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Excessive mechanical stress induces chondrocyte apoptosis through TRPV4 in an anterior cruciate ligament-transected rat osteoarthritis model

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ABSTRACT

Aims: Chondrocyte apoptosis is the most common pathological feature of cartilage in osteoarthritis (OA). Excessive mechanical stress can induce chondrocyte apoptosis and destroy cartilage tissue. Transient receptor potential channel vanilloid 4 (TRPV4) is a mechanosensitive ion channel that mediates chondrocyte response to mechanical stress. Here, we investigated the potential role of TRPV4 in chondrocyte apoptosis induced by excessive mechanical stress.

Main methods: Using a rat OA anterior cruciate-ligament transection (ALCT) model, we detected immunolocalization of calmodulin protein and mRNA and protein levels of TRPV4, calmodulin, and cleaved caspase-8 in articular cartilage. Primary chondrocytes were isolated and cultured in vitro, and Fluo-4AM staining was used to assess intracellular Ca^{2+} levels in order to evaluate TRPV4-mediated Ca^{2+} influx. Flow cytometry and western blot were performed to detect apoptosis and apoptosis-related protein levels in chondrocytes, respectively.

Key findings: TRPV4 was upregulated in ALCT-induced OA articular cartilage, and we found that administration of a TRPV4 inhibitor attenuated cartilage degeneration. Additionally, TRPV4 specifically mediated extracellular Ca^{2+} influx, leading to chondrocyte apoptosis in vitro, which was inhibited by transfection of *TRPV4* small-interfering RNA or administration of a TRPV4 inhibitor. Moreover, increased Ca^{2+} influx triggered apoptosis by upregulating FAS-associated protein with death domain and cleaved caspase-3, -6, -7, and -8 levels, with these effects abolished by *TRPV4* knockdown or TRPV4 inhibition.

Significance: These results indicated that TRPV4 was upregulated in OA articular cartilage, and that excessive mechanical stress might induce chondrocyte apoptosis via TRPV4-mediated Ca^{2+} influx, suggesting TRPV4 as a potential drug target in OA.

1. Introduction

Osteoarthritis (OA) is the most common joint disease and also referred to as “immortal cancer”. Although not fatal, it results in a disability rate of 53%, and its incidence continues to increase. OA can cause joint stiffness, swelling, pain, or disability, and as age and joint-wear increase, OA incidence continues to significantly increase [1]. The knee joint shows the highest incidence of OA, which is characterized by progressive articular-cartilage degeneration [2]. Articular cartilage depends solely on resident cells (chondrocytes) to maintain the extracellular matrix, with chondrocyte apoptosis a central feature of cartilage degeneration in OA [3].

Numerous studies show that OA progression is related to not only to biochemical factors but also mechanical stress [4]. Excessive mechanical-stress loading is associated with articular-cartilage degradation and plays an important role in the occurrence and development of excessive chondrocyte apoptosis [5]. A recent study reported that apoptosis of growth-plate chondrocytes is regulated by mechanical stress, and that appropriate stretch stress can effectively promote cell proliferation and differentiation, whereas excessive stress promotes their apoptosis [6]. Additionally, Li et al. [7] showed that chondrocytes tend to undergo late-stage apoptosis under compressive loading, during which caspase-12 is significantly upregulated. Chondrocytes can convert external mechanical signals into intracellular metabolic signals via

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mechanosensitive channels [8], among which transient receptor potential channel vanilloid 4 (TRPV4) is one of the most important mechanosensitive ion channels and widely distributed in the organs and tissues of various organisms [9]. Mechanical stimulation can regulate metabolic responses by activating TRPV4 and increasing intracellular Ca^{2+} concentrations [10]. Previous studies indicated that TRPV4 protein is highly expressed in articular chondrocytes, and that TRPV4-mediated Ca^{2+} signaling plays a central role in the transduction of mechanical signals to support cartilage extracellular-matrix maintenance and joint health [8,11]. However, the role of TRPV4 in chondrocyte apoptosis induced by excessive mechanical stress remains unknown.

This study determined the potential regulatory mechanism of TRPV4 in mediating Ca^{2+} influx and initiating chondrocyte apoptosis in a rat model of OA induced by mechanical stress.

2. Materials and methods

2.1. Animals and the development of a rat OA model

Male Sprague–Dawley rats (2-months old; 200–260 g) obtained from Beijing Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) were maintained in a specific pathogen-free, laminar-flow housing apparatus under controlled temperature, humidity, and a 12-h light/dark cycle. Rats were randomly assigned to five groups: normal ($n = 5$), OA-7 days ($n = 5$), OA-14 days ($n = 5$), OA-28 days ($n = 5$), and OA-28 days + GSK2193874 ($n = 5$). The rat OA model was used to prepare an anterior cruciate-ligament transection (ALCT) model, as described previously [12]. GSK2193874 (a selective TRPV4 inhibitor) was administered orally at 0.3 mg/kg every 2 days according to our previous study [12]. All animal protocols were approved by the Animal Care and Use Committee of the Nanjing University of Chinese Medicine (ACU171108), and all experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

2.2. Isolation of primary chondrocytes

Primary chondrocytes were harvested from the articular cartilage of male Sprague–Dawley rats, as previously described [13]. Cartilage tissues were washed three times with phosphate-buffered saline (PBS), cut into 2-mm^2 pieces, and incubated at 37 °C with 0.1% type II collagenase (Sigma-Aldrich, St. Louis, MO, USA) for 7 h. The samples were passed through a 70-mm cell strainer, and chondrocytes were pelleted by centrifugation at 500*g* for 5 min, plated on Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Life Technologies, Carlsbad, CA, USA), and incubated under a humidified atmosphere of 5% CO_2 at 37 °C.

