytes is limited due to a lack of blood supply and a poor population of stem cells in the cartilage [5]. Thus, an effective regimen is urgently needed to promote the regeneration of articular cartilage.

OA is usually accompanied by inflammatory changes and the inflammatory microenvironment is considered to accelerate chondrocyte apoptosis and cartilage degeneration [6, 7]. Therefore, the regulation of the articular inflammatory microenvironment might promote the repair and regeneration of articular cartilage. Synovitis is characterized by synovial pathologic hyperplasia, proliferation and infiltration of macrophages, lymphocytes and angiogenesis [7, 8]. In physiologic synovial joints, macrophages are usually maintained in a quiescent state. However, in the inflammatory microenvironment of OA, M1 macrophages activate and produce a large number of inflammatory mediators, accelerating chondrocyte apoptosis and cartilage degradation [7, 9]. It is generally considered that M1 macrophages can secrete pro-inflammatory cytokines, while M2 macrophages can downregulate them and stimulate tissue repairing [10]. Previous studies have shown that the M1/M2 polarization of macrophages can dynamically adapt to changes in the

microenvironment, indicating that macrophages are plastic and can differentiate into M1 or M2 types [9]. Recently, the polarization of macrophages has been reported in response to dynamic mechanical loading in different loading modes, amplitudes and frequencies [11, 12].

Because bone is a mechano-sensitive tissue, temperate mechanical loading can be used to various synovial joints via a type of knee loading, elbow loading and ankle loading [13-15]. Our previous works have shown that mechanical loading can effectively reduce synovial inflammatory thickening and prevent cartilage degeneration in OA [16-18]. However, the unanswered question is whether mechanical loading improves the articular inflammatory microenvironment via regulating the macrophage polarization.

Cytokines, produced by M1 macrophages such as IL-1β and TNF-α, are the main pro-inflammatory factors in OA pathogenesis [19, 20]. The levels of IL-1β and TNF-α in OA patients are increased in synovial membrane, synovial fluid, cartilage and subchondral bone [21]. Many studies have shown that many intracellular signaling pathways are involved in OA development and progression. Among them, NF-κB plays a primary character in the induction of cytokines, inflammatory mediators and MMPs in OA [22, 23]. PI3K/AKT signaling is one of the important pathways. Some studies report that its activation is reported to suppress IL-1β-induced apoptosis of chondrocytes and improve articular cartilage damages [22, 24], whereas others show that the inhibition of PI3K/AKT signaling can prevent chondrocyte apoptosis [25, 26]. Exercise training may regulate PI3K/AKT signaling and affect oxidative stress induced

by IL-1β as well as inflammatory responses in chondrocytes [27, 28]. We addressed a question in this study as to whether mechanical loading affects the polarization of macrophages by regulating PI3K/AKT/NF-κB signaling.

We hypothesized that mechanical loading can mitigate articular inflammation by regulating macrophage polarization via PI3K/AKT/NF-κB signaling, thus decreasing articular cartilage injury. To verify the hypothesis, we evaluate daily loading effects used a mouse OA model and determined cartilage damage degree using the OARSI score and evaluated synovial membrane change by histology with H&E staining. We also examined the lelve of IL-1β, TNF-α and other genes involved in PI3K/AKT/NF-κB signaling by western blot analysis, immunohistochemistry and immunofluorescent analyses. Furthermore, bone marrow-derived cells and a murine macrophage cell line (RAW264.7) were used to assay chondrogenesis and evaluate the polarization of macrophages.

