# *Brief Communication*

# Therapeutic effects of kartogenin on temporomandibular joint injury by activating the TGF-β/SMAD pathway in rats

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### Impact Statement

Temporomandibular dysfunction (TMD) is a common disease affecting 30% of adults. Patients with TMD could suffer from pain and joint dysfunctions, which impacts their quality of life severely. Pathology of TMD included a deterioration and abrasion of articular cartilage and subsequent subchondral bone remodeling. Current treatment of TMD alleviates symptoms but could not reverse the pathology of cartilage and subchondral bone. This study aims to disclose the effects of kartogenin on cartilage and subchondral in TMD with tissue injury via the rat model. Results indicate that kartogenin could repair the injured condylar cartilage, make the condylar surface smooth, promote cell proliferation via activating the TGF-β/SMAD pathway, and strengthen the subchondral condylar bone. Kartogenin may have the ability to cease or correct the ongoing pathology of condylar cartilage by promoting tissue regeneration. Kartogenin may be a promising intra-articular injection agent for the treatment of TMD.

### Abstract

Patients with temporomandibular dysfunction (TMD) usually suffer from pathology or malpositioning of the temporomandibular joint (TMJ) disk, leading to the degenerative lesion of condyles. Kartogenin can promote the repair of damaged cartilage. This study aimed to explore whether intra-articular injection of kartogenin could alleviate the TMJ injury induced by type II collagenase. We measured the head withdrawal threshold and found that kartogenin alleviated the pain around TMD induced by type II collagenase. We observed the morphology of the condylar surface and found that kartogenin protected the integration of the condylar surface. We analyzed the density of the subchondral bone and found that kartogenin minimized the damage of TMJ injury to the subchondral bone. We next explored the histological changes and found that kartogenin increased the thickness of the proliferative layer and more collagen formation in the superficial layer. Then, to further ensure whether kartogenin promotes cell proliferation in the condyle, we performed immunohistochemistry of proliferating cell nuclear antigen (PCNA). The ratio of PCNA-positive cells was significantly increased in the kartogenin group. Next, immunofluorescence of TGF-β1 and SMAD3 was performed to reveal that kartogenin activated the TGF-β/SMAD pathway in the proliferative layer. In conclusion, kartogenin may have a therapeutic effect on TMJ injury by promoting cell proliferation in cartilage and subchondral bone. Kartogenin may be promising as an intra-articular injection agent to treat TMD.

**Keywords:** Temporomandibular joint, dysfunction, kartogenin, cartilage, cell proliferation, collagenase

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## Introduction

Temporomandibular dysfunction (TMD) is a common condition that occurred in up to 60 of the population and women have a higher incidence than men.[1,2] The etiology of TMD remains controversial, whereas it is broadly related to anatomical, pathophysiological, and psychosocial factors.[1,3,4] TMD patients often suffer from pathology or malpositioning of the temporomandibular joint (TMJ) disk, the primary pathology of which appears degenerative lesion along with the morphology changes of condyles. Pathological observations revealed a deterioration and abrasion of articular cartilage and subsequent subchondral bone remodeling.[5] Patients with TMD could suffer from pain and joint dysfunctions, which impacts their quality of life severely.[3,4]

Kartogenin (KGN) is a non-protein small molecule compound, which was first identified by Johnson Kristen and his colleagues in 2012.[6] KGN binds filamin A, disrupts its interaction with the transcription factor core-binding factor β subunit, and induces chondrogenesis by regulating the transcriptional complex of core-binding factor β subunit and RUNX family transcription factor 1. Previous studies demonstrated that KGN could promote cartilage regeneration in knee osteoarthritis (OA).[7,8] The TMJ is a hinge-type synovial joint in which fibrocartilage covers the condyles, unlike the hyaline cartilage in the knee joint. Hence, it remains unclear whether KGN could be applied to the treatment of TMJ degeneration.

