

Hypoxia-inducible Factor Expression Is Related to Apoptosis and Cartilage Degradation in Temporomandibular Joint Osteoarthritis

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Abstract

Background: It remains unclear whether hypoxic conditions affect apoptosis and contribute to degradation of cartilaginous tissues in osteoarthritis (OA) lesions. In this study, we hypothesized that hypoxic conditions induced the accumulation of hypoxia-inducible factor (HIF) and activated apoptosis to contribute to OA cartilage degeneration *in vivo*.

Methods: Malocclusion stress was applied for 2 weeks, 4 weeks and 8 weeks to induce an OA-like lesion animal model (OD) in rats. Histological analysis was performed by H&E staining and safranin O/fast green staining. The expression levels of protein in condylar cartilage were examined by immunostaining to evaluate cartilage degeneration.

Results: We found apparent histological phenotypes associated with degeneration in the occlusion disorder stress (OD) group. The OD group at 4 weeks and 8 weeks had obviously reduced expression of Acan and Col II in cartilage. In contrast, the OD groups had higher levels of Col X, ADAMTS5 and MMP13 in the condylar cartilage than the control group. Moreover, the OD group cartilage had prominent degenerative changes with reduced levels of HIF1 α and increased levels of HIF2 α and the apoptosis factor Caspase3 in condylar cartilage at 8 weeks.

Occlusion disorder stress results in cartilage degeneration. HIF1 α and HIF2 α are involved in temporomandibular joint (TMJ) cartilage homeostasis by regulating chondrocyte apoptosis, which contributes to TMJ cartilage degeneration.

Conclusion: Thus, abnormal hypoxic conditions inducing opposite expression patterns of HIF1 α and HIF2 α could be involved in the pathogenesis of condylar cartilage degeneration. HIF2 α may provide a potential negative feedback mechanism for HIF1 α during cartilage damage.

Introduction

The temporomandibular joint (TMJ) is characterized by cartilage destruction and subchondral bone abnormal bone remodelling(1). However, the aetiology of TMJ osteoarthritis (TMJOA) remains to be elucidated. The TMJ has different morphological, functional, biomechanical and biological features compared with other joints in the body (2). The most superficial cellular layer of the TMJ is fibrocartilage, which consists of type I collagen (Col I) and type II collagen (Col II) (3). Mandibular condylar cartilage acts as avascular connective tissue that functions autonomously to bear loads. Mechanical stress is associated with the pathogenesis condylar cartilage homeostasis disruption and the initiation of the catabolic pathway (4). Cartilage degeneration occurs when abnormal mechanical stress continues to occur, which causes an imbalance in cartilage anabolism and catabolism (5). Chondrocyte apoptosis and catabolic enzymes induce cartilage destruction to contribute to disease pathogenesis (6).

Condylar cartilage a tissue that lacks blood vessels and nerves. Chondrocytes are located in a hypoxic or anoxic environment (7). Hypoxia-inducible factor (HIF)1 and HIF2 appear to be the major regulators of the

hypoxic response (8). HIF1 α exists only in hypoxic environments and exerts cytoprotective effects. HIF1 α translocates to the nucleus and interacts with hypoxia-sensitive target genes to regulate angiogenesis, energy metabolism and cell proliferation and apoptosis under hypoxic conditions (9). HIF2 α is mainly expressed in highly differentiating chondrocytes and acts as a key catabolic transcription factor that mediates the hypertrophic differentiation of chondrocytes and cartilage degradation in osteoarthritic cartilage in humans and mice (10). HIF2 α is an essential catabolic factor in the pathophysiology of OA (11). Moreover, HIF2 α is involved in the initiation of blood vessel formation accompanied by increased VEGF expression and the upregulation of multiple degradative enzymes, including matrix metalloproteinase 13 (MMP13) (12). When HIF2 α was silenced, ROS and HIF1 α expression was elevated in prehypertrophic cells (13). This result suggested that HIF1 α and HIF2 α were negatively regulated.