2. Materials and methods

2.1 Animals and materials preparation

Sixty 14-week-old C57BL/6 female mice were obtained from the Animal Center of Academy of Military Medical Sciences, China. All animal researches were performed according to the National Institutes of Health Guide for Care and Use of Laboratory Animals and approved by the Ethics Committee of the Tianjin Medical University. M-CSF, recombinant mouse IL-4, bacterial LPS of Escherichia coli and IFN-γ were purchased from PeproTech (Cranbury, NJ, USA). Triptolide (PG490; Catalog No. S3604), an NF-κB inhibitor, was purchased from Selleck Chemicals. The DAB substrate kit and immunohistochemical staining kit were acquired from ZSGB-BIO (Beijing, China). MEM-α, DMEM, fetal bovine serum (FBS), streptomycin, penicillin, and trypsin were obtained from Invitrogen (Carlsbad, CA, USA). Other chemicals were obtained from Sigma (St. Louis, MO, USA) unless other clarifications.

2.2 Experimental design

Sixty mice were randomly divided into three groups with twenty mice in each group. The mice in the control group were treated as the sham group. The OA group received OA surgery on both knees. Group OAL was designated as the OA + Loading group and received OA surgery as well as mechanical loading on two knees. The mechanical loading was administered daily for 2 weeks and mice were sacrificed (Fig. 1A).

2.3 OA surgery

We used a sterile technique and under anesthetic with 1.5% isoflurane (IsoFlo,

Abbott Laboratories, USA) at 1.0 L/min flow rate to perform OA surgery. The bilateral hindlimb was hairless and sterilized befer expose the knee joint by cutting a 20-mm incision. We transected the medial collateral ligament and opened the articular cavity to remove the medial meniscus. Then we washed surgery site use sterile saline to remove tissue debris, and closed the incision[16].

2.4 Mechanical loading

Mechanical loading was performed to both knees for 2 weeks (Fig. 1B, C) [17]. Dynamic loads were sinusoidal at 1 N (peak-to-peak) and the frequency was 5 Hz for 6 min/day (each knee, 3 min). The sham group and the OA group were given sham loading without any dynamic force.

2.5 Histological analysis

Knee joints were collected and fixed in 10% of buffered formalin for 2 days and decalcified in 14% ethylene diamine tetra-acetic acid for 14 days. Decalcified tissues were fixed in paraffin in preparation for histopathological analysis. We cut 5μm-thick coronal sections by a Leica RM2255 microtome. To detecting the histological parameters of the synovium and cartilage, we stained knee joint sections with H&E and Safranin O [29]. Two blinded researchers assessed each section and the average score was used for statistical analysis.

2.6 Bone marrow-derived cells isolate

We flushed the femur and tibia to collect bone marrow-derived cells with DMEM consisting of 2% FBS after euthanasia. Low-density gradient centrifugation was to separated cells and the cells were cultured in DMEM or MEM-α supplemented with 10%

FBS and 1% penicillin/streptomycin [30].

2.7 Chondrogenic assays

For the induction of chondrogenesis, bone marrow-derived cells were plated in 6-well plates at 1×10^6 cells/ml with the chondrogenic differentiation medium (MesenCult proliferation kit supplemented with 10 mmol/l β-glycerophosphate, 10^{-8} mol/l dexamethasone, 50 µg/ml ascorbic acid 2-phosphate and 10 ng/ml TGF-β3) for 4 weeks. Then, cells were fixed in 10% buffered formalin at room temperature for 30 min, and then incubated with 1% Alcian Blue in 0.1M HCl (pH 1.0) for 2 h. Blue-stained cells were chondrogenic cells [16].

2.8 Macrophages induction

We collected bone marrow-derived cells from the mice and cultured it in DMEM supplemented with 1% penicillin/streptomycin, 10% heat-inactivated FBS and M-CSF (40 ng/ml, Cranbury, NJ, USA) over 7 days in a humidified atmosphere with 5% CO_2 at 37°C for macrophages differentiation. Cells were then activated to reach the M1 condition with LPS (100 ng/ml, Cranbury, NJ, USA) and IFN-γ (20 ng/ml, Cranbury), or activated to the M2 condition with IL-4 (20 ng/ml, Cranbury, NJ, USA) for 1 day [31].