Transforming growth factor-β (TGF-β) is a regulator for articular cartilage metabolic homeostasis and structural

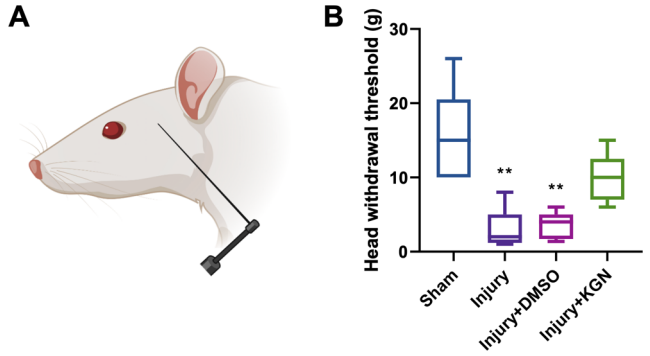


**Figure 1.** Pain in the TMJ region in rats treated with KGN. (A) Illustration of head withdrawal measurement by von Frey hairs. (B) The head withdrawal threshold of rats at four weeks ($n = 5$ in each group). ***$P < 0.001$ by comparing Sham group or Injury + KGN group to Injury group or Injury + DMSO group.

integrity.[9] TGF-β pathway is essential for chondrogenesis in all biological phages in normal conditions,[10] and its regulatory role in cartilage matrix proteins and metalloproteases also prevents hypertrophy of chondrocytes.[11] Cumulative evidence supported the protective role of TGF-β in cartilage, while some evidence suggested that the TGF-β pathway may perform a deleterious effect and could be contributed to the development of OA.[12] Any interruption of the TGF-β signaling pathway would impair cartilage integrity and subsequently cause a pathological process. Whether KGN affects the TGF-β/SMAD pathway also remains unclear.

Type II collagenase could cause cartilage matrix degradation as well as subchondral bone degradation.[13,14] Its application has greatly provided an ideal option for researching changes in cartilage and subchondral bone in temporomandibular joint osteoarthritis (TMJOA) in different animals, including rats.[14] Therefore, this study aimed to explore the treatment effect of KGN on type II collagenase–induced TMJ injury and the relationship between the effect and the TGF-β/SMAD pathway.

## Materials and methods

### Animal model establishment

All procedures were approved by the ethics committee, West China School of Stomatology, Sichuan University, under document number WCHSIRB-D-2019–092. Eight-week-old male Sprague Dawley rats weighing an average of 200 g (purchased from Dossy Experimental Animal Limited Company, Chengdu, China) were used in this research. Pentobarbital of 0.1% was used by 0.4 mL per 100 g weight for anesthesia. Type II collagenase of 0.05 mL (C2-BIOC, Sigma, St. Louis, MO, USA) with 2% w/v was injected into the articular cavity unilaterally to establish the TMJ injury model as previously described.[15] Saline of 0.05 mL was used in the sham injection. In the KGN (B5626; APExBIO, Houston, TX, USA) or dimethylsulfoxide (DMSO, D8371; Solarbio, Beijing, China) group, 0.05 mL 100 μM KGN dissolved in 5.2‰ DMSO or 0.05 mL 5.2‰ DMSO was injected into the articular cavity at one week and two weeks after the injection of type II collagenase. Bilateral TMJs were harvested for subsequent analysis four weeks after the model establishment.

### Head withdrawal threshold measurement

The von Frey test was used to measure the pain experienced by the rats at four weeks.[16] The hard-plastic tip was used to stimulate the midpoint of the connection between the eyes and ears of the rat's head and face (Figure 1A). The rat behavior was observed with the mechanical stimulation intensity increased. When reactions such as rubbing the mouth or scratching the head were observed, the stimulation intensity was recorded. The operation was repeated five times every 30 s, and the head withdrawal threshold (HWT) was defined as the average of measured stimulation intensity.

### Stereomicroscope observation

Bilateral TMJs were dissected, fixed by 4% paraformaldehyde solution for 24 h, and transferred in 70% ethanol at 4°C before analysis. The morphology of the TMJ specimens was observed under a stereomicroscope (OLYMPUS, SXZ16, Japan).

