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GSDMD inhibitor protects chondrocyte from mechanical injury in human articular cartilage

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ABSTRACT

Traumatic arthritis is caused by mechanical injury and results in the degeneration of articular cartilage, but it is unclear whether it is related to the pyroptosis of chondrocyte (CHs). Thus, this study was designed to investigate the role of GSDMD, the executor of pyroptosis, in the human cartilage during mechanical injury. We collected the human hip joint and used a loading apparatus to produce compression on the cartilage disc. After one hour of 15 MPa or 25 MPa injury, the acute and chronic effects of the mechanical injury on the cartilage were tested. We stained the CHs in the cartilage with calcein and DAPI to calculate the live-cell rate. The chondrogenic phenotype was determined by analyzing the mRNA levels of type II collagen alpha 1 (Col2A1), type I collagen alpha 2 (Col2A1), and SOX9. Besides, the pyroptosis process was determined by the mRNA levels of caspase-1/5, GSDMD, IL-1β, and IL-18. We also explored the preventive role and therapeutic role of GSDMD inhibitors in mechanical injury via culturing the cartilage before and after the compression, respectively. Mechanical compression injured the viability and function of CHs in cartilage partly based on the pyroptosis. The pretreatment of GSDMD inhibitor in cartilage before injury could maintain the live cells and Col2A1 expression and prevent pyroptosis after injury. Besides, supplying the cartilage with GSDMD inhibitor after injury also alleviated the cell death and dysfunction of CHs, and suppressed the pyroptosis. Using an inhibitor of GSDMD can play a preventive role and play a therapeutic role in the mechanical injury of cartilage.

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Introduction

Traumatic arthritis is one of the common diseases of bones and joints. It is a series of pathological changes such as cartilage degeneration and secondary cartilage hyperplasia and hypertrophy caused by external forces around the joints, which is the most common complication of joint fractures or instability (1,2). The clinical symptoms of patients with traumatic arthritis are mainly joint pain and stiffness, as well as joint deformities and dysfunction. The pathogenesis of traumatic arthritis is related to the direct mechanical environment changes in articular cartilage. The acute extreme shear and compression forces directly act on the cartilage's surface, which can penetrate the cartilage matrix and even extend to the subchondral bone, causing chondrocyte (CH) damage and death (3). The mechanical damage caused by abnormal CHs death is bound to affect the dynamic balance of cartilage decomposition and anabolism, which further reduces the number of functional CHs and weakens cartilage's ability to maintain or repair the extracellular matrix (ECM) (4).

Previous studies have elucidated that injurious compression induces CHs death by apoptosis and necrosis, and the remaining cells lose their biosynthetic activity[2]. D'Lima et al. reported the mechanical injury of 14 MPa for 500 ms resulted in CHs apoptosis and GAG release from the matrix (5). In vitro, mechanical unloading also significantly promoted the apoptosis rate and decreased the proliferation rate of CHs in rabbits (6). Unlike apoptosis and necrosis, pyroptosis is a new way of programmed cell death proposed in recent years. It mainly manifests as cell membrane formats 1~2.4 nm pores to make the cells the intracellular osmotic pressure surges and release many inflammatory factors. There are two types of pyroptosis pathways, the classical pathway mediated by caspase-1 and the non-classical pathway mediated by caspase-11 (human homologous cysteine protease is caspase-4/5) (7).

The morphological feature of pyroptosis is the formation of pores in the cell membrane. The cell membrane loses its integrity, and the barrier function of the membrane disappears, causing the cell to swell and rupture, but the nucleus remains intact (8). Gasdermin D (GSDMD) is the final executor in pyroptosis (9), which is related to pores' formation in the cell membrane (10). In the classical pathway, activated caspase-1 cuts GSDMD to form gasdermin pores in the membrane (11). Meanwhile, it activates the precursors of IL-18 and IL-1β to produce mature IL-18 and IL-1β, which flow out from the gasdermin pores, causing inflammation (12). Besides, caspase-11 is also stated to play a role in the GSDMD activation and leads to pyroptosis by forming pores in the membrane-containing fraction of immortalized macrophages (13).