A murine macrophage cell line (RAW264.7, ATCC) was cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Cells were stimulated with LPS (100 ng/ml) and IFN-γ (20 ng/ml, Cranbury) or IL-4 (20 ng/ml, Cranbury) for 24 h at 37°C. For an inhibition study, an NF-κB inhibitor (PG490, Catalog No. S3604) was incubated with macrophages for 1 h before the addition of polarizing

stimuli.

2.9 Immunohistochemistry staining

Immunohistochemical staining was made using a DAB substrate kit and immunohistochemical kit in line with the manufacturer protocol. The sections prepared on slides were pretreated with 3% H_2O_2 to inactivate endogenous peroxidases at room temperature for 10 min, followed by washing with PBS. The sections were then incubated with primary antibodies against p-P65 (ImmunoWay; dilution 1:100), p-AKT (Cell Signaling Technology; dilution 1:100), collagen II (Proteintech; dilution 1:800), MMP13 (Abcam; dilution 1:200), IL-1β, and TNF-α (ImmunoWay; dilution 1:200) overnight at 4°C. The slides were incubated with a secondary antibody and the reaction products were visualized using diaminobenzidine (DAB). The ratio of the area stained by the primary antibodies to the total field area was determined in a blinded fashion.

2.10 Immunofluorescent staining

For immunofluorescence staining, tissue-paraffin sections were blocked with 5% goat serum at 37°C for 30 min and stained with primary antibodies overnight at 4°C. Then, sections were stained with Alexa 488 or Alexa 594 dye-labeled secondary antibodies (Life Technologies, Carlsbad, CA, USA). Nuclei were labeled with 4, 6-diamidino-2-phenylindole (DAPI) and images were obtained using a U-RFL-T fluorescence microscope (Olympus, Tokyo, Japan). We counted the number of positively stained cells per specimen and measured three sequential specimens per mouse in each group. Cells, cultured on coverslips, were fixed in ethanol, followed by treatment with 0.5% TritonX-100 (Sigma Aldrich, Germany) in PBS. Ten-percent

blocking serum was used to block non-specific binding and the same procedure for tissue immunofluorescence was employed [32].

2.11 Western blot analysis

Tissues and cells were lysed in a radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitors and phosphatase inhibitors (Roche Diagnostics GmbH, Mannheim, Germany). Following electrophoresis, proteins were blotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA) in a transfer buffer with a constant current of 350 mA at 4℃ for 2 h. The PVDF membranes were successively washed with 1×TBST, blocked with 5% nonfat milk and incubated with the primary antibodies specific to PI3K, p-PI3K, AKT, p-AKT (Cell Signaling, Danvers, MA, USA), P65, p-P65 (ImmunoWay, Suzhou, China), MMP-13 (Abcam, Cambridge, MA, USA), IL-1β, TNF-α, iNOS, CD206 (ImmunoWay, Suzhou, China), and β-actin overnight at 4°C. The membranes were then incubated with secondary IgG antibodies conjugated with horseradish peroxidases for 1.5 h with shaking. We detected signals via enhanced chemiluminescence and repeated the experiments at least three times.

2.12 Statistical analysis

Data were expressed as mean ± standard error of the mean (SEM). Statistical significance among groups was examined using one-way ANOVA, and a post-hoc test was conducted using Fisher's protected least significant difference. All comparisons were two-tailed and statistical significance was assumed at $p < 0.05$. The asterisks (*, **, *** and n) represent $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p > 0.05$, respectively.

3. Results

3.1 Mechanical loading reduced articular cartilage damage in vivo and in vitro

Histological section staining of articular cartilage showed that in the sham group, the cartilage surface was smooth and unbroken. But, the OA group presented severe proteoglycan loss and apparent hypo-cellularity at 2 weeks after OA surgery (Fig. 2A). Compared to the sham group, the OARSI scores of articular cartilage in the OA group were significantly inceresed ($p < 0.001$; Fig. 2B). But, the application of mechanical loading considerably decreased the OARSI scores in 2 weeks' endpoints after OA surgery ($p < 0.01$; Fig. 2B).