### Micro-computed tomographic scanning

Micro-computed tomographic (micro-CT) scanning for the changes of subchondral bones was applied by Micro-CT (SCANCO Medical AG, Switzerland) with the following parameters: 70 kV/200 μA, 20 μm voxel size. Data analysis was performed by SCANCO Evaluation application, including bone volume to tissue volume (BV/TV), trabecular number (Tb. N), trabecular thickness (Tb. Th), and trabecular space (Tb. Sp).

### Hematoxylin and eosin staining

Each TMJ was fully fixed with 10% neutral buffered formalin, decalcified in 10% ethylene diamine tetraacetic acid, dehydrated with alcohol, cleared with xylene, and embedded in paraffin. The TMJ specimens were cut into 10-μm-thick sections for histological analysis. For hematoxylin and eosin (H&E) staining, the sample sections were rehydrated with alcohol and stained with hematoxylin for 10–15 min, followed by eosin for 5–10 min, and finally dehydrated and sealed. The OARSI-modified Mankin score was calculated as previously described.[17]

### Safranin O and toluidine blue O staining

The preprocessing of TMJ specimens was as described above. Safranin O (SO) (G1371; Solarbio) and toluidine blue O (TBO) staining (G2543; Solarbio) were performed according to manufacturers' protocols.

### Immunohistochemistry staining

The sections were deparaffinized with xylene, treated with hydrogen peroxide, and then treated with citric acid at 70°C for antigen retrieval. Diluted primary antibodies against proliferating cell nuclear antigen (PCNA) (10205-2-AP; Proteintech, Rosemont, IL, USA) were added for incubation with the sections at 4°C overnight. Then, horseradish peroxidase conjugated secondary antibody (SE134; Solarbio) was added and incubated with the sections at 37°C. Subsequently, DAB staining (DA1016; Solarbio) was performed to show