2.3. Small-interfering (si)RNA preparation and transfection

To inhibit TRPV4 expression in chondrocytes, TRPV4 short interfering RNA (siRNA) was used. Chondrocytes were transfected with siRNA transfection reagent (Lipofectamine 2000) with TRPV4 siRNA (Invitrogen, Carlsbad, CA, USA) diluted into siRNA transfection medium at a final concentration of 20 pM according to the manufacturer's instructions. Chondrocytes that were transfected with scrambled control siRNA at a concentration of 20 pM were served as the negative control. Cells were transfected for 6 h.

2.4. Treatment of chondrocytes

Chondrocytes were seeded onto BioFlex plates (Flexcell International, Burlington, NC, USA) and grown to 80% confluence. BioFlex plates were subjected to 20% surface elongation at a frequency of six cycles/min using a computer-controlled vacuum stretch apparatus (FX-4000T Tension Plus System; Flexcell International) and harvested after 6 h, 12 h, and 24 h, respectively. Additionally, chondrocytes were treated with cyclic stretching following pretreatment with 100 μM GSK2193874 or *TRPV4* siRNA in order to evaluate the role of TRPV4 in chondrocyte apoptosis.

2.5. Hematoxylin-eosin (H&E) staining

Cartilage was collected after sacrificing the rats and fixed with 4% paraformaldehyde, soaked in ethylenediaminetetraacetic acid (EDTA), embedded in paraffin, and cut into slices for routine H&E staining.

2.6. Immunohistochemistry

Cartilage tissue was fixed with 4% paraformaldehyde for 2 weeks and decalcified in EDTA decalcification liquid for 3 weeks, followed by paraffin embedding and cutting into 4-μm sections. After antigen retrieval and blocking of endogenous peroxidase activity, the sections were incubated with the anti-calmodulin antibody (1:2000; Bioss, Beijing, China) at 4 °C overnight. The secondary antibody was then added for 30 min at room temperature, and staining was detected with 3,3′-diaminobenzidine tetrahydrochloride. Sections were counterstained with hematoxylin for 5 min to stain the nucleus and then dehydrated with an ethanol gradient, cleared with xylene, and mounted onto a coverslip.

2.7. Enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in rat serum were estimated using a rat ELISA kit (FcMACS, Nanjing, China) according to manufacturer instructions.

2.8. Measurement of Ca^{2+} influx

Treated cells were washed three times with PBS and loaded with 2 μmol/L Fluo-4AM (Beyotime Biotechnology, Shanghai, China) for 30 min at 37 °C in the dark, followed by two washes with PBS to remove extracellular Fluo-4/AM. Samples were observed by fluorescence microscopy (Leica, Wetzlar, Germany) and analyzed with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD, USA).

2.9. Western blot

Western blot was performed, as previously described [13]. Blots were incubated with primary antibodies, including anti-TRPV4 (1:2000; Affinity Biosciences, Cincinnati, OH, USA), calmodulin (1:1000; Bioss, Beijing, China), FAS-associated protein with death domain (FADD; 1:500; Bioss), cleaved-caspase-8 (1:500; Bioss), cleaved-caspase-3 (1:500; Bioss), cleaved-caspase-6 (1:2000; Proteintech, Rocky Hill, NJ, USA), and cleaved-caspase-7 (1:2000; Affinity Biosciences).

2.10. Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was extracted from chondrocytes using Trizol reagent (Invitrogen), and RNA concentration and purity were measured with a spectrophotometer, with a 260 nm/280 nm threshold ratio of between 1.8 and 2.0. cDNA was synthesized from 2 μg of total RNA using random primers and the M-MLV reverse transcriptase (Invitrogen). RNA expression was measured with a SYBR Green PCR kit using an ABI 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA) according to manufacturer instructions and using the following cycling: 3 min at 95 °C, followed by 40 cycles for 15 s at 95 °C and 15 s at 60 °C. The relative expression level of the target gene was calculated using the $2^{-\Delta\Delta CT}$ method.

2.11. Flow cytometry

After treatment, cells were double-stained with annexin V-fluorescein isothiocyanate and propidium iodide (Annexin V-Alexa647 apoptosis detection kit; FcMacs) according to manufacturer instructions. Flow cytometry was performed using Cell Quest software (Beckman Coulter, Brea, CA, USA).

2.12. Statistical analysis

All experiments were performed in triplicate, and statistical analyses were performed using GraphPad Prism 6.0 software (San Diego, CA, USA). All data represent the mean + standard deviation. Data were analyzed by one-way or two-way analysis of variance with Bonferroni's or Dunnett's post-test to compare multiple columns (as appropriate).

