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Original Article

The effect of TNF-α on the development of degenerative changes in anterior disc displacement of the temporomandibular joint

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Abstract: This study aimed to use immunohistochemical analysis of the expression of tumor necrosis factor-α (TNF-α) in the temporomandibular joints (TMJs) of patients with anterior disc displacement without reduction (ADDwoR) and rabbits to examine the effects of TNF-α on the development of degenerative changes in ADD. Six temporomandibular discs from 5 patients with articular disc perforations caused by ADDwoR were examined using immunohistochemistry. Tissues distant to the perforated area served as controls. The histological changes were observed, and elevated expression levels of TNF-α were detected. To investigate the changes in the mandibular condyle, rabbits were used in animal experiments because the mandibular condyles of patients with ADD cannot be resected. The discs of the TMJs were surgically displaced anteriorly in 20 adult rabbits. Five additional rabbits served as non-surgical controls. The animals were sacrificed at 2, 4, 8, and 12 weeks after surgery, and the histological changes were observed. The TNF-α expression levels in the cartilage were detected by immunohistochemistry. The expression of TNF-α in the cytoplasm of the chondrocytes of normal condyles exhibited sporadic scattering in the hypertrophic zone. Two and four weeks after ADD, the TNF-α-positive cells were aligned in the hypertrophic zone and located near the proliferating zone and the center of the hypertrophic zone. Then, after eight and twelve weeks, brown staining was observed in both the cytoplasm and nucleus of chondrocytes in the outer hypertrophic zone. The expression of TNF-α in the cartilage of the condyle after ADD was different relative to the normal cartilage and was altered during the ADD period. TNF-α may be related to the process of cartilage degeneration during ADD.

Keywords: temporomandibular joint, anterior disc displacement, tumor necrosis factor-α, animal model

Introduction

Temporomandibular disorders (TMDs) are a significant health problem, and the most common abnormality that is encountered in patients with signs and symptoms of a TMD is disc displacement, primarily major anterior disc displacement (ADD) [1]. Several inflammatory mediators play important roles in the pathogenesis of TMDs, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), prostaglandin E2 (PGE2), and matrix metalloproteinases (MMPs) [2, 3].

TNF-α is one of the most important pro-inflammatory cytokines that is related to immune and inflammatory responses. TNF-α has various biological functions, such as the production of antibodies, induction of cytokines, production of PGE2 with macrophage activation, production of collagenase in synovial cells, and induction of bone degeneration. In the cartilage degeneration process, the induction of cytokines, such as TNF-α, and the production of proteases that target the extracellular cartilage matrix play important roles in the progression of cartilage degradation [4]. Examinations of the synovial fluid have demonstrated increased amounts of TNF-α in patients with osteoarthrosis (OA) [5, 6]. However, the cartilage cells themselves secreted TNF-α, the changes in the

Table 1. Baseline clinical characteristics of the study group

Patient	Gender	Age (yrs)	Affected side	
			Right	Left
1	F	41	X	
2	F	38	X	
3	M	50		X
4	M	26		X
5	M	55	x	X

expression of TNF-α during ADD are still unknown.

In our study, the perforated articular discs from patients with ADD without reduction (ADDwoR) were examined immunohistochemically, and high levels of TNF-α expression were observed. To observe the condylar cartilage changes after ADD, we created a rabbit ADD model and we investigated the sources and the role of the pro-inflammatory cytokine TNF-α.

Material and methods

Sample selection

A sample of 6 perforated temporomandibular discs from 5 patients (mean age 42 years old, range: 18 to 56 years) recruited for the study from the patient pool at the Department of Oral and Maxillofacial Surgery, Oral Biomedicine of Ministry of Education, School & Hospital of Stomatology, Wuhan University, Wuhan, was used (**Table 1**). Articular disc perforations were confirmed by radiographs or computed tomography (CT) scans. Each subject provided informed consent before participation. In this study, normal TMJ discs were not collected, and tissues distant from the perforated area served as controls.