Pyroptosis has been verified to be associated with the development of arthritis (14), and the inhibition of pyroptosis is also useful in alleviating the process of arthritis (15,16). However, whether the suppression of GSDMD protects the CHs from pyroptosis in traumatic arthritis remains unclear. In this study, we applied the mechanical

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injury on the human articular cartilage and used the inhibitor of GSDMD to explore 1) whether the pretreatment of GSDMD would prevent the CHs pyroptosis from the acute mechanical injury; 2) whether the treatment of GSDMD would alleviate the mechanical injury caused CHs pyroptosis.

Materials and Methods

Cartilage disc preparation

Articular cartilage was collected from the patients (n=8, from 43 to 62 years) of femur neck fracture, approved by the Third Affiliated Hospital of Soochow University Ethics Committee. All patients signed informed consent before surgery. We conserved the hip joint tissues in a culture medium immediately after removing them from patients. We drilled perpendicular to the cartilage surface to achieve a 4mm–diameter cartilage cylinder using a dermal punch. This cylinder was then placed in a microtome holder to obtain the sequential 2-mm thick slices using a brain matrix (TM-1000; ASI Instruments, Warren, MI, USA). These disks were then washed with PBS and precultured for 24 h in culture medium (low-glucose DMEM medium containing 10% FBS, 10 mM HEPES buffer, and 1% penicillin-streptomycin, Gibco, Rockville, MD, USA) in a 37°C, 5% CO_2 incubator for the following injurious compression.

Injurious compression and drug treatment

After 24 h preculture, the round cartilage disc (4 mm diameter, 2 mm thick) underwent mechanical compression in an incubator-housed loading apparatus (17) (Figure 1A). Cartilage dice to be injured were placed individually into a polysulfone chamber (Figure 1B), allowing unconfined compression of the disk by impermeable platens. Experiments used dynamic compression protocols with a 25% static offset compression (0.5 mm displacement) or 50% static offset compression (1 mm displacement) followed by immediate removal of the compression. Compression was performed non-continuously with cycles containing 5 s periods of compression followed by 1 min of free swelling culture (resulting in an average peak stress of 15 MPa for 0.5 mm displacement and 25 MPa for 1 mm displacement). The total loading period was 1 h, which has previously been shown a significant decrease in cell viability and ECM production (18,19). After compression, the disk was transferred into a fresh culture medium for instant measurement or extra culture.

Additionally, we used the inhibitor of GSDMD, LDC7559 (LDC, 1 and 5 μM, MedChemExpress, Monmouth Junction, NJ, USA), to pretreat the disc before injurious compression or treat the disc after compression. We set the control disc as no compression and drug treatment.

Live-cell staining

To measure the live-cell rate after compression, we stained the disc with DAPI and calcein. Briefly, the cartilage disc was changed to the fresh medium containing 10% calcein (ab141420, Abcam, Cambridge, UK) and 0.1% DAPI (Beyotime, Shanghai, China) and cultured for 45 min in the dark. Then, the disc was washed one time with PBS and replaced in the new culture medium. Under the fluorescence microscope, the cytoplasm of live cells was stained in green, and the nuclei of the total cells were stained in blue. Because the entire disc area is too large to image once, we use the microscope's continuous camera function to divide the whole plane into several small pictures and form the completed one. The rate of live cells was calculated by dividing the number of calcein-stained cells with the DAPI-stained cells from the disc's integrated image using ImageJ software.

Real-time Polymerase Chain Reaction (PCR) analysis

The relative gene expression was determined by the mRNA expression level. Briefly, total RNA was isolated from the cartilage disks by first pulverizing the samples and then immersed in TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA was isolated from the liquid by the RNeasy Mini Kit (Qiagen, Duesseldorf, Germany) according to the manufacturer's instructions. Then, equal quantities of RNA (2.5 g) was reverse-transcribed into cDNA with a reverse transcription kit (Applied Biosystems, Foster City, CA, USA). The real-time PCR analysis was performed using the SYBR Green Master (TOYOBO, Osaka, Japan). Gene expressions were achieved by GAPDH normalization according to the method of $2^{-\Delta\Delta Ct}$. The primers used for PCR are listed in Table 1.