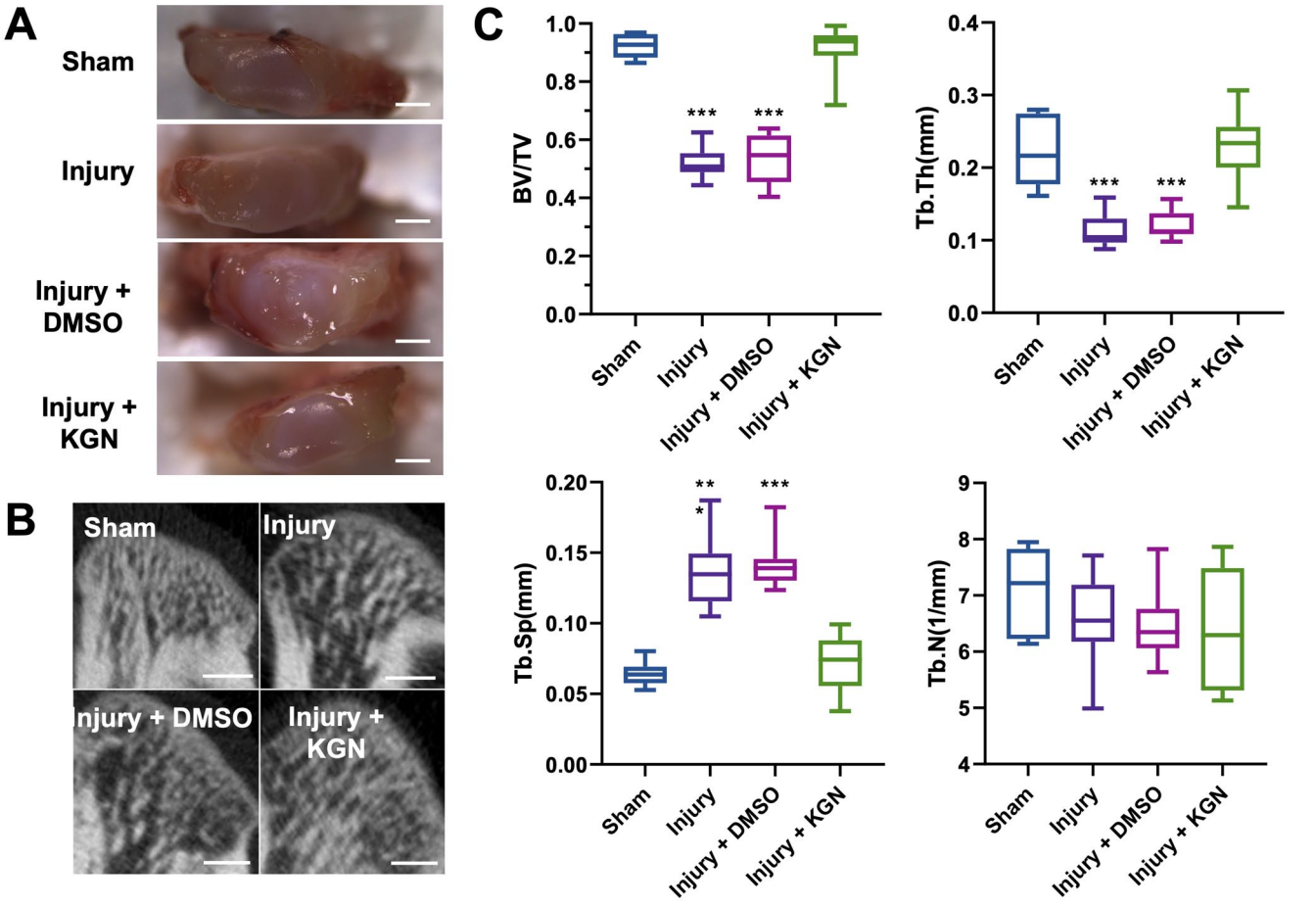


**Figure 2.** Stereomicroscope and micro-CT analysis of TMJ treated with KGN. (A) Representative images of gross observation of TMJ specimens. Scale bar = 1 mm. (B) Representative images of sagittal plane images of micro-CT analysis for the changes of subchondral bones. Scale bar = 1 mm. (C) Bone parameter analysis of BV/TV, Tb. N, Tb. Th, and Tb. Sp ($n = 5$ in each group). ***$p < 0.001$ by comparing Sham group or Injury + KGN group to Injury group or Injury + DMSO group.

color, and hematoxylin counterstaining was performed to visualize the nucleus.

### Immunofluorescence staining

Bilateral TMJs were harvested and fixed by 4% paraformaldehyde solution for 24 h and transferred in 70% ethanol at 4°C before analysis. The specimens were sliced into 4-μm-thick sections. Sections were incubated with specific primary antibodies for TGF-β-1 (1:200, PA1-29032; Invitrogen, Carlsbad, CA, USA) and SMAD3 (1:200, 9513; CST, Danvers, MA, USA). Secondary antibodies including FITC-conjugated (1:1000, ab6785; Abcam) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594, 1:1000, ab150080; Abcam, Cambridge, UK) were used followed by primary antibodies. The nuclei were stained with DAPI (C0065; Solarbio). Then the sections were observed on an Olympus microscope.

### Statistical analysis

All the data were analyzed using GraphPad Prism 8.3.0 (GraphPad Software, Inc., San Diego, CA, USA). When parametric test assumptions were met, the significant difference of multiple groups was compared by one-way analysis of variance followed by the Dunnett $t$ test. The difference was considered statistically significant with $P < 0.05$.

## Results and discussion

We first observe the rat behavior by the von Frey test. We found that the HWT in the injury or DMSO group was significantly lower than that in the sham group, whereas the threshold also seemed a little lower in the KGN group but the difference did not in a significant level (Figure 1B). Then, we observed the morphology of the condylar surface (Figure 2A). The surface of the condyle in the sham group was smooth and continuous, while there were large defects in the injury group induced by type II collagenase. DMSO group was set to exclude the potential impacts of solvents for KGN. Results showed that the morphology in the DMSO group has no significant difference from the injury group. With the application of KGN, the surface of the fibrocartilage became less rough and showed smaller pits.