Based on the anabolic role of HIF1 α and the catabolic role of HIF2 α , HIF1 α and HIF2 α have spatiotemporal expression differences during the pathologic process of OA (14). However, the expression of HIF1 α and HIF2 α during the development of TMJOA is not well understood. Thus, we used OD rat models to show that the regulated spatiotemporal expression pattern of HIF1 α and HIF2 α results in apoptosis during TMJOA lesion development *in vivo*.

Material And Methods

Animals

All animal experiments complied with the and approved by Ethical Committees of Kunming Medical University. Eight-week-old male Sprague–Dawley (SD) rats (weighing 160–180 g) were obtained from the Experimental Animal Department of Kunming Medical University and were randomly divided into the control (n=10 rats) and OD groups (n=30 rats/group). In the experimental group (OD group), disordered occlusion was created by abnormal dental occlusion force based on a previous report (15). TMJ samples from OD rats were collected after disordered occlusion for 2 weeks, 4 weeks, and 8 weeks.

Histological staining

At the time of euthanasia, TMJ samples were dissected and fixed in 4% paraformaldehyde overnight. After be decalcified in 10% EDTA (pH 7.2–7.4), the samples were processed, embedded in paraffin and cut into 5 μ m sections using a microtome (Leica, RM2235, Germany). Standard haematoxylin and eosin (H&E) staining was used to examine tissue histology. Safranin O and fast green staining were performed to determine proteoglycan changes, and the histological data were further analysed by assessing the Mankin scores (16). Semiquantitative Mankin scores were significantly correlated and positively associated with exercise duration.

Immunohistochemistry

Immunohistochemical analyses of sections of each construct were performed using an anti-rabbit/mouse HRP-DAB cell & tissue staining kit (R&D Systems, USA). Sections were subjected to epitope recovery in citrate buffer at 99 °C for 30 min. Once the samples reached room temperature, the slides were washed in triethanolamine-buffered saline, and nonspecific immunoglobulin binding was blocked with 5% (V/V) bovine serum albumin for 30 min at room temperature. The sections were incubated overnight at 4 °C with the primary antibodies against Aggrecan (Acan, Santa Cruz Biotechnology, 1:100, USA); type collagen II (Col II, ab34712, Abcam, 1:100), type collagen X (Col X, ab58623, Abcam, 1:100); ADAM metalloproteinase with thrombospondin type 5 (ADAMTS5, ab182795, Abcam, 1:100), MMP13 (ab219620, Abcam, 1:100), Caspase3 (ab179519, Abcam, 1:100), HIF1 α , (PA3-16521, Thermo Fisher, 1:100) and HIF2 α (ab109616, Abcam, 1:100). All sections were incubated with a biotinylated secondary antibody, stained using an R&D HRP-DAB Staining Kit and counterstained with haematoxylin. After being mounted, the slides were photographed with an Olympus BX53 microscope (Olympus, Japan). The numbers of positive cells in the cartilage layer were determined by ImageJ (National Institutes of Health). The percentages of positive cells among all chondrocytes are shown. All sections were placed on one slide and processed together under the same conditions.

Statistical analysis

Comparisons between groups were evaluated with one-way analysis of variance (ANOVA) followed by Tukey's test for multiple comparisons using SPSS 16.0 software (IBM, Armonk, NY, USA). Values of * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, and **** $P < 0.001$ were considered to indicate a significant difference between groups.

Results

Condylar cartilage degeneration in the OD rat models

H&E staining indicated that the mandibular condylar cartilage was divided into a fibrocartilage layer and subchondral bone. Compared with that of the controls, condylar cartilage with abnormal dental occlusion had structural and degradation changes. The 2-week, 4-week and 8-week OD groups had irregular surfaces. Starting at 2 weeks, the OD group had small superficial clusters. The 8-week OD group included fissures into the radial layer and had slight disorganization in the superficial cartilage layer (Fig. 1A). Safranin O fast staining showed that the distribution of proteoglycans in the controls was even and rich, whereas the OD group exhibited time-dependent cartilage degradation accompanied by extensive loss of proteoglycan areas and the total number of chondrocytes (Fig. 1B). Mankin scores based on H&E staining and safranin O staining were increased in the OD groups. In the OD groups, the Mankin scores (Fig. 1C) and OARSI scores (Fig. 1D) increased with time. These histomorphological staining results confirmed that the degeneration of condylar cartilage became serious with the extension of animal modelling time.