Statistical analysis

The data were expressed as mean ± standard deviation (SD) of 3 replicate experiments analyzed by the Statistic Package for Social Science (SPSS) Version 22.0 software package (IBM, Armonk, NY, USA). We used a one-way ANOVA to compare the differences between the groups. P-value<0.05 was found to be statistically significant.

Table 1. Primer sequences for PCR.

Gene name	Forward (5'-3')	Reverse (5'-3')
Col2A1	CCTGGCAAAGATGGTGAGACAG	CCTGGTTTTCCACCTTCACCTG
Col1A2	CCTGGTGCTAAAGGAGAAAGAGG	ATCACCACGACTTCCAGCAGGA
SOX9	AGGAAGCTCGCGGACCAGTAC	GGTGGTCCTTCTTGTGCTGCAC
Caspase-1	GCTGAGGTTGACATCACAGGCA	TGCTGTCAGAGGTCTTGTGCTC
Caspase-5	ACAACCGCAACTGCCTCAGTCT	GAATCTGCCTCCAGGTTCTCAG
GSDMD	ATGAGGTGCCTCCACAACTTCC	CCAGTTCCTTGGAGATGGTCTC
IL-1β	CCACAGACCTTCCAGGAGAATG	GTGCAGTTCAGTGATCGTACAGG
IL-18	GATAGCCAGCCTAGAGGTATGG	CCTTGATGTTATCAGGAGGATTCA
MMP13	ACTGAGAGGCTCCGAGAAATG	GAACCCCGCATCTTGGCTT
GAPDH	ACAACTTTGGTATCGTGGAAGG	GCCATCACGCCACAGTTTC

Results

Mechanical compression injures CHs viability

To induce mechanical injury in the cartilage, we assembled the cartilage disc (4-mm diameter, 2-mm thick) in the specialized chamber of incubator-housed loading apparatus (Figure 1A, B) with a uniaxial unconfined compression. We applied 15 MPa in the cartilage disc with 0.5 mm displacement and 25 MPa with 0.5 mm displacement in uniaxial unconfined compression. The detailed dynamic compression protocol was described in the section on methods. We tested the acute injury of cartilage instantly after the mechanical compression. Additionally, the developed effects of the mechanical compression were analyzed after three days of culture. As shown in Figure 1C, the estimation of total cell number in the cartilage was done by DAPI fluorescence (cell nuclei staining), and the live cell population was determined by the calcein staining (20). After one hour of 15 MPa or 25 MPa mechanical injury, the amount of live cells was significantly decreased compared to the control, which was further aggravated three days later. Besides, there was no significant difference between the energy of 15 MPa and 25 MPa in the acute injury instantly after the compression. However, the live cell rate in the 25 MPa compression group was lower than the 15 MPa compression group (Figure 1D). Therefore, the higher energy of compression leads to lower cell viability in the long time run.