ADD animal models

Twenty 4-month-old white rabbits (Oryctolagus cuniculus) weighing 2.5 to 3 kg underwent surgical ADD. The experimental rabbits were divided into four groups consisting of five rabbits each. The right side of the TMJ served as the surgery sample, and the left side served as the surgical control. Additionally, five rabbits served as normal controls. After the induction of general anesthesia with pentobarbital sodium, the skin over the right side preauricular area was shaved and prepared with antiseptic solution. A 2-cm incision was made in the skin over 1 cm of the lateral and inferior margins of the orbit. Blunt and sharp dissections were used to expose the zygomatico-squamosal suture and the zygomatic arch. The periosteum was reflected superiorly, which allowed entrance into the floor of the orbit. Blunt separation was used to expose the outer capsule of the anterior attachment of the disc. The temporal extension of the anterior band of the disc was sutured using a 1-0 silk and pulled anteriorly. When the disc was pulled anteriorly, clicking could be heard. A hole was drilled in the zygomatic arch, and the suture was passed through the hole. The disc was fixed in the anterior position, and the surgical site was closed in layers.

The left side of the rabbit TMJ was approached in a similar manner, with the exception of the suturing of the temporal extension of the anterior band of the disc and the repositioning of the disc anteriorly. After the operation, the rabbits received antibiotics for two days, and the animal weights were monitored.

Specimen preparation

The patients' perforated discs and the rabbit TMJs were excised, and placed in 10% neutral-buffered formalin (pH 7.4) for 24 hours. After fixation, decalcification was completed in 10% ethylene-diaminetetraacetic acid (EDTA) solution for 4 to 6 weeks, and the specimens were dehydrated in ascending grades of ethanol, embedded in paraffin, and sectioned into 5-μm thick sections.

Hematoxylin and eosin staining

Deparaffinized sections were stained with hematoxylin and eosin (H&E) for histological observation. In brief, the sections were incubated in Mayer's hematoxylin (0.75% w/v) for 12 min, then immersed in acid alcohol for 30 s and in tap water for 2 min, and finally stained with 1% (w/v) aqueous eosin for 5 min. The sections were washed with running tap water before and after each solution, dehydrated in serial alcohol concentrations, and mounted with gum.

Immunohistochemistry

The sections were deparaffinized in xylene two times for 30 min each, hydrated gradually using