We also calculated chondrocytes and vacuolar cells in the area of medial tibial plateau (Fig. 2A). Compared to the sham group, chondrocytes in the OA group was considerably decreased ($p < 0.001$). After mechanical loading, however, it was significantly increased ($p < 0.001$; Fig. 2C). In the OA group, the number of vacuolar cells was significantly increased ($p < 0.001$), while their number was decreased after application of mechanical loading ($p < 0.001$; Fig. 2D). Two weeks after OA surgery, calcified cartilage thickness (CC) was increased while hyaline cartilage (HC) was decreased and tidemark (black dotted line) moving towards the articular surface. After two weeks mechanical loading treatment, however, the calcified cartilage thickness was decreased (Fig. 2A). Compared to the sham group, the value of CC/TAC in the OA group was incrased ($p < 0.001$). But, after 2-week mechanical loading, the value of CC/TAC was decreased ($p < 0.001$; Fig. 2E).

We using bone marrow mesenchymal cells performed a chondrocyte

differentiation assay (Fig. 2F, G). The number of differentiated chondrocytes, derived from the OA group, was significantly decreased as compared to the sham group ($p < 0.001$). After mechanical loading, differentiated chondrocytes derived from the OAL group was restored ($p < 0.001$; Fig. 2G).

3.2 Mechanical loading decreased the level of MMP13 and increased the level of collagen II

To investigate the effect of mechanical loading on the extracellular matrix of the cartilage, we examined the level of MMP13 and collagen II in the articular cartilage via IHC and IF (Fig. 3A-F). Both IHC and IF analysis showed that two weeks after OA induction, the MMP13 positive cells in the OA group was remarkablely increased (both $p < 0.001$), but they were remarkably reduced by mechanical loading (both $p < 0.001$; Fig. 3A, B, D, E). Both IHC and IF analysis indicated that collagen II positive cells in the OA group were remarkablely reduced compared to the sham group (both $p < 0.001$). However, the number of collagen II positive cells in the OAL group was increased by 2-week mechanical loading (both $p < 0.001$; Fig. 3A, C, D, F).

Western blot analysis indicated that two weeks after OA surgery, the expression of MMP13 was greatly increased compared to the sham group ($p < 0.01$), but, it was decreaed by two-week mechanical loading ($p < 0.05$; Fig. 3G, H).

3.3 Mechanical loading reduced synovial inflammation in the mouse OA model

In the H&E stained sections, the synovium thickening was present in the OA group two weeks after surgery (Fig. 4A). The OA group revealed the considerably increased synovitis score compared to the sham group, whereas the synovitis score was decreased

after two-week mechanical loading ($p < 0.001$; Fig. 4B).

To further investigate the joint inflammation process, we assessed the presence of pro-inflammatory cytokines (IL-1β, TNF-α) by IHC and western blot (Fig. 4C-H). IHC analysis exhibited that the positive cells of IL-1β and TNF-α in the OA group were noticeably increased compared to the sham group (both $p < 0.001$). However, mechanical loading reduced the number of positive cells (both $p < 0.001$; Fig. 4C-E). Western blot analysis showed that the expression of IL-1β and TNF-α in the OA groups was significantly increased ($p < 0.001$ and $p < 0.01$). However, their expression levels were reversed by mechanical loading (both $p < 0.001$; Fig. 4F-H).

3.4 Mechanical loading reduced M1 and increased M2 macrophage polarizations

To investigate the inflammatory process of synovium, we accessed the macrophage polarization in the synovial tissue (Fig. 5A-E). IF analysis displayed that iNOS-positive cells in the OA group were significantly increased compared to the sham group, indicating that the number of synovial M1 macrophages in the development of OA was accumulated ($p < 0.001$; Fig. 5A, C). However, the number of iNOS-positive cells in the OAL group was decreased after two-week mechanical loading ($p < 0.05$; Fig. 5A, C). The number of CD206-positive cells in the OA group was decreased, but in the OAL group it was remarkably increased (both $p < 0.001$; Fig. 5B, D). Moreover, the ratio of M1/M2 was significantly increased in the OA group, while in the OAL group it was decreased (both $p < 0.01$; Fig. 5E).