Lower expression of condylar cartilage matrix protein in the OD rat models

The expression of cartilage matrix proteins in the TMJ was assessed by immunohistochemistry (Fig. 2A). In the 2-week and 4-week OD groups, the level of Acan in condylar cartilage was close to that in the control group. At 8 weeks, the expression of Acan in the OD group was lower than that in the control group and the 2-week and 4-week OD groups (Fig. 2B). These results indicated that occlusion disorder for 8 weeks induced a decrease in Acan expression in the cartilage layer. The trend in the expression of Col II in cartilage was similar to that of Acan (Fig. 2C). In addition, Col X expression at 4 weeks and 8 weeks in the OD group was greater than that in the control group and 2-week group (Fig. 2D). Consequently, cartilage degeneration decreased the expression of synthesis proteins in the OD group.

Increased expression of extracellular matrix (ECM) degradation proteins in OD rat models

The protein expression of MMP13 and ADAMTS5 was lower in the control group than the OD groups. The expression of MMP13 began to increase at 2 weeks and continued to increase in the 8-week group. There were significant differences in protein expression among the control group, 2-week group, 4-week group and 8-week group (Fig. 3A). ADAMTS5 began to increase at 2 weeks after modelling and maintained its expression level at 8 weeks (Fig. 3B). There was no significant difference in MMP13 expression between the control group and the 2-week model group. Protein expression was significantly different from that of the 2-week group and the 8-week group (Fig. 3C). The catabolic enzymes ADAMTS5 and MMP13 contributed to condylar cartilage destruction.

Chondrocyte apoptosis and expression of HIF1 α and HIF2 α in the OD condyle

Immunohistochemical staining for apoptosis-related markers in condylar cartilage was performed. Occlusion disorder induced an increase in caspase-3 in the OD group at 2 weeks, 4 weeks and 8 weeks. The 8-week group had a significant increase in cartilage among the OD groups. After malocclusion stress, the 4-week and 8-week groups had higher levels of Caspase 3 in the condylar cartilage hypertrophic layer (Fig. 4A). The 8-week group had the highest expression of Caspase3 among the OD groups (Fig. 4B). Malocclusion stress-induced condylar cartilage destruction, catabolic enzymes and chondrocyte apoptosis are associated with pathogenesis. HIF1 and HIF2 regulate apoptosis. Thus, we used immunohistochemical staining to examine the expression of HIF1 α and HIF2 α in the cartilage of the TMJ (Fig. 4A). Malocclusion stress significantly increased the expression of HIF1 α at 2 weeks, while that of HIF1 α was obviously decreased at 8 weeks compared with that at 2 weeks (Fig. 4C). HIF2 α , which is a

catabolic factor in chondrocytes, began to increase in the 2-week group and reached its highest expression level in the 8-week group (Fig. 4D).

Discussion

Condylar cartilage is maintained in a low oxygen environment throughout life. Chondrocytes are therefore adapted to these hypoxic conditions (17). However, the effects of pathological hypoxic conditions on OA lesions remain unclear. In this study, we explored whether malocclusion stress induces pathological hypoxic conditions in the TMJ that contribute to OA development. The condylar cartilage surface is covered by fibrocartilage, which is stress-sensitive (18). Cartilage degradation is a key factor that induces excessive mechanical stress (19). TMJOA rat models were established by unilateral molar occlusal elevation. This method is reproducible and can simulate the pathogenesis of TMJ disease (15, 20). Excessive mechanical loading on the normal condylar cartilage initiates the disruption of cartilage (21). We observed that the condylar cartilage structure changed after 2 weeks of malocclusion stress. Condylar cartilage degradation was obvious in the 8-week group. The phenotype of cartilage degradation in the OD group was an early lesion of TMJOA, which was defined by Mankin scores and histological staining. Animal models are a critical tool to investigate the pathogenesis of TMJOA (22). Therefore, rat OD models are suitable for investigating the disruption of cartilage matrix homeostasis and related pathogenic factors.