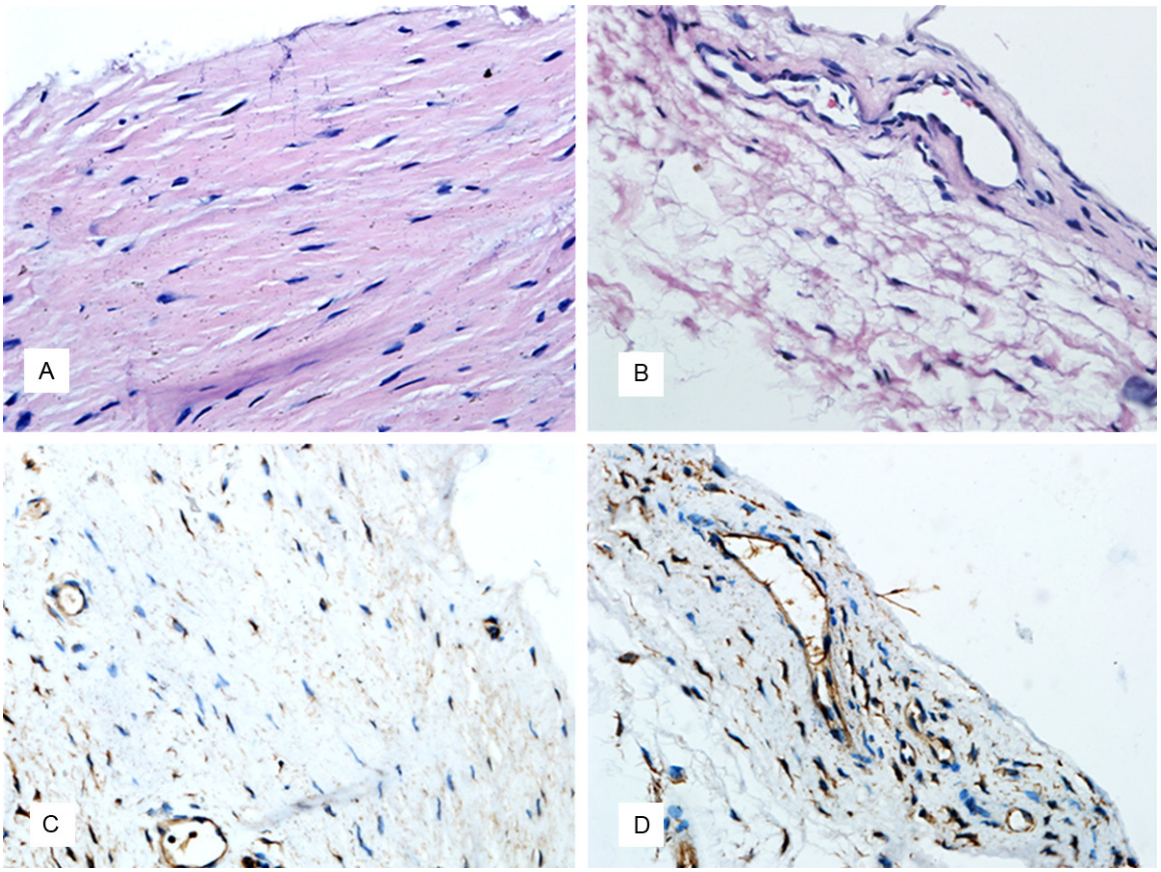

Figure 1. A: Tissue distant from the perforated area. The H&E stain shows the regular arrangement of collagen and fibrochondrocytes. B: Tissue near the perforated area. The H&E stain shows a disorderly arrangement of fibrochondrocytes and the destruction of collagen. C: Immunostaining shows a disc with a relatively normal histological structure and reduced expression of TNF-α. D: Immunostaining shows a perforated disc with relatively increased expression of TNF-α.

a graded series of alcohols (100% ethanol two times for 5 min each, then 95%, 85%, and 75% ethanol for 5 min each) and rinsed in distilled water for 1 min. The deparaffinized sections were treated with 3% H_2O_2 for 30 min at 37°C to quench the endogenous peroxidase activity and then rinsed in 0.01% PBS three times for 5 min each. Next, the slides were incubated with normal goat serum for 1 h at 37°C to block the non-specific binding of antibodies. The slides were incubated separately with antibodies against TNF-α (bsm-0387M, Boster Bioengineering Limited Company, China; 1:50), incubated for 12 h at 4°C, and then rinsed (0.01% PBS, 5 min × 3). Next, the slides were incubated separately with secondary antibodies (goat anti-mouse IgG-B, SC-2039, Santa Cruz Biotechnology, Inc. USA; 1:100) for 15 min and then incubated in an avidin-peroxidase complex (Zhongshan Golden Bridge Biotechnology Co., Ltd.) at 37°C for 15 min. Antibody staining was performed using a peroxidase/diaminobenzidine (DAB) kit (Fuzhou Maixin Biotechnology Development Co., Ltd., China). The sections were lightly counterstained with hematoxylin, dehydrated using an ethanol series, cleared in xylene, and coverslipped. For control experiments, the sections were incubated without the primary antibodies.

Image and statistical analyses

Histological and immunohistochemically stained sections were examined under a light microscope (Leica DM 2500, Wetzlar, Germany). Image acquisition was performed with the Leica DFC490 system (Leica, Wetzlar, Germany). Measurements were performed within defined areas of the condylar cartilage; five squares located at the condylar cartilage immediately opposite to the eminence were chosen, and the