Magnitude and frequency of jaw loading influence stem and fibrocartilage cell chemistry, causing alterations in osteogenesis of subchondral bone.[18] Hence, we would like to observe changes in the subchondral bone (Figure 2B and C). Both the injury and DMSO groups showed significantly decreased BV/TV and Tb. Th, and increased Tb. Sp ($P < 0.05$), indicating the possible influence of overloading in the TMJ. The KGN group showed no difference in these parameters compared to the sham group, which indicates

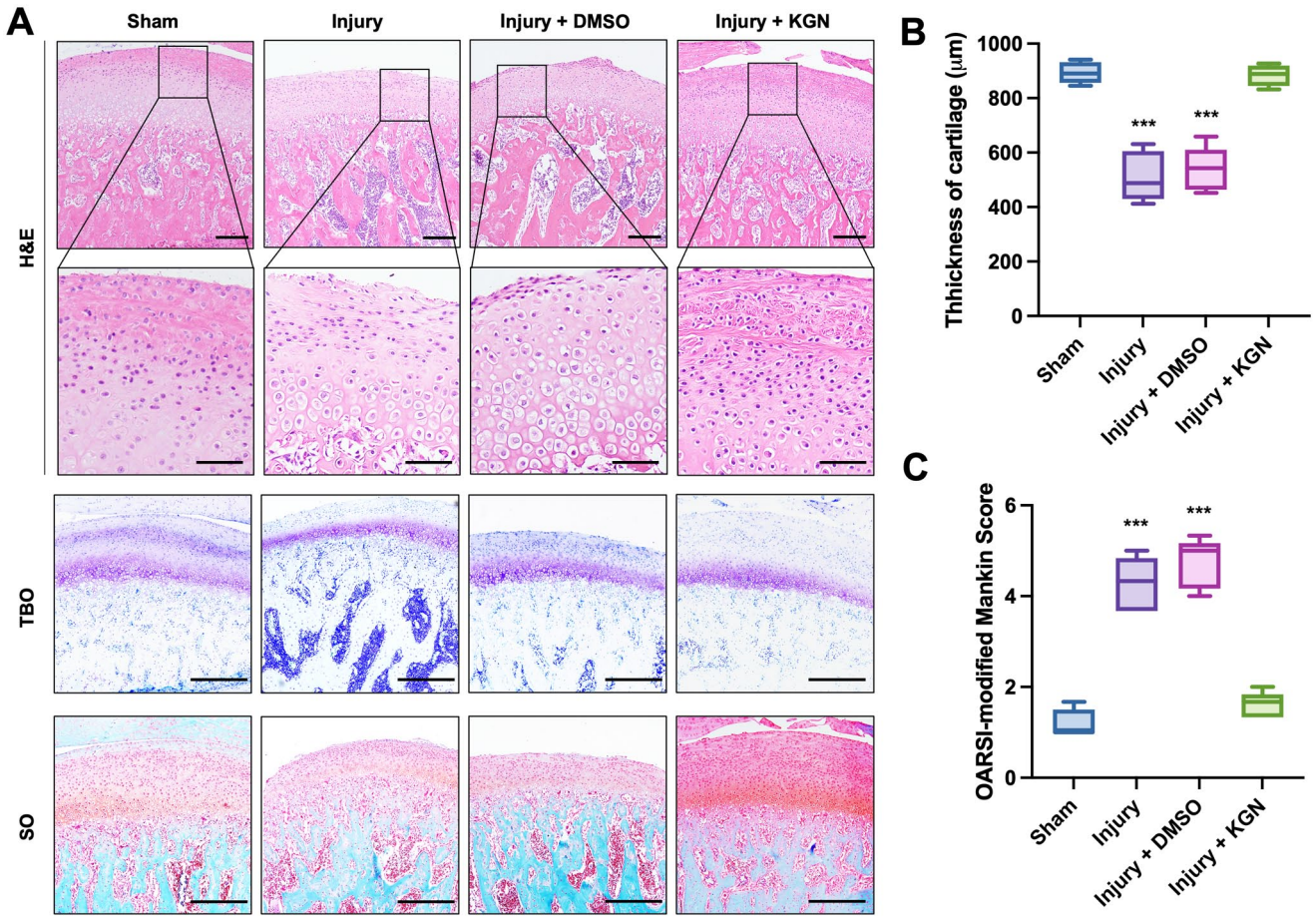


**Figure 3.** Histological structure of TMJ treated with KGN. (A) Representative images of H&E, TBO, SO staining of TMJ treated with KGN. Scale bar = 400 μm for upper panel of H&E, TBO, and SO staining. Scale bar = 50 μm for lower panel of H&E staining. (B) Quantitative analysis of OARSI-modified Mankin score ($n = 5$ in each group). (C) Quantitative analysis of cartilage thickness ($n = 5$ in each group). ***$P < 0.001$ by comparing Sham group or Injury + KGN group to Injury group or Injury + DMSO group.

that KGN minimized the secondary damage of type II collagenase to the subchondral bone.

To verify the findings in the stereomicroscopic observation and micro-CT analysis, we performed histological staining to detect the histological alterations (Figure 3A). The injury group and DMSO group showed a decreased thickness of cartilage and deficiency of the superficial zone compared to the sham group and KGN group. Moreover, below the superficial zone, mesenchymal cells in the proliferative layer are responsible for chondrocyte proliferation.