3. Results

3.1. TRPV4 inhibitor attenuated cartilage degeneration in rat OA models

The cartilage tissue in rat OA models showed progressive aggravation of cartilage degeneration as observed by hematoxylin-eosin staining, which was markedly inhibited by the selective TRPV4 inhibitor (Fig. 1A). The results of ELISA showed that the level of IL-6 and TNF-α in serum increased gradually (Fig. 1B).

3.2. TRPV4 expression was confirmed in normal articular cartilage and TRPV4 was upregulated in OA articular cartilage

TRPV4 mRNA was detected by RT-PCR and TRPV4 protein was identified by western blot in articular cartilage. TRPV4 was weakly expressed in normal cartilage. Accordingly, high TRPV4 mRNA expression was detected in the OA articular cartilage (Fig. 2C), which is consistent with results obtained via western blot (Fig. 2A; B). TRPV4 levels were significantly greater in the OA cartilage, and the degree of up-regulation was positively correlated with the degree of OA lesions.

3.3. TRPV4 inhibitor inhibited the upregulated expression of calmodulin and cleaved caspase-8 in the OA articular cartilage

Calmodulin, and apoptosis marker cleaved caspase-8 protein levels were detected by western blot and found to be weakly expressed in normal cartilage but showed high expression in the OA articular cartilage (Fig. 2A; B). Calmodulin, and cleaved caspase-8 mRNA levels as detected by RT-qPCR were consistent with the western blot results (Fig. 2C). The TRPV4 inhibitor inhibited the high expression of

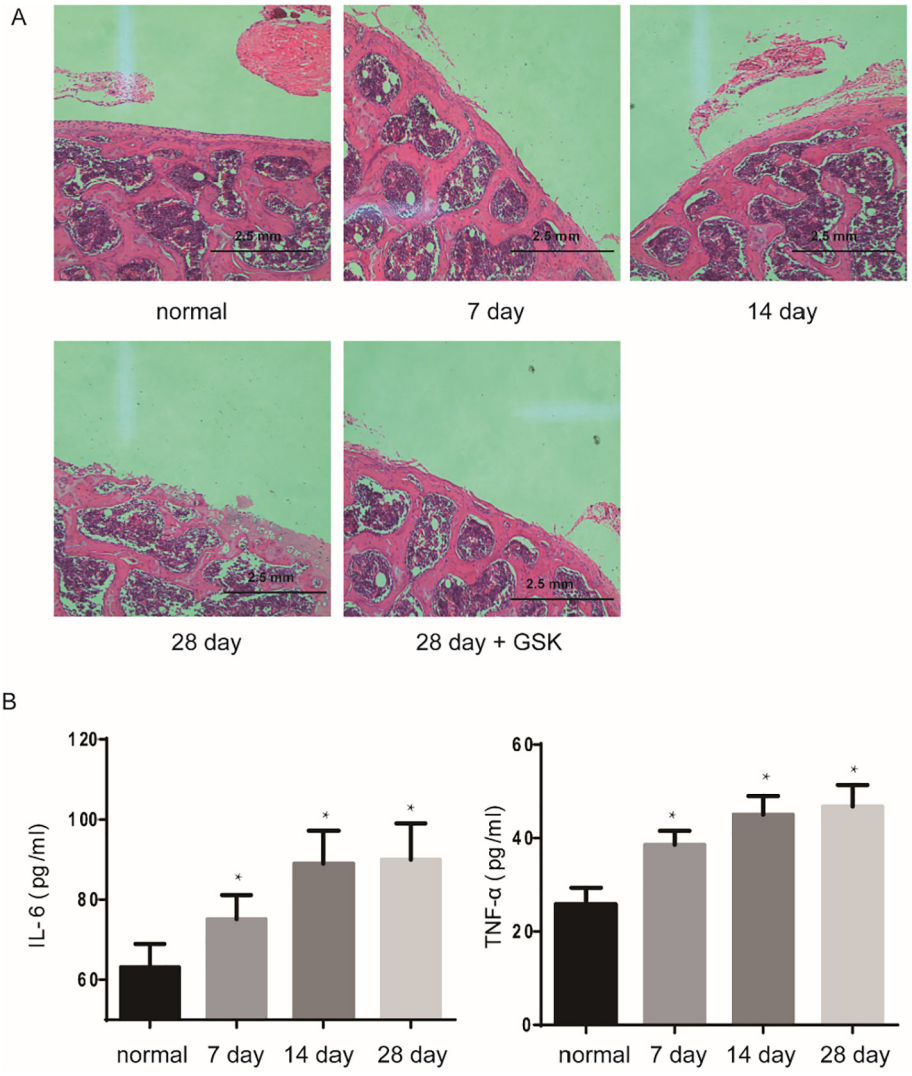

Fig. 1. TRPV4 inhibitor attenuated cartilage degeneration in rat OA models. (A) Representative cartilage tissues of each group stained with HE, 40×, scale bar = 2.5 mm; (B) The levels of IL-6 and TNF-α in the rat serum were detected by ELISA. Data are expressed as the mean ± SD. *P < 0.05 vs. normal group.