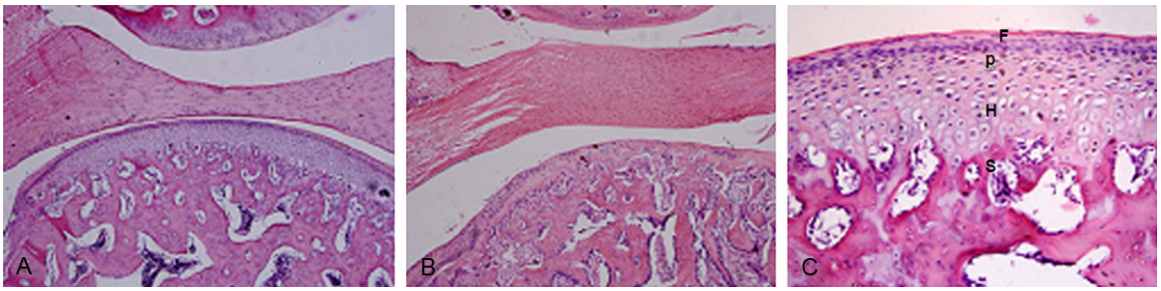

Figure 2. (A) The normal position of the disc. (B) The position of the disc was displaced anteriorly. (C) The normal histological structure of the condyle of the rabbit. The cartilage of the condyle can be divided into 4 zones: the fibrous zone (F), proliferating zone (P), hypertrophic zone (H), and subchondral zone (S). Stained by H&E. Scale bars: 50 μm in (A) and 20 μm in (B).

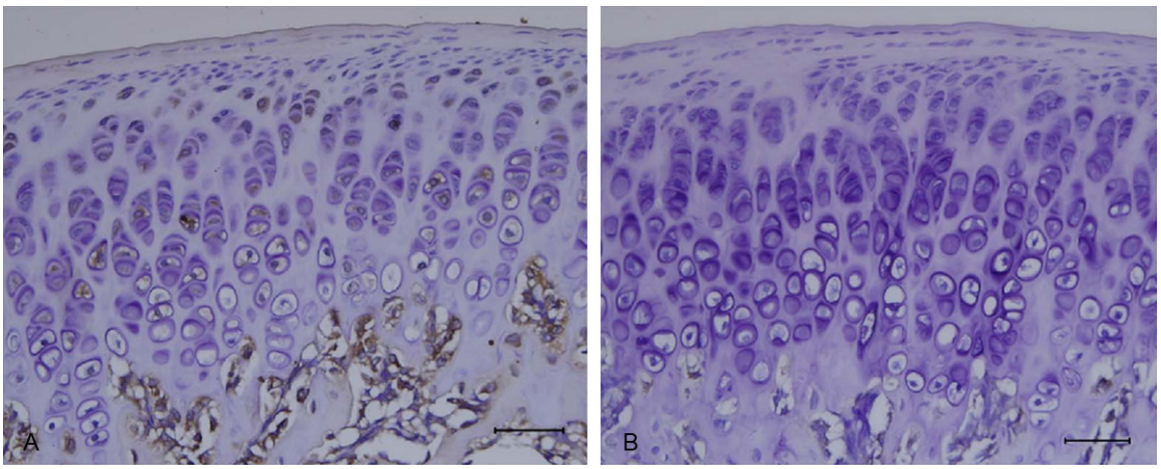

Figure 3. Immunostaining showed TNF-α expression in the normal condylar cartilage of rabbits. A: Positive expression. B: Negative expression. Scale bars are 50 μm.

number of positive cells in the selected frame was determined by two independent observers with no knowledge of the group of origin.

The sample of human TMJ discs was not sufficient for statistical analysis; therefore, statistical analysis was performed only on the ADD animal model. SPSS software, version 11.0 (SPSS, Chicago, IL, USA) was used. All data acquisition and analyses were performed blindly. The numbers of positive chondrocytes were expressed as the means ± standard deviations (SD) for each group. When significant main effects or an interaction between main effects was found, specific comparisons between subgroups were performed using a Student-Newman-Keuls (SNK-q) post-test. In all cases, *P* values less than 0.05 were considered statistically significant.

Results

Perforated temporomandibular discs from patients

H&E staining showed the disorderly arrangement of fibrochondrocytes in discs and the destruction of collagen. A high expression level of TNF-α was observed, especially around the perforated area (**Figure 1**).