[19] KGN significantly increased the thickness and cell numbers in the proliferative layer, showing more undifferentiated cells (Figure 3B). To further confirm the effects on chondrogenesis of TMJ, SO staining and TBO staining were performed (Figure 3A). Compared to the sham group, the injury group and DMSO group showed a thinner layer of cartilage matrix, while the KGN group showed a similar layer to the sham group. The OARSI-modified Mankin score was calculated and a significant increase in the KGN group compared to the DMSO group was found (Figure 3C).

As KGN could promote the cell proliferation of chondrocytes, we would like to ensure whether intra-articular injection of KGN could achieve the promotion of cell proliferation, and we performed immunohistochemistry to label PCNA-positive cells (Figure 4A). The results showed that the ratio of PCNA-positive cells in the proliferative layer decreased in the injury group and DMSO group compared to the sham group (Figure 4B). However, the KGN group showed increased PCNA-positive cells indicating that KGN may improve the ability of cell proliferation via the intra-articular injection. Another report showed that KGN could improve cell proliferation of mesenchymal stem cells and attenuate intracellular reactive oxygen species, thus promoting osteogenic differentiation of mesenchymal stem cells.[20] This may further explain the increased bone density in the KGN group.

To further evaluate the mechanisms of KGN in protecting articular cartilage, we evaluated the expression of TGF-β1 and SMAD3 in the proliferative layer of cartilage (Figure 5A). TGF-β1 and SMAD3 were innately expressed in the Sham group. While in the OA group, there was an obvious decrease of TGF-β1 and SMAD3, indicating that TMJ injury caused by type II collagenase may impair the normal activation of the TGF-β signaling pathway. With the application of KGN, the expression of TGF-β1 and SMAD3 was significantly improved, together with the increased cartilage thickness compared to the DMSO group (Figure 5B and C). We could further observe more nuclear translocation of SMAD3 in the KGN group.