Unlike most hyaline articular cartilage in the appendicular joints, TMJ cartilage is classified as fibrocartilage (23). Condylar fibrocartilage is histologically composed of the force-absorbent, proteoglycan-rich nonmineralized portion and the rigid mineralized region that abuts the subchondral bone (24). The hypertrophic zone is characterized by Col X-producing cells that lie beneath the chondrocytic zone (25). With OA onset, chondrocytes undergo multiple changes in states including proliferation, viability and secretory profiles (26). Condylar cartilage includes two main ECM molecules, Col II and Acan. Acan is the major proteoglycan in articular cartilage, and the loss of Acan is a known characteristic of early OA (27). Decreased Acan expression induced by cartilage aggrecanases and MMPs is often evident in OA cartilage (28). ADAMTS5 is the principal aggrecanase found in animal and human OA articular cartilage (29). The hypertrophic phenotype that produces aberrant Col X and the catabolic protease MMP13 contributes to OA development (30). MMP13 is an interstitial Col II enzyme that has a particular relevance to the degradation of articular cartilage (31). In our study, abnormal occlusion decreased the expression of Acan and Col II and enhanced the expression levels of MMP13, Col X and ADAMTS5 in the cartilage layer of the OD group. This result suggested that malocclusion promoted the loss of cartilage ECM in the TMJ.

Cartilage breakdown in OA is related not only to ECM degeneration but also to chondrocyte apoptosis. An increase in the rate of apoptosis in articular cartilage could play an important role in OA pathogenesis (31, 32). Apoptosis clearly occurs in OA cartilage and subsequently disrupts cartilage homeostasis (33). Caspases, which are key molecules in apoptosis, are known to regulate apoptosis. Caspase 3, the main executioner of apoptosis, is activated by intrinsic and extrinsic apoptotic pathways in cells. Our results

showed that occlusion disorder induced high Caspase 3 expression in chondrocytes, which indicated that chondrocyte apoptosis occurred in cartilage in the OD group (34). Some studies have suggested that HIF1 α contributes to apoptosis in hypertrophic growth plate chondrocytes by liberating proapoptotic factors from blood vessels (35). Hypoxia and HIF activation are regarded as important stimuli of OA development. HIF2 α is highly expressed in OA cartilage (36). HIF2 α enhances the promoter activities of Col X and MMP13 (37). Our study showed increases in HIF1 α expression after 2 weeks and 4 weeks of abnormal occlusion stress and a decrease at 8 weeks. HIF2 α expression in condylar cartilage was significantly increased in the OD group at 8 weeks. HIF1 α and HIF2 α play specific roles in the cellular response to a lack of oxygen, effectively regulating gene expression in response to disturbances in oxygen homeostasis. It was suggested that abnormal occlusion stress aggravated the hypoxic environment in cartilage, and HIF1 α increased its reactivity due to impaired TMJ cartilage damage. However, the TMJ continues to bear occlusion stress, and HIF2 α but not HIF1 α may be essential for oxygen homeostasis in the TMJ. With increasing HIF2 α expression, chondrocyte apoptosis occurs in the TMJ to contribute to cartilage destruction.

Conclusion

Our results suggested that the expression of HIF1 α and HIF2 α in the TMJ is a vital stimulatory of cartilage degeneration. HIF2 α may provide a negative feedback mechanism for HIF1 α during OA development. However, further studies are needed to elucidate the molecular mechanisms between HIF1 α and HIF2 α in OA in vitro.

Abbreviations

HIF1 α /2 α hypoxia-inducible factor1 α /2 α ; OA: Osteoarthritis; OD: Occlusion disorder;TMJ: Temporomandibular joint; Col I/II/X: The type collagen I/II/X; MMP13: Matrix metalloproteinase 13; Acan: Aggrecan; ECM: Extracellular matrix; ADAMTS5: Recombinant a disintegrin and metalloproteinase with thrombospondin 5.