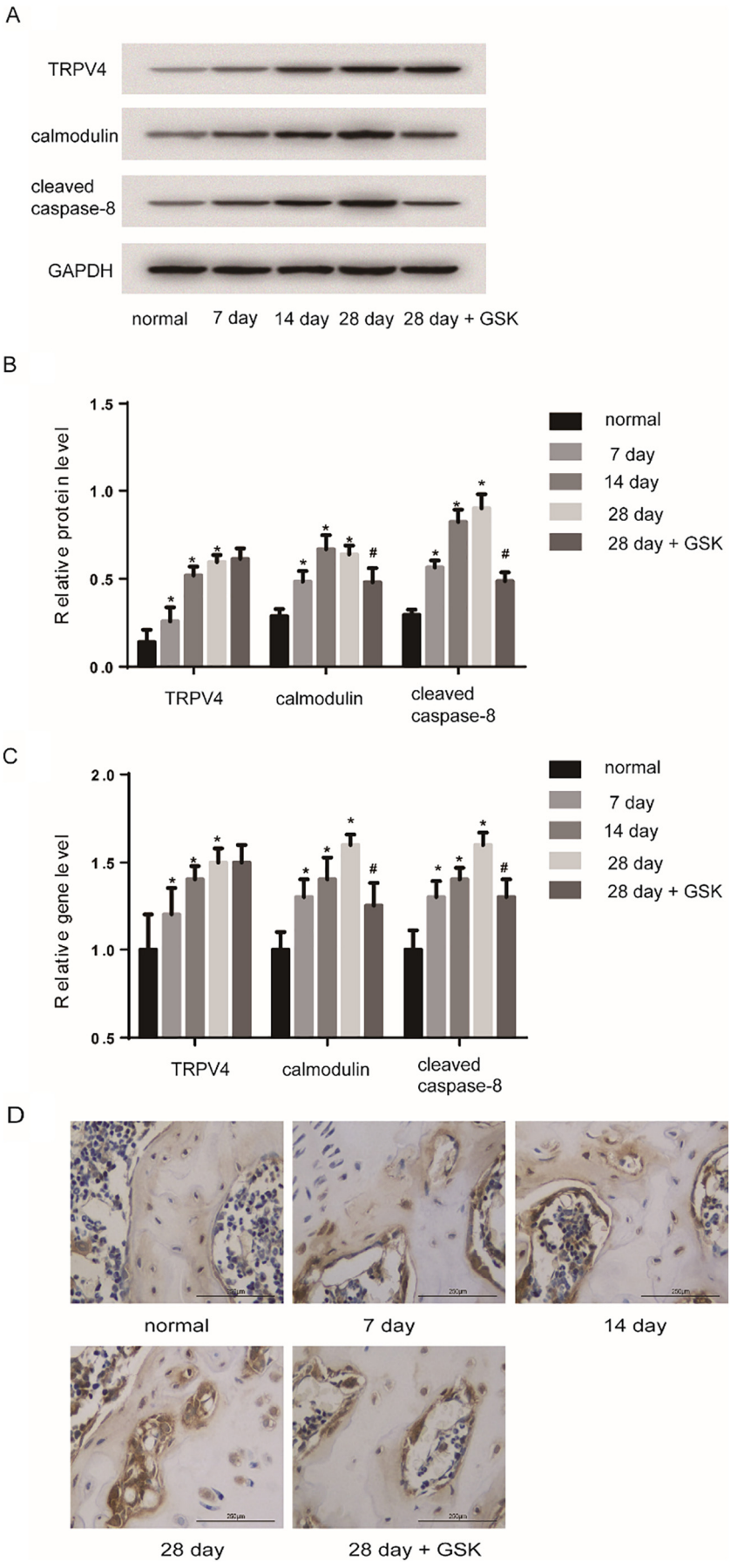

Fig. 2. TRPV4 inhibitor inhibited the high expression of calmodulin and cleaved caspase-8. (A) TRPV4, calmodulin, cleaved caspase-8 protein expressions were detected by western blotting. (B) Bar graph showing the levels of TRPV5, calmodulin, and cleaved caspase-8 proteins in different groups. (C) RT-qPCR showing the levels of TRPV4, calmodulin, and cleaved caspase-8 mRNA in different knee OA time points. (D) Calmodulin protein in articular cartilage was immunolocalized by immunohistochemistry. Brown staining indicates specific calmodulin protein, while blue staining indicates the nucleus, 400×, scale bar = 250 μm. Data are expressed as the mean ± SD. *P < 0.05 vs. normal group; #P < 0.05 vs. 28-day group. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