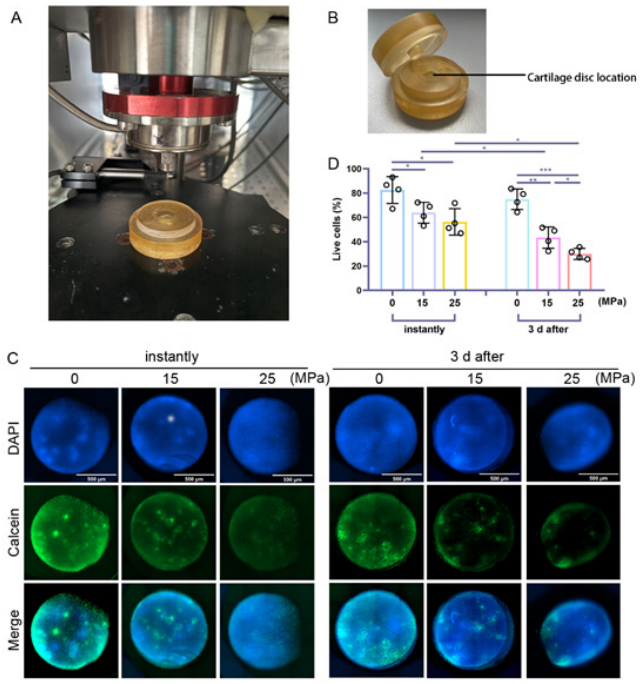

Figure 1. Mechanical compression injures CHs viability. Loading device used to induce injurious compression of human cartilage disc. (A) An incubator-housed loading apparatus was used to apply injurious compression in displacement control to individual cartilage disks. The load and displacement were recorded by transducers during loading. (B) Polysulfone chamber used to hold cartilage disks during loading. 15 MPa and 25 MPa unconfined compression were applied to the cartilage disc. We tested the acute injury instantly after the mechanical compression, and the developed effects were analyzed after three days' culture. (C) The DAPI and calcein staining of the cell nuclei and cytoplasm. (D) The live cell rate was calculated by dividing the calcein-stained cell number by the DAPI-stained cell number. Results are expressed as mean ± SD. (n=4, *P < 0.05, **P < 0.01, ***P < 0.001).

Mechanical compression triggers pyroptosis and affects the CHs function

To determine the effects of mechanical compression on the CHs phenotype, we tested the type II collagen alpha 1 (Col2A1), type I collagen alpha 2 (Col2A1), and SOX9 expression. In the acute phase after mechanical compression, 25 MPa but not 15 MPa effectively induced the reduction of Col2A1 and SOX9 and increased the Col1A2 expression, which was further decreased three days later. The 15 MPa compression suppressed the Col2A1 and promoted the Col1A2 expressions 3 days after injury (Figure 2A). Additionally, compression activated the caspase-1, caspase-5, and GSDMD mRNA expression, which was more evident in the 25 MPa group beginning instantly after the injury. In the 15 MPa group, the caspase-1 and GSDMD expression were instantly affected, and caspase-5 was increased three days later (Figure 2B). In terms of the pyroptosis-related inflammatory factors, high energy of compression was sufficient to increase the IL-1β, IL-18, and MMP-13 t instantly after injury. However, the low energy of 15 MPa compression only affected the MMP13 expression in the acute phase, and it also triggered the activation of them all after three days' culture. These data indicated that the mechanical compression could activate the pyroptosis and affect the chondrogenic phenotype of CHs, which was more evident under the 25 MPa compression.

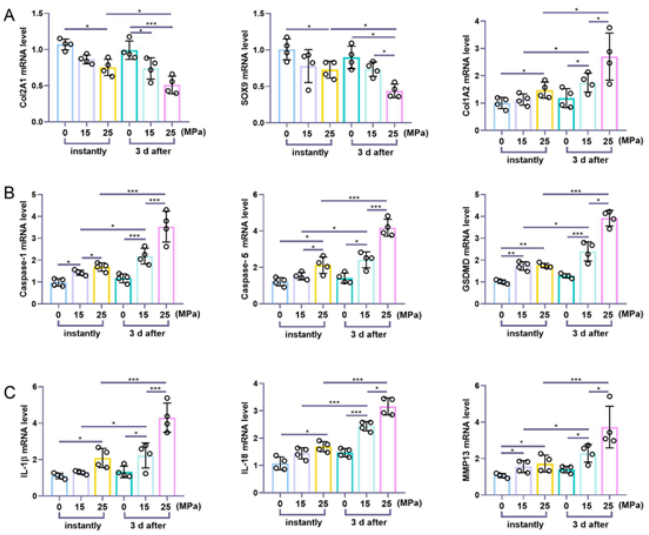

Figure 2. Mechanical compression triggers pyroptosis and affects the CHs function. 15 MPa and 25 MPa unconfined compression were applied to the cartilage disc. We tested the acute injury instantly after the mechanical compression, and the developed effects were analyzed after three days' culture. (A-C) RT-PCR analysis for Col2A1, SOX9, Col1A2, caspase-1, caspase-5, GSDMD, IL-1β, IL-18, and MMP-13 mRNA expression by normalization to GAPDH. Results are expressed as mean ± SD. (n=4, *P < 0.05, **P < 0.01, ***P < 0.001).