Declarations

Ethical approval and consent to participate

All animal experiments approved by Ethical Committees of Kunming Medical University (Local Ethics Committee of Kunming Medical University, No. kmmu2020036), which complies with the Guide for the Care and Use of Laboratory Animals, 8th edition, published by the United States National Institutes of Health (NIH Publication, 2011). All methods in the present study were designed and performed in accordance with ARRIVE guidelines and the other relevant guidelines and regulations.

Consent for publication

Not applicable.

Availability of data and materials

All of the data in this study are obtained from experiments. The data used and analysed in this study are available from the corresponding author on reasonable request.

Competing interests

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

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Author's contribution

Jun Zhang contribute to investigation, formal analysis, writing, review & editing. Yu Hu contribute to data acquisition, validation. Zihan Wang and Xuelian Wu contribute to methodology, visualization. Chun Yang contribute to project administration, funding acquisition, supervision, review and editing. Hefeng Yang contribute to conceptualization, resources, funding acquisition, supervision, review and editing.

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Figures

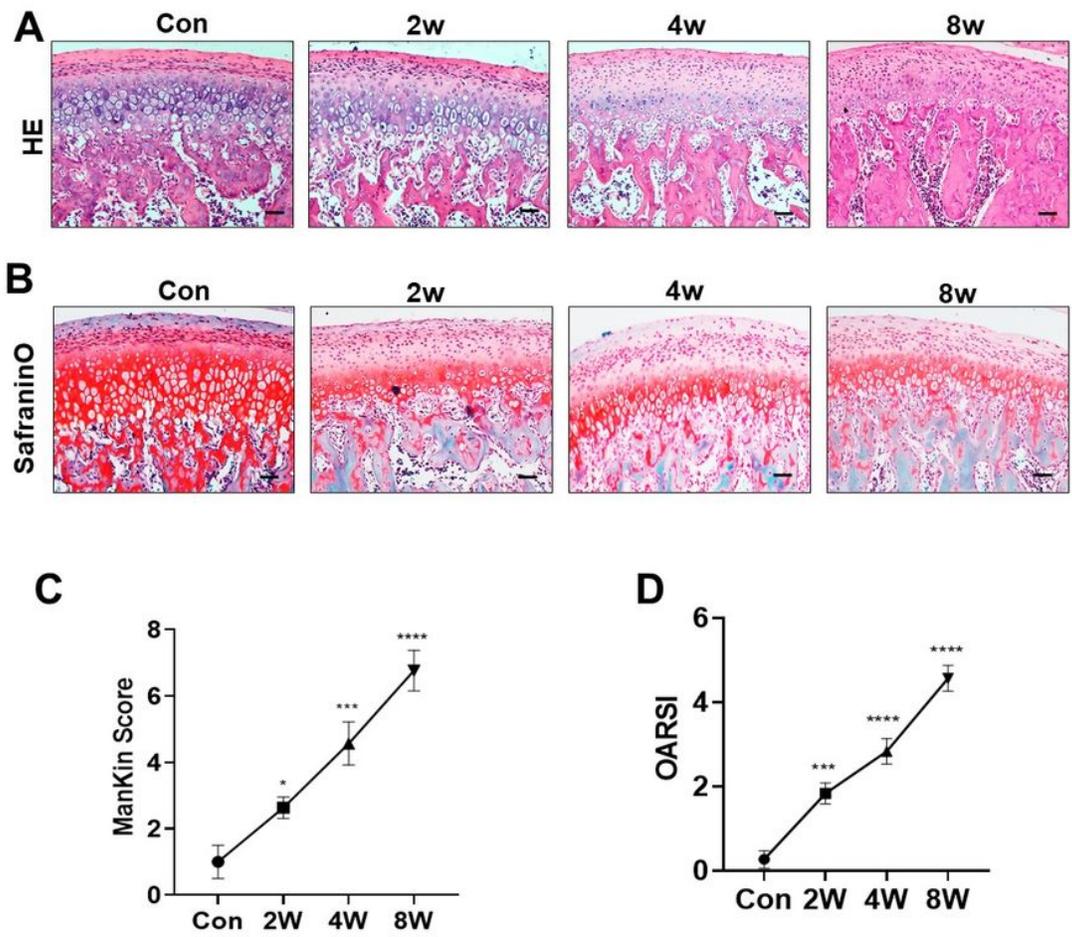


Fig 1

Figure 1

Condylar cartilage degeneration in rat models. Representative images of the first molar occlusion relationship in control and OD rats. H&E staining (A) and safranin O and fast green (B) analyses of glycosaminoglycans (red) in sagittal sections of the TMJ mandibular condylar cartilage layers. Scale bars: 50 μ m. (C) Mankin scores and OARSI scores (D) in the control and OD groups. * $P < 0.05$, *** $P < 0.005$, **** $P < 0.001$.

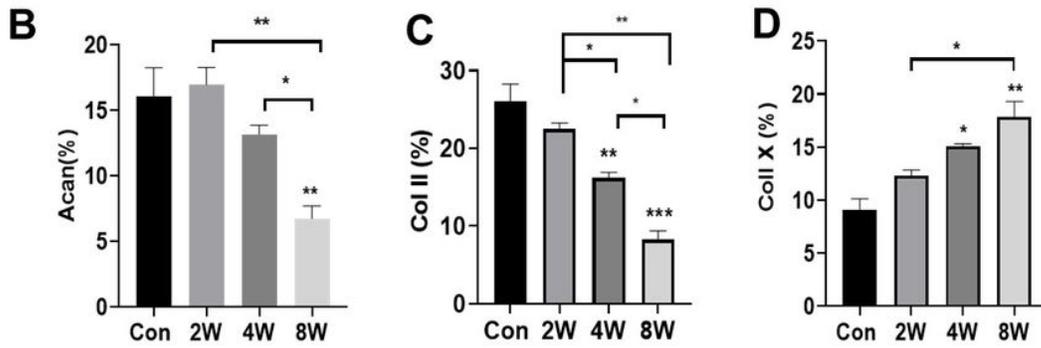
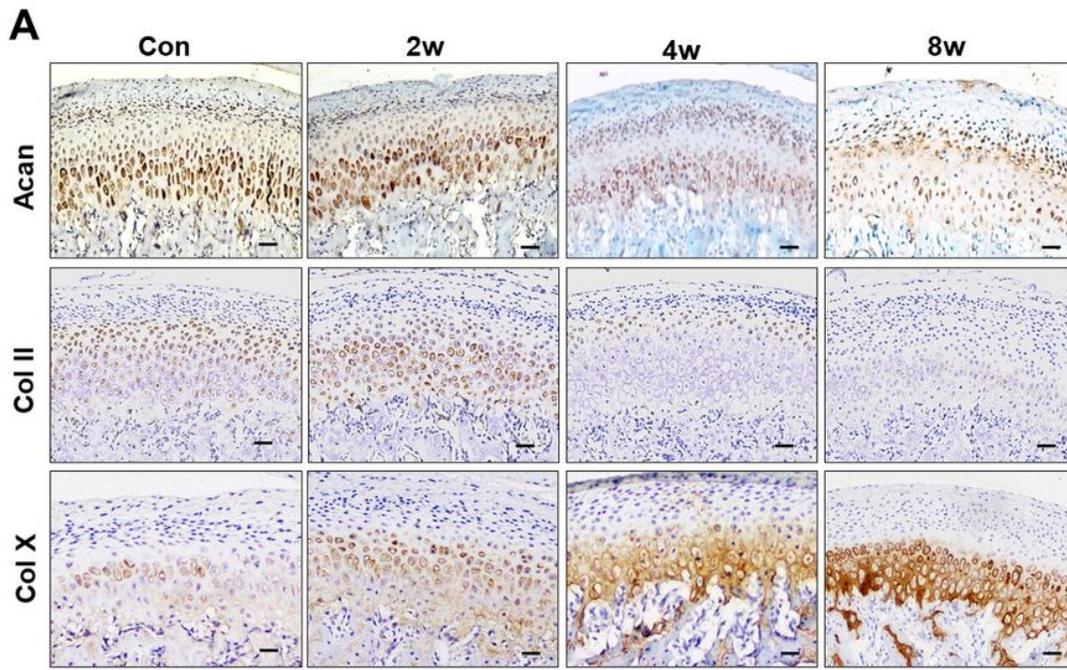