Temporomandibular joints from ADD rabbits

The non-operated and surgical control groups showed no evidence of pathological changes. The posterior band of the disc was positioned directly beneath the shallow fossa on the posterior aspect of the eminence; the disc was located between the condyle and the eminence. The position of the disc was displaced

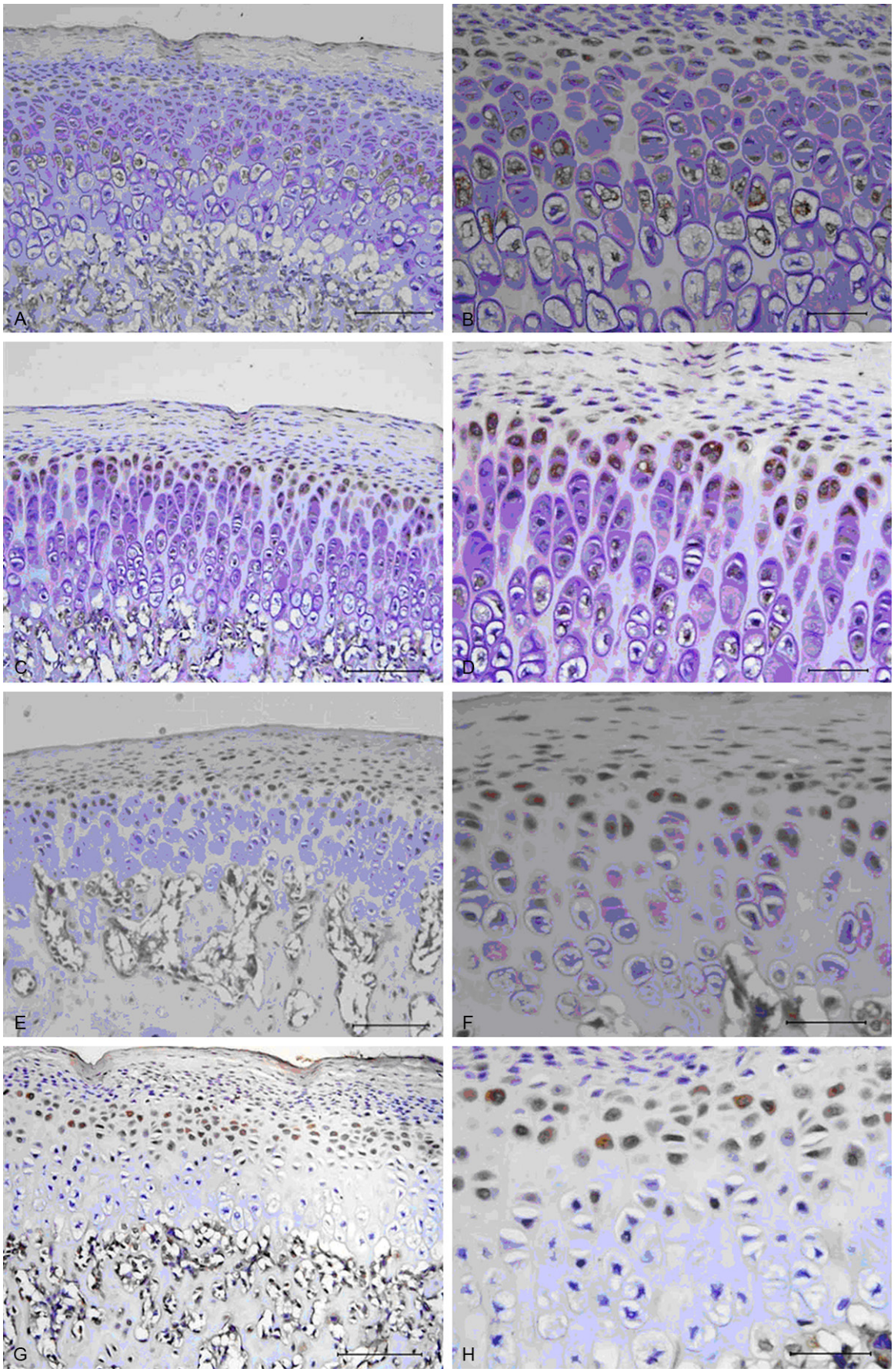
A
B
C
D
E
F
G
H

Figure 4. Immunostaining showed TNF-α expression in the condylar cartilage after disc displacement in rabbits. (A, B) 2 weeks after disc displacement; (C, D) 4 weeks after disc displacement; (E, F) 8 weeks after disc displacement; (G, H) 12 weeks after disc displacement. Scale bars: 50 μm in (A, C, E, G) and 20 μm in (B, D, F, H).