Inhibition of GSDMD delays the mechanical injury to the CHs

The degree of mechanical injury continued to aggravate in the next three days after the compression. We wondered whether the pharmaceutical interruption of GSDMD would delay the development of mechanical injury in the cartilage. Similarly, we applied two LDC concentrations (1 and 5 μM) in the following three days' cartilage culture

after injury. The control group was the cartilage cultured for three days without any compression and drug treatments, and the injury group was the cartilage that suffered the one-hour 25 MPa compression and continued to culture for three days. As shown in Figures 3A and B, the live cell rate in the cartilage was improved when the cells were supplied with 5 μM LDC. Apart from this, a higher concentration of LDC also prevented the loss of Col2A1. However, the SOX9 and Col1A2 expression were not affected by the LDC treatment (Figure 3C). Furthermore, the LDC was insufficient to influence the caspase-1 and caspase-5 expression, but it significantly decreased the GSDMD expression without a dose effect (Figure 3D). Besides, we found the IL-1β and IL-18 gene expressions were obviously suppressed when the cartilage was cultured at a higher level of LDC (Figure 3E). Thus, after injury, the viability and Col2A1 production of CHs were improved, and Col1A2 expression and inflammation were inhibited after using the GSDMD inhibitor, potentially with a dose dependence.

Pretreatment of GSDMD inhibitor prevents acute mechanical injury to the CHs

To further explore whether LDC pretreatment would prevent the cartilage from acute injury in advance, we cultured the cartilage disc with LDC (1 and 5 μM) for two days before mechanical compression. The control group was the cartilage cultured for two days without any drug treatments and compression, and the injury group was the cartilage cultured for two days and then suffered the one-hour 25 MPa compression. All the cartilage was tested instantly after the mechanical injury. As shown in Figures 4A and B, the live cell rate was protected compared to the injury group when the cartilage was precultured with 5 μM LDC. The reduction of Col2A1 expression was alleviated from the LDC pretreatment, which was more significant in the 5 μM LDC group. Besides, 5 μM LDC also suppressed Col1A2 expression compared to the injury group, but the SOX9 expression was still not observably changed (Figure 4C, 4D). Apart from this, a higher LDC level was efficient in preventing the IL-1β, IL-18, and MMP13 gene upregulation compared to the injury group (Figure 4E). Therefore, pretreated with enough doses of LDC could protect the CHs viability and suppress the pyroptosis progress.

Discussion

CHs are the only cellular component in cartilage, synthesizing matrix components such as type II collagen and proteoglycans (PGs). The loss of functional CHs induces cartilage matrix metabolism disorders and accelerates the progression of arthritis (21). Customarily, the stu-