Fig 2

Figure 2

The expression of matrix proteins in condylar cartilage. Immunohistochemical analysis of Acan, Col II and Col X in mandibular condylar cartilage (A). Scale bars: 50 μ m. Acan- (B), Col II- (C) and Col X-positive (D) cells were counted in the cartilage layer. * $P < 0.05$, ** $P < 0.01$. *** $P < 0.005$.

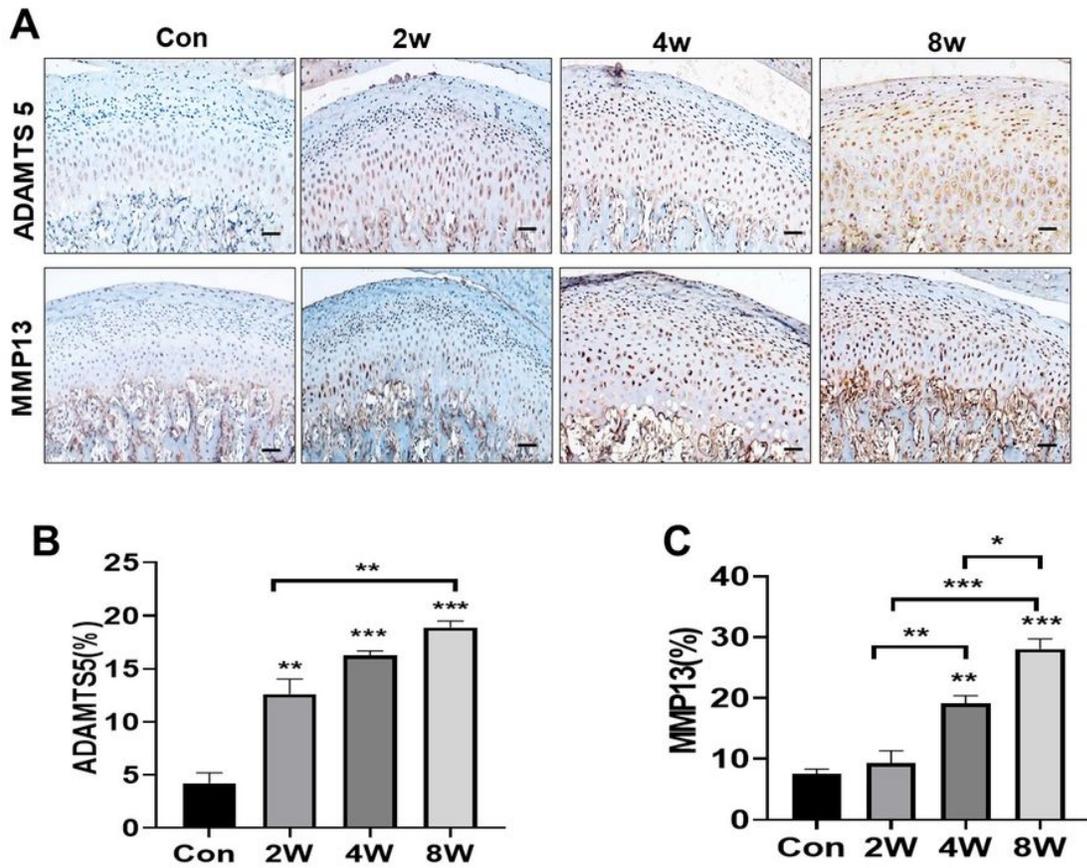


Fig 3

Figure 3

The expression of matrix degeneration-related proteins in the OD rat model. Immunohistochemical analysis of ADAMTS5 and MMP13 in mandibular condylar cartilage (A). Scale bars: 100 μ m. ADAMTS5- (B) and MMP13-positive (C) cells were counted in the cartilage layer. *P < 0.05, **P<0.01, ***P<0.005.