To further investage the influence of mechanical loading on macrophage polarization, we accessed the polarization of mononuclear macrophages from the bone

marrow cavity (Fig. 5F-H). IF analysis showed that iNOS-positive cells in the OA group were considerably increased ($p < 0.001$; Fig. 5F, G). However, the number of iNOS-positive cells in the OAL group was decreased($p < 0.001$). The number of CD206-positive cells in the OA group was significantly decreased, but it was increased in the OAL group (both $p < 0.001$; Fig. 5F, H).

We also evaluated the expression of iNOS and CD206 in the synovial tissue by western blot. Two weeks after OA surgery, the level of iNOS in the OA group was significantly increased compared to the sham group ($p < 0.01$), and it was decreased by mechanical loading ($p < 0.05$; Fig. 5I, J). The levels of CD206 in the OA group were significantly decreased compared to the sham group ($p < 0.01$). However, it was increased in response to mechanical loading ($p < 0.05$; Fig. 5I, K).

3.5 Mechanical loading inhibited phosphorylation of PI3K, AKT and P65

To evaluate the change of PI3K/AKT/NF-κB signaling in response to mechanical loading, we examined the phosphorylation level of PI3K, AKT and P65 in the knee joint (Fig. 6A-J). IHC and IF study exhibited that the positive cells of p-P65 and p-AKT in the OA group were considerably increased compared to the sham group (all $p < 0.001$). But, those positive cells were reduced by mechanical loading (all $p < 0.05$; Fig. 6A-F).

Western blot study displayed that the levels of p-PI3K, p-AKT and p-P65 in the OA group were significantly increased compared to the sham group (all $p < 0.01$). However, their levels were reduced by mechanical loading (all $p < 0.01$; Fig. 6G-J).

3.6 Inhibition of NF-κB decreased the polarization of M1 macrophages

To determine whether the change of PI3K/AKT/NF-κB signaling is related to macrophage polarization, we inhibited the expression of NF-κB in RAW264.7 cells and observed the polarization of macrophages. Western blot showed that with an increase in the concentration of NF-κB inhibitor, PG490, the level of p-P65 gradually decreased (both $p < 0.001$; Fig. 7A, B). Also, in RAW264.7 cells stimulated with LPS and IFN-γ, an increase in PG490 decreased the level of iNOS (both $p < 0.001$; Fig. 7A, C).

4. Discussion

In this study, we presented the beneficial role of mechanical loading, a form of physical rehabilitation, in the mitigation of OA symptoms by analyzing the inflammatory microenvironment in the mouse model of OA. Our results revealed that mechanical loading decreased the M1 polarization of synovial macrophages with an elevated M2 polarization *in vivo* and *in vitro*. Mechanical loading reduced the inflammatory responses in the arthritic knee joint and suppressed the degradation of articular cartilage.

Degenerative OA in the joint is associated with joint inflammation and cartilage disintegration [33]. A growing body of evidence suggests that OA is a low-grade inflammatory disease involving the activation of innate inflammatory pathways [34, 35]. In the local pathological changes, macrophages play a critical role. They may become pro-inflammatory macrophages (M1 macrophages), releasing excessive pro-inflammatory cytokines and inducing chondrocyte hypertrophy and cartilage matrix destruction [33, 36, 37]. To date, many attempts have been conducted to decrease articular inflammation and reduce cartilage erosion. However, no effective treatment exists and little concensus is present regarding the role of macrophages and their polarization in the development of OA [32]. Some studies show that synovial macrophages are crucial during the progression of OA and they stimulate pro-inflammatory cytokines IL-1β and TNF-α secrete and aggravate synovial inflammation [35, 38, 39]. A specific phenotype of Macrophages is suggested to regulate the occurrence or progression of OA via the anabolic or catabolic reactions with different