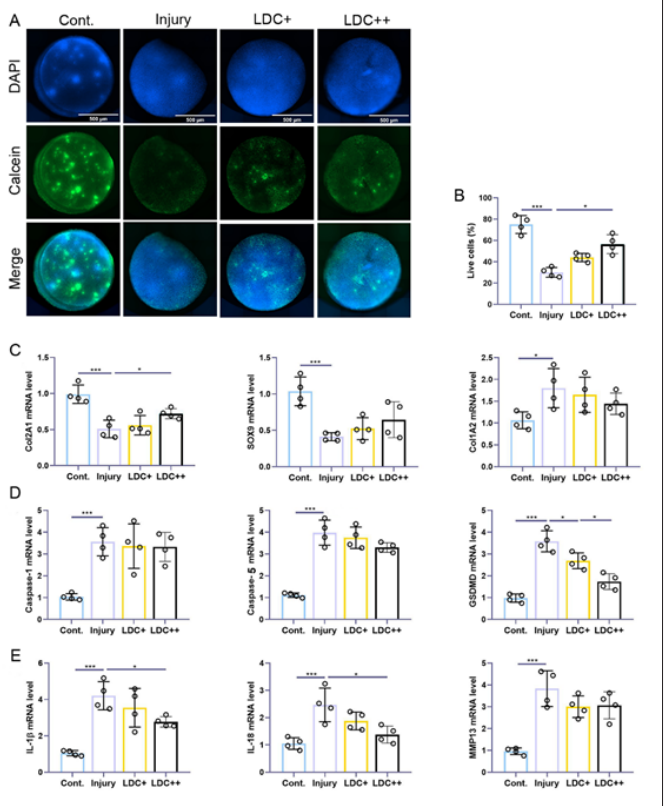

Figure 3. Inhibition of GSDMD delays mechanical injury to the CHs. 25 MPa unconfined compression was applied to the cartilage disc. The cartilage was cultured with LDC+ (1 μM) or LDC++(5 μM) and then tested the developed effects after three days of culture. (A) The DAPI and calcein staining of the cell nuclei and cytoplasm. (B) Live cell rate was calculated by dividing the calcein-stained cell number by the DAPI-stained cell number. (C-E) RT-PCR analysis for Col2A1, SOX9, Col1A2, caspase-1, caspase-5, GSDMD, IL-1β, IL-18, and MMP-13 mRNA expression by normalization to GAPDH. Results are expressed as mean ± SD. (n=4, *P < 0.05, ***P < 0.001).

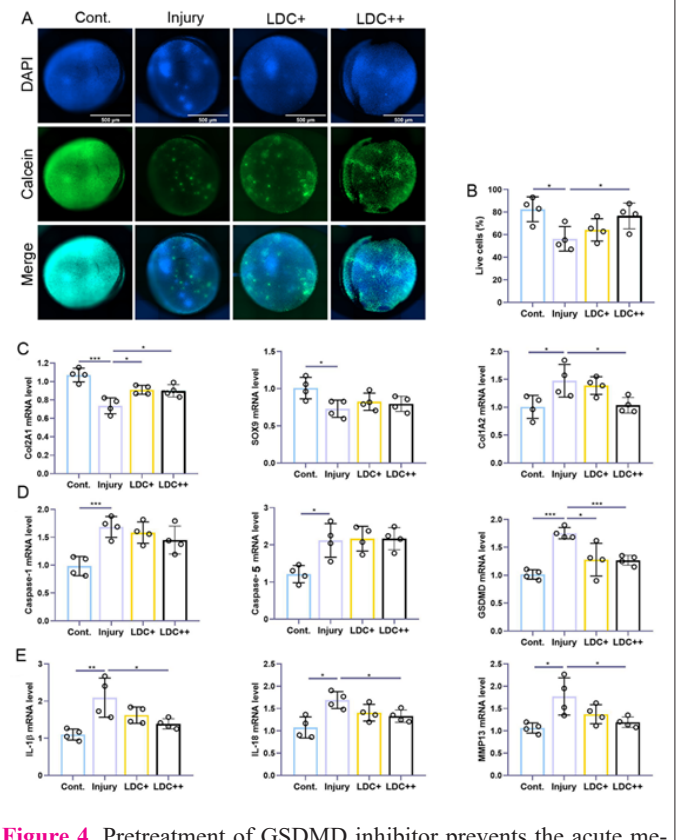

Figure 4. Pretreatment of GSDMD inhibitor prevents the acute mechanical injury to the CHs. The cartilage disc was pretreated with LDC+ (1 μM) or LDC++(5 μM) for two days and then applied 25 MPa unconfined compression. We tested the acute injury instantly after the mechanical compression. (A) The DAPI and calcein staining of the cell nuclei and cytoplasm. (B) The live cell rate was calculated by dividing the calcein-stained cell number by the DAPI-stained cell number. (C-E) RT-PCR analysis for Col2A1, SOX9, Col1A2, caspase-1, caspase-5, GSDMD, IL-1β, IL-18, and MMP-13 mRNA expression by normalization to GAPDH. Results are expressed as mean ± SD. (n=4, *P < 0.05, **P < 0.01, ***P < 0.001).