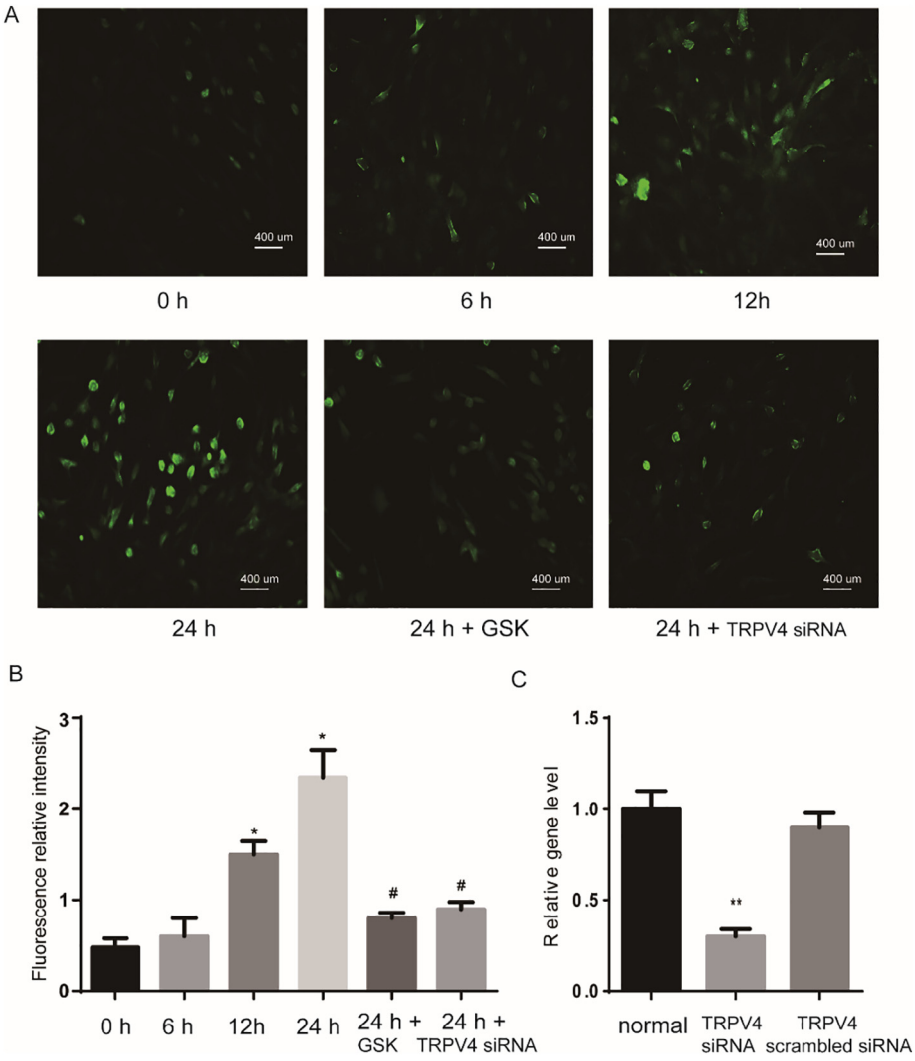

Fig. 3. Excessive mechanical stress increased Ca^{2+} influx mediated by TRPV4.
(A) Relative Ca^{2+} fluorescence intensity in different groups (200×, scale bar = 400 μm). (B) Bar graph showing relative fluorescence intensity. (C) The silencing effect of the TRPV4 siRNA was confirmed by PCR. Data are expressed as the mean ± SD. *P < 0.05 vs. 0 h group; #P < 0.05 vs. 24 h group; **P < 0.05 vs. normal group.

calmodulin and cleaved caspase-8.

Additionally, calmodulin protein was immunolocalized in articular cartilage according to immunohistochemistry analysis. In normal cartilage, staining was very light. Staining levels progressively increased with increasing knee OA severity (Fig. 2D).

3.4. Excessive mechanical stress induced chondrocytes apoptosis by activating TRPV4

The results showed that relative fluorescence intensity increased gradually along with increased cyclic stretch time, with fluorescence intensity significantly reduced following treatment with either the TRPV4 inhibitor or *TRPV4* siRNA (Fig. 3). Additionally, flow cytometric analysis indicated significant increases in the percentage of apoptotic cells according to cyclic stretch time, with these increases also attenuated following administration of the TRPV4 inhibitor or transfection of *TRPV4* siRNA (Fig. 4). These results suggested that TRPV4 played an important role in chondrocyte Ca^{2+} influx and apoptosis induced by mechanical stress.

3.5. Excessive mechanical stress stimulated caspase-8-dependent apoptotic signaling in chondrocytes via TRPV4 activation

To investigate the specific mechanism associated with mechanical-stress-related induction of chondrocyte apoptosis via TRPV4, core protein levels were detected by western blot. The results showed that levels of calmodulin, FADD, and cleaved caspase-8, -3, -6, and -7 gradually increased along with increases in cyclic stretch time, with these increases significantly attenuated following treatment with either the TRPV4 inhibitor or transfection of *TRPV4* siRNA (Fig. 5).