cell types [40]. Macrophages play crucial roles in innate immunity and exhibit a high degree of plasticity [9]. They express varying cell surface receptors, which may contribute to polarizing macrophages into pro-inflammatory-type M1 and anti-inflammatory type M2 [33, 41]. The activation state of macrophages and the M1/M2 ratio are highly associated with OA severity. One study disclosed that the polarization of synovial macrophage M1 aggravated experimental collagenase-induced osteoarthritis whereas M2 polarization decreased OA development [42]. In this study, we found that M1/M2 ratio increased significantly in the OA group and this increase was reversed by mechanical loading. The result herein demonstrates that mechanical loading can change the direction of macrophage polarization in the progression of OA.

IL-1β and TNF-α, a sort of pro-inflammatory cytokines, play a crucial role in the pathogenesis of OA. Our study found that the level of IL-1β and TNF-α in the OA group increased significantly. At the same time, we found that the loss of ECM in articular cartilage elevated. The destruction of cartilage is thus closely related to the inflammatory microenvironment. Synovial macrophages are involved in the production of pro-inflammatory cytokines and matrix metalloproteinases (MMPs) [43]. According to clinical and experimental data, there is strong evidence that MMP-13 has a degradative effect on the extracellular matrix and is considered to be an important cofactor or disease mediator in OA [44, 45]. In this study, we found that consistent with the increase in M1 macrophages, MMP13 increased significantly in the OA group. After two-week mechanical loading, with the reduction in M1 macrophages the level of MMP13 decreased.

Several signaling pathways, including PI3K/AKT, TGF-β and hypoxia-dependent intracellular pathways, are involved in macrophage reprogramming, leading to the transition of polarization between M1 and M2. However, the polarization mechanism in the pathogenesis and progression of OA remains unclear [42, 46]. Some studies have shown that activating PI3K/AKT can inhibit the apoptosis of chondrocytes induced by IL-1β and improve the damaged knee cartilage in rats [47, 48]. However, other studies have shown that inhibiting PI3K/AKT signaling can prevent chondrocyte apoptosis [49]. We have previously shown that diverse signal pathways are involved in mechanical transduction and bone metabolism. PI3K signaling was highlighted in the prediction with Pathway-Express as an important pathway by mechanical loading [50]. In this experiment, we explored the role of PI3K/AKT signaling in OA. We found in the OA group that PI3K/AKT signaling was greatly activated, while this activation was inhibited and the destruction of articular cartilage was reduced in response to mechanical loading. It is generally considered that NF-κB signaling plays a role in regulating inflammation related to the pathogenesis of OA [25, 51]. Our results showed that mechanical loading suppressed NF-κB activation and reduced the ratio of M1/M2 in OA mice. Interestingly, our data indicated that that NF-κB inhibite reduced M1 macrophages polarization but it had no detectable effect on the polarization of M2 macrophages. We thus interpreted that mechanical loading can inhibit the polarization of M1 macrophages by repressing PI3K/AKT/NF-κB signaling. However, the increase of M2 polarization can be associated with the change in M1 macrophages or caused by other signaling pathways (Fig. 7D).

This study has limitations. Our data showed that mechanical loading inhibited M1 polarization and increased M2 polarization. However, the mechanism for the increase of M2 polarization is unclear and needs further analysis. In summary, this study demonstrates that mechanical loading can mitigate OA symptoms by decreasing the ratio of M1/M2 and suppressing the inflammatory microenvironment. The results indicate the possibility of the use of mechanical loading as an adjuvant option for the treatment of OA.

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6. Author contributions

W. Zheng and P. Zhang designed research; W. Zheng, X. Li, J. Li and X. Wang conducted research, D. Liu, L. Zhai, G. Li and Y. Sun collected the data; W. Zheng, X. Li, J. Li and H. Yokota analyzed the data; W. Zheng and P. Zhang wrote the manuscript; P. Zhang approved the final manuscript as submitted; P. Zhang accepted responsibility for the integrity of data analysis.