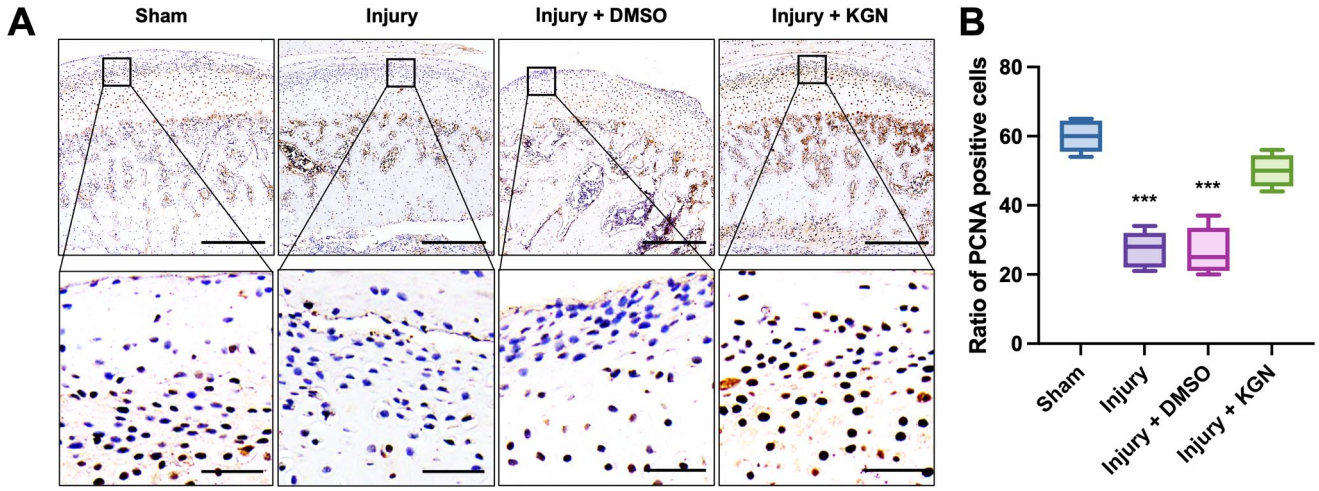


**Figure 4.** Immunohistochemistry staining of PCNA in TMJ treated with KGN. (A) Representative images of immunohistochemistry staining of PCNA in the proliferative layer. Scale bar = 400 μm for upper panel and 50 μm for lower panel. (B) Quantitative analysis of the number of PCNA-positive cells in proliferative layer ($n = 5$ in each group). ***$P < 0.001$ by comparing Sham group or Injury + KGN group to Injury group or Injury + DMSO group.