4. Discussion

OA is a common degenerative arthropathy characterized by progressive articular cartilage degeneration, which might be largely driven by elevations in chondrocyte apoptosis [14]. Moreover, abnormal mechanical stress associated with obesity, trauma, and joint instability alters joint loading and is closely related to cartilage degeneration and chondrocyte apoptosis [4]. Therefore, exploring the specific mechanisms associated with mechanical-stress-induced chondrocyte apoptosis is important to promote the development of targeted therapies for OA. In this study, we found that excessive mechanical stress increased levels

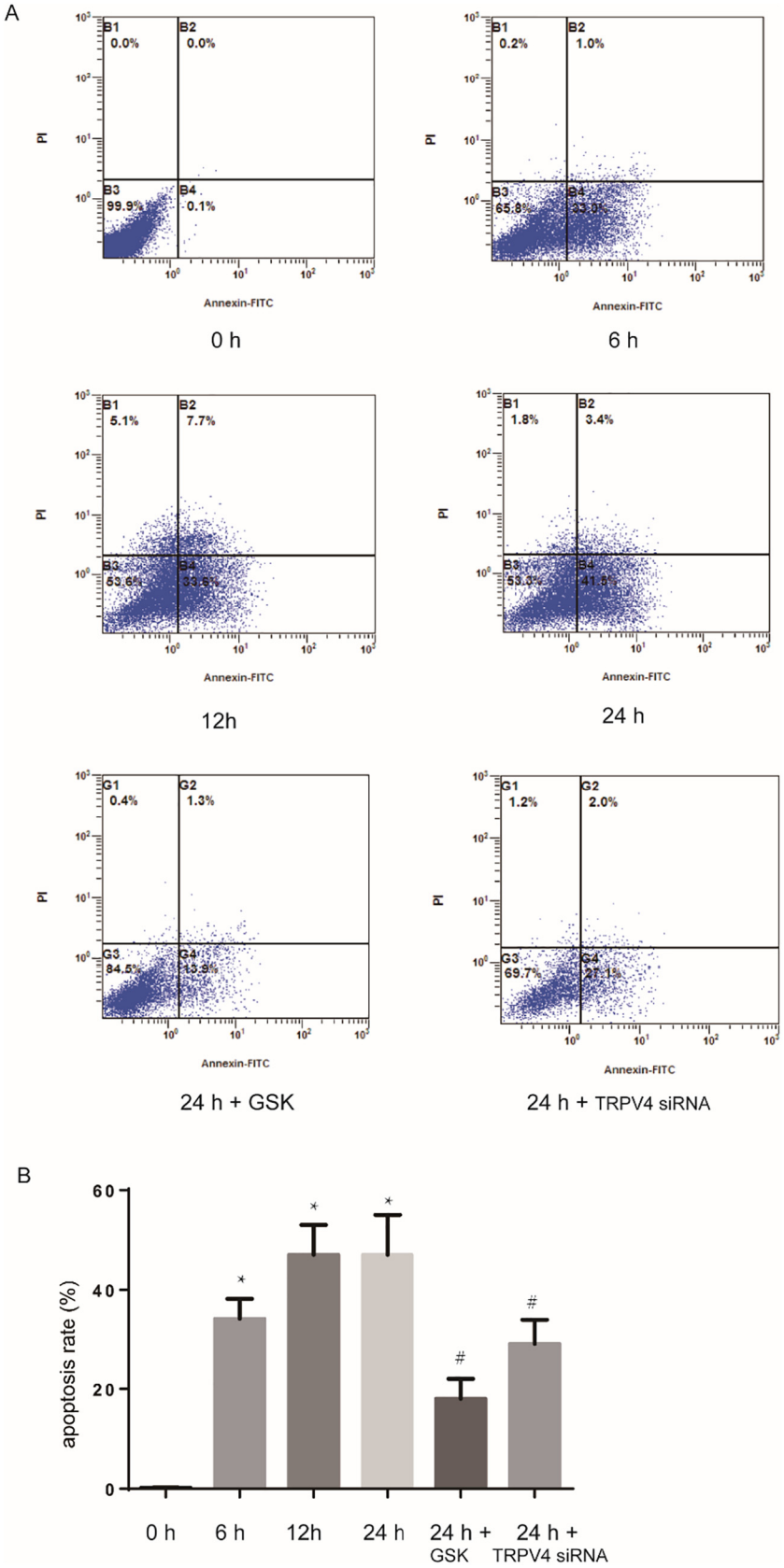

Fig. 4. Excessive mechanical stress induced chondrocyte apoptosis mediated by TRPV4. (A) Flow cytometry with Annexin V-FITC/PI staining of chondrocytes. (B) Bar graph showing the apoptosis rate in each group. Data are expressed as the mean ± SD. *P < 0.05 vs. 0 h group; #P < 0.05 vs. 24 h group.