7. Disclosures

The authors have nothing to disclose.

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Figure legends

Fig. 1. Experimental timeline and setting of mechanical loading. (A) Experimental timeline. (B) Loading was laterally applied to both knees successively (1 N at 5 Hz) with 6 min/day (each knee, 3 min) for 2 weeks. (C) Schematic diagram, illustrating the loading equipment.

Fig. 2. Mechanical loading reduced articular cartilage damage. (A) Safranin O stained images. (B-E) Statistical analysis of OARSI scores, chondrocyte numbers, vacuolar cell and CC/TAC in the area of subchondral bone. Black arrows showed vacuolar cells, Bar = 500 μm (upper) and Bar = 50 μm (under), n = 10 per group. (F) Images of chondrocyte from mouse-derived bone mesenchymal cells. (G) Statistical analysis of the chondrocyte numbers. Bar = 50 μm, n = 3 per group. *P < 0.05, **P < 0.01 and ***P < 0.001.

Fig. 3. Mechanical loading decreased the expression of MMP13 and increased the expression of collagen II. (A) Immunohistochemistry staining of MMP13 and collagen II in the coronal sections of articular cartilage. Bar = 50 μm. (B, C) Statistical analysis of MMP13 and collagen II positive cells in articular cartilage. (D) Immunofluorescence staining of MMP13 and collagen II in the coronal sections of articular cartilage. Bar = 50 μm. (E, F) Statistical analysis of MMP13 and collagen II positive cells in articular cartilage. (G, H) Western blot revealed the protein level of MMP13 and the relative statistical analysis of the protein level of MMP13. n = 3 per group. *p < 0.05; **p < 0.01 and ***p < 0.001.

Fig. 4. Influence of mechanical loading on the inflammation of OA mice joint. (A, B) H&E stained images and statistical analysis of synovitis scores, Bar = 50μm, n = 10 per group. (C) Immunohistochemistry staining of IL-1β and TNF-α in the coronal sections of articular cartilage. Bar = 50 μm. (D, E) Statistical analysis of IL-1β and TNF-α positive cells in articular cartilage. (F-H) Western blot revealed the protein levels of IL-1β and TNF-α and the relative statistical analysis of the protein levels of IL-1β and TNF-α. n = 3 per group. *p < 0.05; **p < 0.01 and ***p < 0.001.

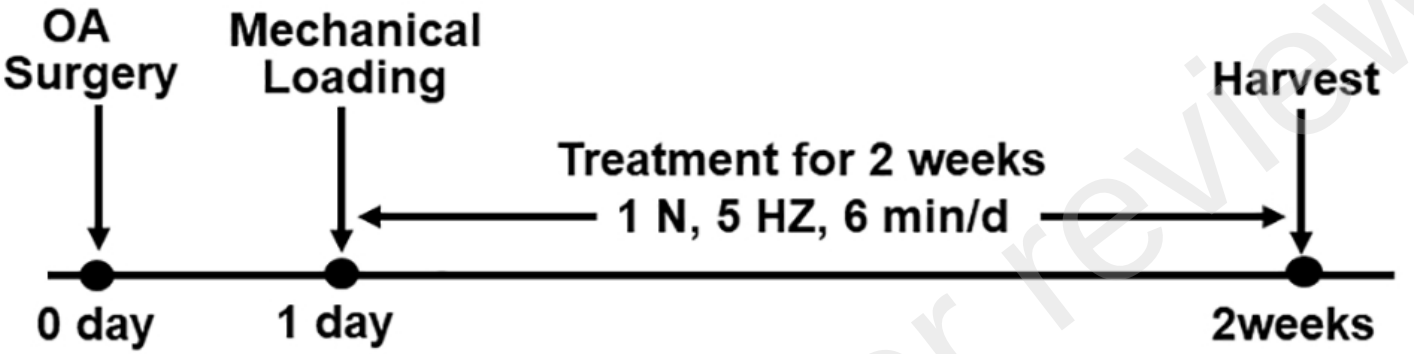
Fig. 5. Mechanical loading decreased synovial macrophage M1 polarization and increased M2 polarization. (A, B) Immunofluorescence staining of iNOS and CD206