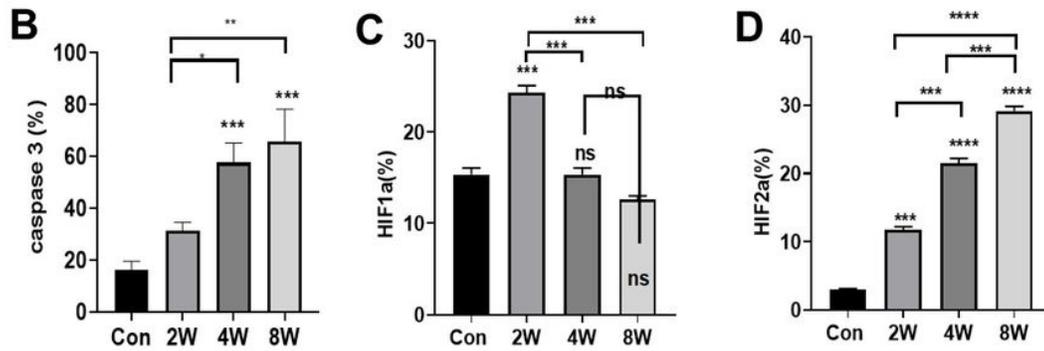
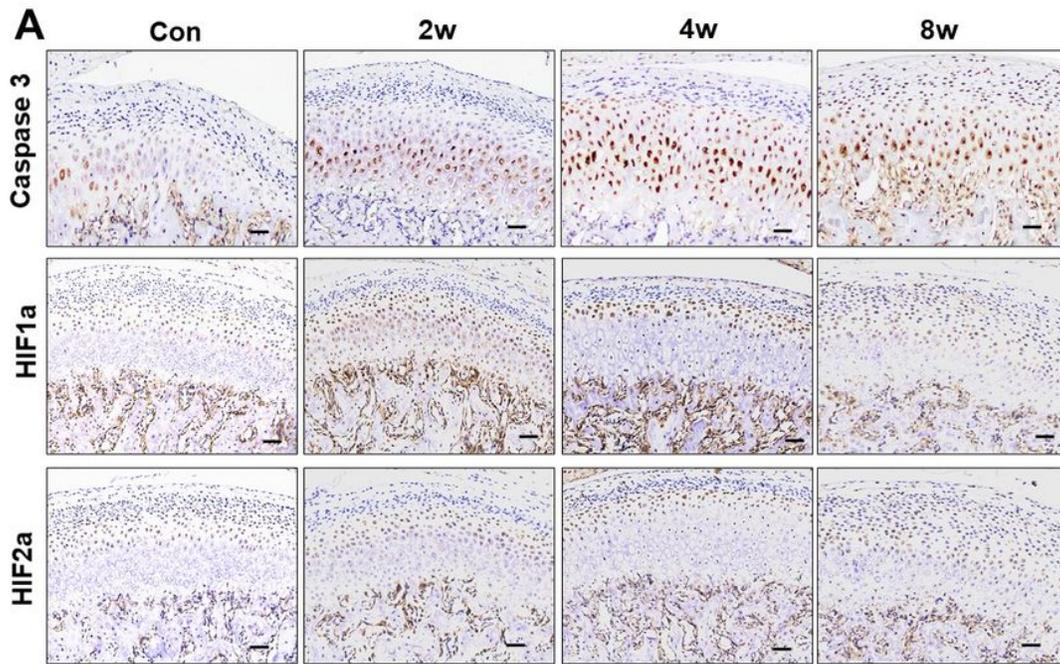


Fig 4

Figure 4

The expression of Caspase3, HIF1α and HIF2α in condylar cartilage in the condylar cartilage of the rat models. Immunohistochemical analysis of Caspase3, HIF1α and HIF2α in mandibular condylar cartilage (A). Caspase3- (B) cells, HIF1α- (C) and HIF2α-positive (D) cells were counted in the cartilage layer.

P<0.005, *P<0.001.

Reference