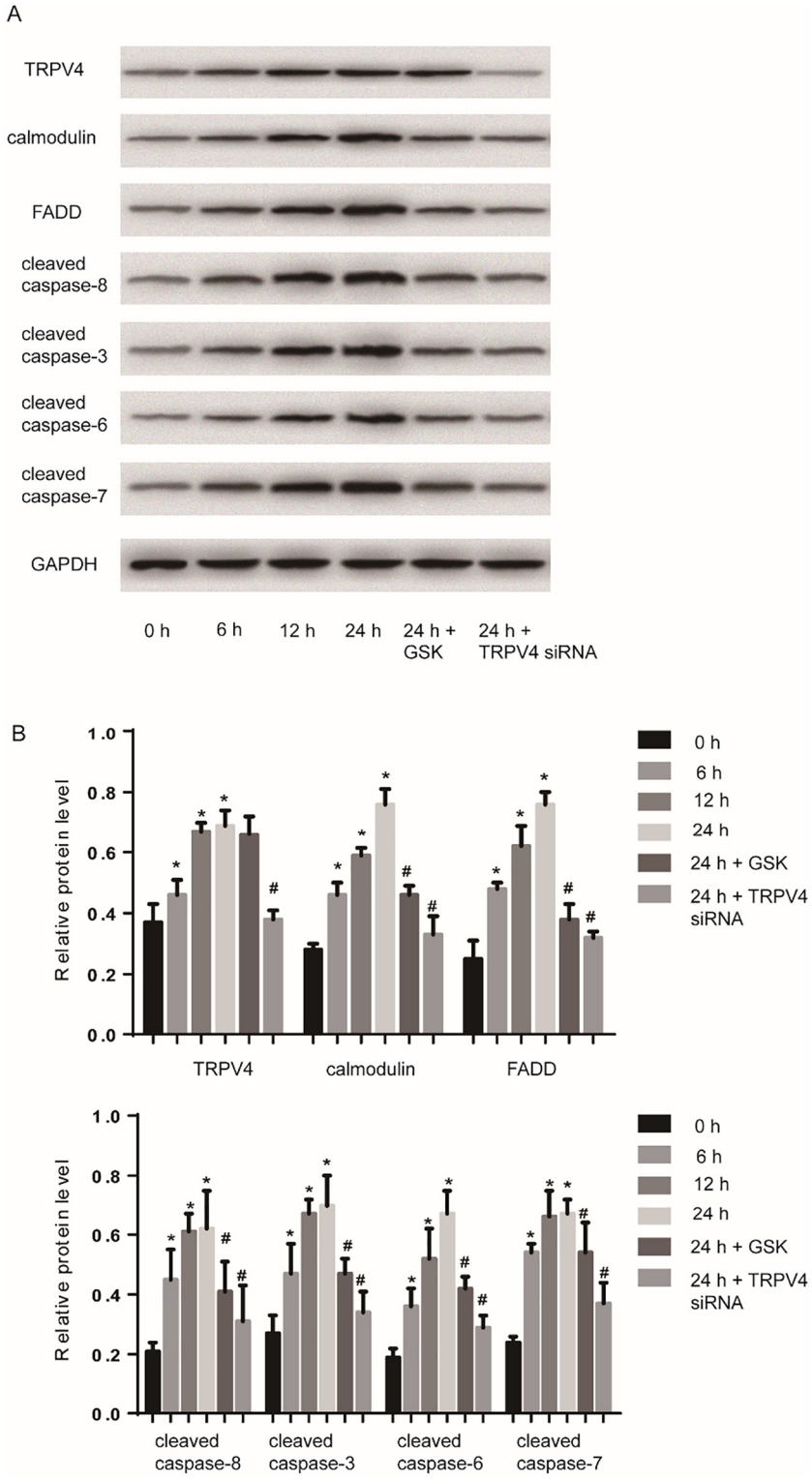

Fig. 5. Excessive mechanical stress stimulated caspase-8-dependent apoptotic signaling pathway in chondrocytes by activating TRPV4. (A) TRPV4, calmodulin, FADD, cleaved caspase-8, cleaved caspase-3, cleaved caspase-6, and cleaved caspase-7 protein expression was detected by western blotting. (B) Bar graph showing the relative levels of TRPV4, calmodulin, FADD, cleaved caspase-8, cleaved caspase-3, cleaved caspase-6, and cleaved caspase-7. Data are expressed as the mean ± SD. *P < 0.05 vs. 0 h group; #P < 0.05 vs. 24 h group.

of TRPV4, which contributed to excessive Ca^{2+} Influx and initiation of apoptosis. Attenuation of TRPV4 levels or inhibition of TRPV4-related activity produced a protective effect against cartilage degeneration and chondrocyte apoptosis.

Surgical disruption of joint ligaments results in excessive mechanical stress and simulates chronic OA after trauma, with this widely used model more accurately mimicking the pathological process of human OA, particularly chronic OA after trauma [15]. In the present study, we successfully constructed an ALCT-induced OA model, which displayed cartilage degeneration and elevated levels of inflammatory