in the coronal sections of the synovium. Bar = 50 μm. (C-E) Statistical analysis of iNOS and CD206 positive cells in the synovium and the ratio of M1/M2. (F) Immunofluorescence staining of iNOS and CD206 in bone marrow-derived mononuclear macrophage. Bar = 50 μm. (G, H) Quantification of iNOS and CD206 positive cells. (I-K) Western blot revealed the protein levels of iNOS and CD206 and the relative statistical analysis of the protein levels of iNOS and CD206. n = 3 per group. *p < 0.05; **p < 0.01 and ***p < 0.001.

Fig. 6. Mechanical loading inhibited the phosphorylation of PI3K, AKT and P65. (A) Immunohistochemistry staining of p-P65 and p-AKT in coronal sections of articular cartilage. Bar = 50 μm. (B, C) Statistical analysis of p-P65 and p-AKT positive cells in articular cartilage. (D) Immunofluorescence staining of p-P65 and p-AKT in the coronal sections of articular cartilage. Bar = 50 μm. (E, F) Quantification of p-P65 and p-AKT positive cells in articular cartilage. (G-J) Western blot revealed the expression of p-PI3K, p-AKT and p-P65 and the relative quantification of the protein levels of p-PI3K, p-AKT and p-P65. n = 3 per group. *p < 0.05; **p < 0.01 and ***p < 0.001.

Fig. 7. Inhibition of NF-κB decreased the polarization of M1 macrophages. (A) Western blot revealed the expression of p-P65 in RAW264.7 cells with different doses of an NF-kB inhibitor, PG490, and the expression of iNOS in RAW264.7 cells stimulated with LPS + IFN-γ, with different doses of an NF-kB inhibitor, PG490. (B, C) Relative quantification of the protein levels of p-P65 and iNOS. n = 3 per group. (D) The mechanism of mechanical loading, altering the inflammatory microenvironment for the treatment of OA. *p < 0.05; **p < 0.01 and ***p < 0.001.

A



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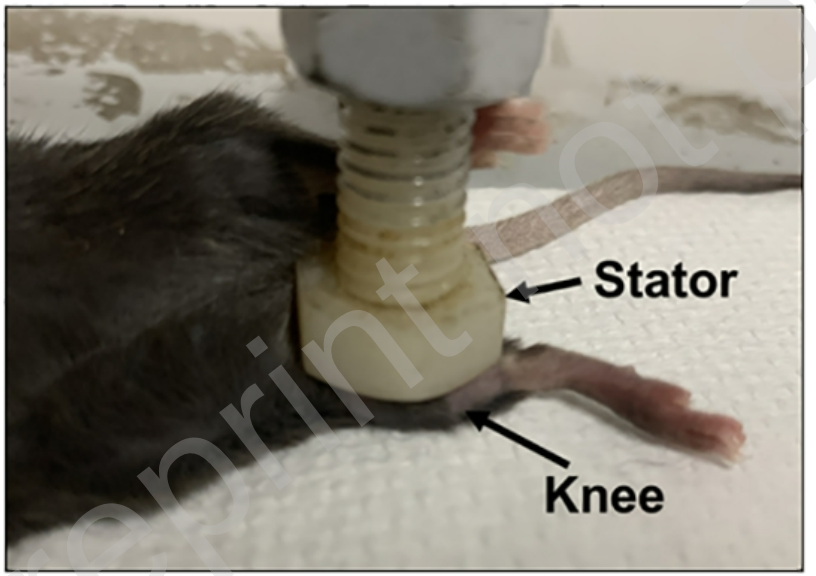

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