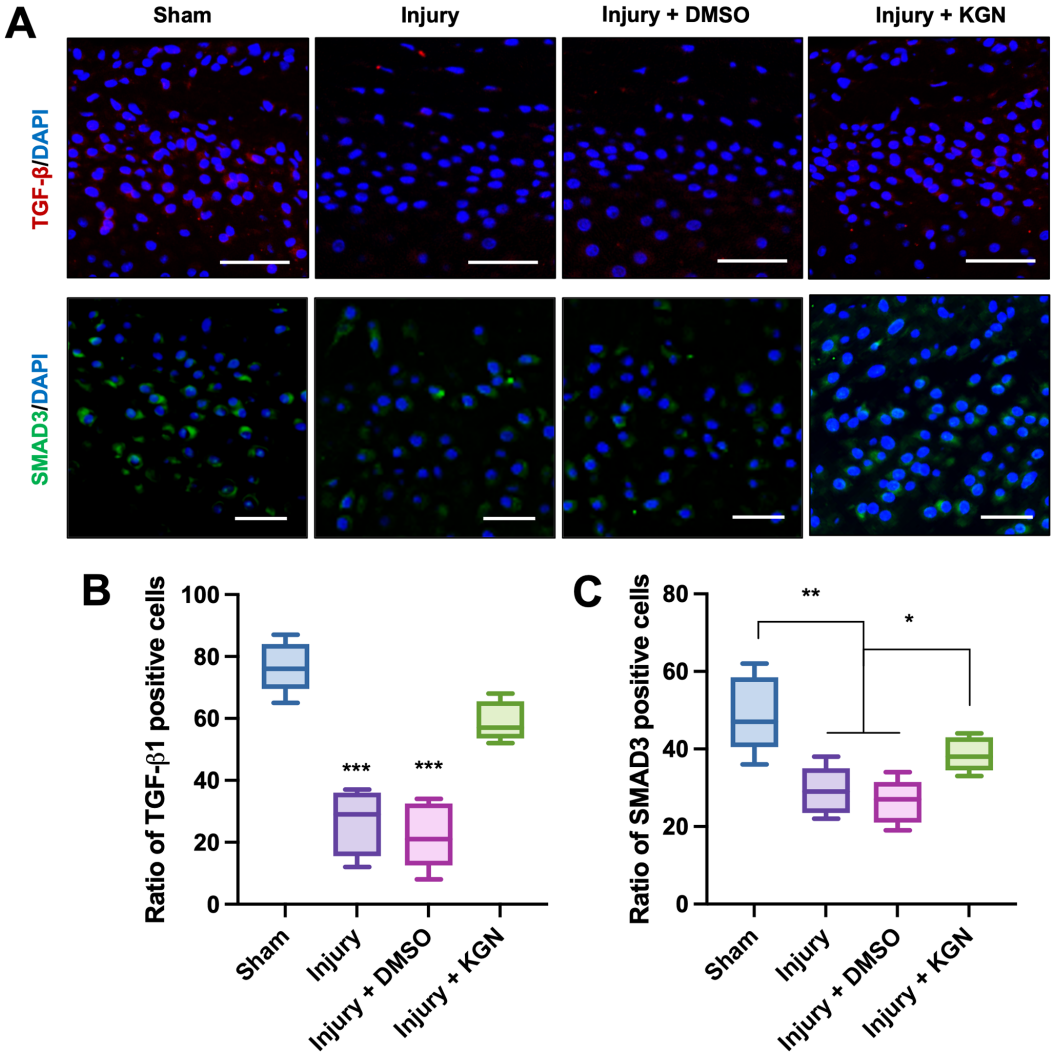


**Figure 5.** Immunofluorescent staining of TGF-β1 and SMAD3 in TMJ treated with KGN. (A) Representative images of immunofluorescent staining of TGF-β1 and SMAD3 in the proliferative layer. Scale bar = 50 μm for upper panel and 25 μm for lower panel. (B) Quantitative analysis of the number of TGF-β1-positive cells in proliferative layer ($n = 5$ in each group). ***$P < 0.001$ by comparing Sham group or Injury + KGN group to Injury group or Injury + DMSO group. (C) Quantitative analysis of the number of SMAD3-positive cells in proliferative layer ($n = 5$ in each group). **$P < 0.01$ by comparing Sham group to Injury group or Injury + DMSO group. *$P < 0.05$ by comparing Injury + KGN group to Injury group or Injury + DMSO group.

However, we did not observe an obvious difference in TGF-β1 and SMAD3 expression in the subchondral bone. A previous study showed that overexpressed TGF-β in the subchondral bone of transgenic mice leads to mandibular condyle degradation.[21] We did not exclude the possibility that the little difference could not be observed by immunofluorescence, so further quantitative analysis of proteins may be needed to identify the changes in the TGF-β signaling pathway in the bone. The difference may be also related to the origin and dosage of TGF-β, which may need further clarification.

There are some limitations in this study. First, the poor solubility of KGN hindered its intracellular delivery and caused an obstacle in the application. Several studies reported nano-controlled release systems loaded with KGN could achieve efficient intra-delivery, alleviate drug cytotoxicity, and thus enhance cartilage repair and regeneration.[22–24] Our study may consider a similar delivery system for the long-term controlled release and transmembrane delivery to mesenchymal cells to expand the application potential of KGN treating TMD in the future. Second, the pathological characteristics may be different in population and young females are the most affected population. To validate the results in animal models with different gender or age should be considered in the future. Third, although other studies have demonstrated the effect of KGN on synovial fluid-derived mesenchymal stem cells or chondrocytes *in vitro*,[25,26] it remains impractical in TMJ under our current techniques. We may consider extracting primary TMJ chondrocytes or stem cells in the future.

In conclusion, KGN may have a therapeutic effect on TMJ injury by promoting cell proliferation in cartilage and subchondral bone. KGN may be promising as an intra-articular injection agent to treat TMD.

## AUTHORS' CONTRIBUTIONS

All authors participated in the design, interpretation of the studies and analysis of the data and review of the manuscript; ZCZ, YL, and CZW performed the experiments, acquired, analyzed, interpreted the data, and wrote the manuscript; TX and YL analyzed and interpreted the data; ZCZ, YL, and CZW drafted the manuscript; CYB and PJ supervised the study and revised the manuscript. All authors approved the final version of the manuscript.

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## DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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