dy concerning cell death has been referred to as the available dying cells based on apoptosis and necrosis. Recent literature has pointed out some new types of cell death, such as pyroptosis, parthanatos, ferroptosis, and necroptosis (22-24). A caspase-1/4/5/11 involved proinflammatory programmed cell death terms pyroptosis, which has been elucidated to participate in osteoarthritis (14). Cartilage breakdown is the typical pathological change in osteoarthritis, especially traumatic arthritis. To date, no literature has described the role of pyroptosis in traumatic arthritis. For the first time, our study found that mechanical injury could trigger the release of caspase-1, caspase-5, and GSDMD in the cartilage. In the classical pathway, activated caspase-1 can promote the cleavage of IL-1β and IL-18 precursors to form mature IL-1β and IL-18[7]. Meanwhile, it also cleaves GSDMD to form a GSDMD-N terminal, combining with phosphatidylinositol phosphate on the eukaryotic cell membrane to create pores. As the membrane pores accumulate, the cell membrane's osmotic pressure appears to unbalance, the plasma membrane ruptures, and eventually leads to cell lysis and death and IL-1β and IL-18 release outside the cell (9).

Typical joint weight-bearing is necessary to maintain standard articular cartilage composition and structure. When the intensity and frequency of weight-bearing exceed or fall below this range, the synthesis and degradation of cartilage are out of balance, resulting in changes in cartilage ultrastructure (25). Within one week after knee joint trauma, various inflammatory factors, matrix-degrading enzymes, and oxygen-free radicals appeared in the local joint cavity, which caused abnormal CHs death and became the key to initiating articular cartilage degeneration (26). Therefore, one week after trauma is the “prime time” for preventing and treating mechanical injury-induced arthritis. In our study, the mechanical compression of 25 MPs for one hour caused acute injury and more serious chronic damage to CHs, with a significant decrease in cell viability, chondrogenic phenotype, and massive pyroptosis gene expression. However, when the cartilage was treated with the inhibitor of GSDMD for three days, the loss of cell viability was delayed. Though the caspase-1/5 expression was not affected, the Col2A1 content was maintained, and the Col1A, IL-1β, and IL-18 levels were suppressed. Therefore, the inhibition of GSDMD alleviated the pyroptosis process and protected the CHs phenotype in the following chronic phase.

The cartilage matrix is composed of a collagen mesh arch structure and organically combined with PGs, which can withstand an acute 25 MPa impact without damage, but after the cartilage matrix is subjected to a pressure load of 5 MPa for 1 hour (26), CHs will undergo necrotically and the loss with an increase of PG degradation. To test whether the pretreatment of GSDMD inhibitor would enhance the ability to resist mechanical injury. We cultured the cartilage disc with lower or higher LDC doses, and the higher amount was efficient in resisting the effects of 25 MPa compression compared to the non-drug treated group. Maintaining the viability and function of the CHs are the primary treatment strategy for traumatic arthritis to keep the balance of ECM metabolism and alleviate the inflammatory response. Our data revealed that inhibiting GSDMD prevented the coming effects of mechanical injury and weakened the mechanical injury that already existed in the cartilage. This study is an in vitro tissue experiment, which is somewhat different from the pathological process in vivo. The following shortcomings may exist (1) Because of the limitation of the sample size obtained from each modeling, we only analyzed the expression of genes from mRNA's perspective. (2) The duration and dose of GSDMD inhibitor still need to be compared. (3) The impact of mechanical injury's time and intensity is more complicated, and the biological potency requires further verification.

In conclusion, the mechanical compression caused cartilage degradation is associated with the pyroptosis process. Using the inhibitor of GSDMD can not only play a preventive role but also play a therapeutic role in the development of pyroptosis-related cartilage mechanical injury. We believe that GSDMD will become a new target in the treatment of traumatic arthritis in the future.

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Conflict of interests

The authors declared no conflict of interest.

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