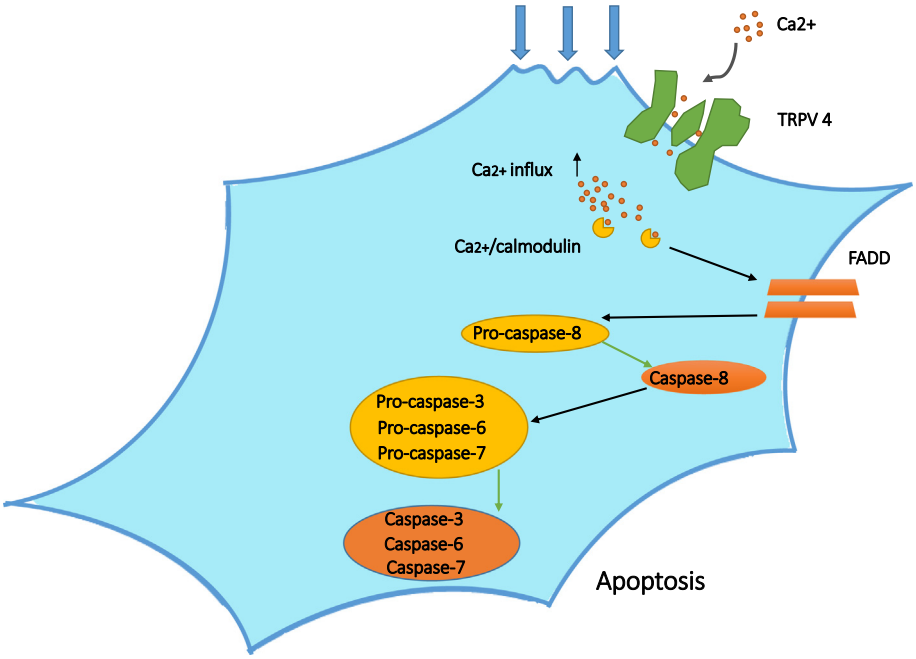

Fig. 6. Signaling cascade involved in the effect of TRPV4-mediated Ca^{2+} influx on apoptosis in mechanical stress-induced chondrocytes. TRPV4 may exert a core initiation effect to mediate the induction of apoptosis signaling via cytosolic Ca^{2+} accumulation.

factors.

The TRP channel is a non-selective cation channel located on the cell membrane and capable of converting Ca^{2+} influx, temperature changes, and mechanical, chemical, and other stimuli into intracellular signals [16]. TRPV4 is present in various tissues and mediates responses to pathological changes, such as pain and inflammation [17]. Following discovery of TRPV4 in bone, cartilage, and the synovium, its role as a receptor associated with mechanical stress in OA was confirmed [18], with O'Conor et al. [8] showing that TRPV4 regulates mechanical-stress-induced chondrocyte dissimilation [8]. Another study reported that abnormal mechanical stimulation increases and activates TRPV4 levels on chondrocytes, thereby increasing the expression of inflammatory factors. [19]. However, the potential role of TRPV4 in chondrocyte apoptosis induced by mechanical stress had not been studied. Here, we found that excessive mechanical stress increased functional expression of TRPV4 on the chondrocyte surface, which is consistent with findings reported previously [8,20]. Interestingly, we also found that TRPV4 levels positively correlated with cartilage degeneration and apoptosis, with these changes mitigated by inhibition of TRPV4-related activity or *TRPV4* knockdown.

In vitro evaluation of TRPV4-related responses to mechanical stress in chondrocytes suggested its roles in the initiation of apoptosis. Ca^{2+} influx through Ca^{2+}-selective channels mediate apoptosis and DNA fragmentation [21,22]. In the present study, we found that increased mechanical stress applied to chondrocytes also increases levels of TRPV4-mediated Ca^{2+} influx, which induced apoptosis. This finding agreed with previous studies reporting that TRPV4 inhibition or deficiency reduces apoptosis of cardiomyocytes neurons [23] and oligodendrocytes [24]. Ca^{2+} is a major intracellular second messenger, with a previous study showing that elevated intracellular Ca^{2+} levels activate calmodulin to form Ca^{2+}/calmodulin complexes [25], which promote FADD recruitment and participation in exogenous apoptotic pathways [26]. In the present study, we found that Ca^{2+} influx via TRPV4 upregulated a caspase-8-dependent apoptosis-related signaling pathway in chondrocytes, and that inhibition of TRPV4-related activity inhibited activation of this pathway (Fig. 6).

TRPV4 plays an important role in many physiological and pathophysiological processes. Results from animal studies suggest that TRPV4 antagonism has therapeutic potential in oedema, pain, gastrointestinal disorders, and lung diseases such as cough, bronchoconstriction, pulmonary hypertension, and acute lung injury [27,28]. Our results suggested that TRPV4 is closely related to cartilage degeneration and targeting TRPV4 may be a beneficial therapeutic target for the treatment of OA.

There were limitations with this study. First, this conclusion has not been validated in humans and other animals, with results in a TRPV4-knockout animal model required for validation of these findings. Second, as a non-selective cation channel, TRPV4 also can transport Na^+ and K^+; therefore, we cannot rule out roles for Na^+ and K^+ flux in chondrocyte status [29]. The role of TRPV4-mediated Na^{2+} and K^+ flux in chondrocyte apoptosis requires further investigation.

5. Conclusion

In summary, based on the data obtained from rats and in vitro cells, we found that TRPV4 was functionally expressed in normal cartilage and upregulated in OA articular cartilage. Additionally, excessive mechanical stress induced chondrocyte apoptosis via TRPV4-mediated increases in Ca^{2+} influx. These results suggest TRPV4 as a potential drug target in OA.

Acknowledgments

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Conflict of interest statement

The authors declare that there are no conflicts of interest.

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