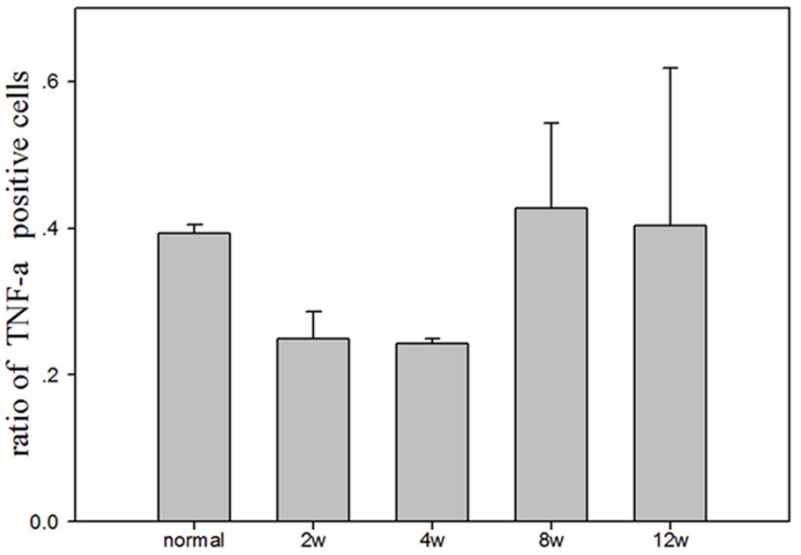

Figure 5. The ratio of TNF-α positive cells to all cartilage cells. The ratio of TNF-α positive cells at eight and twelve weeks increased compared to that at two or four weeks.

anteriorly relative to normal controls in the surgical groups. The condyle consisted of four zones: the fibrous zone, proliferating zone, hypertrophic zone, and subchondral zone (**Figure 2**). Two weeks after ADD, the thickness of the condylar cartilage remained consistent or became slightly thinner, the proliferating zone became broader, and the cell array in the hypertrophic zone lost its regular pattern. After four weeks, the proliferating zone was broadened, and the irregular cell array decreased in the surgical group. However, the condylar cartilage became obviously thinner than the normal controls at eight weeks after surgery, and the cartilage matrixes were decreased. Twelve weeks after ADD, the thicknesses of the total cartilage and each zone were similar to normal joints, but the cells were arrayed in a disorganized manner.

TNF-α expression in the condylar cartilage from ADD *rabbits*

Typical expression of TNF-α was observed in normal condyles, sporadic scattered expression was observed in the hypertrophic zone, and slight brown staining was observed in cytoplasm of chondrocytes (**Figure 3**). The ratio of TNF-α positive cell was approximately 40% of all cartilage cells.

Two weeks after ADD, the TNF-α positive cells were aligned in the hypertrophic zone located near the proliferating zone and the center of hypertrophic zone (**Figure 4A**, **4B**). Four weeks after ADD, the TNF-α positive cells were also aligned in the hypertrophic zone, mainly in the outer hypertrophic zone immediately behind the proliferating zone, forming 3~4 lines (**Figure 4C**, **4D**). The brown staining in the remaining area of the hypertrophic zone had nearly disappeared. Compared to normal condyles, the brown staining in the cytoplasm of chondrocytes was much more dense. The positive cell ratio decreased to almost half that of normal cartilage (approximately 25%) due to the limited area of TNF-α-expressing cells (**Figure 5**). Then, eight weeks after ADD, brown staining was present in both the cytoplasm and nucleus of chondrocytes (**Figure 4E**, **4F**). The TNF-α positive cells were present only in the outer hypertrophic zone as the cartilage became thinner eight weeks after surgery. Twelve weeks after ADD, the expression of TNF-α was localized in 2 or 3 layers of cells in the outer hypertrophic zone (**Figure 4G**, **4H**). Brown staining was present in both the cytoplasm and nucleus of chondrocytes. The ratio of TNF-α positive cells at eight and twelve weeks increased compared to that at two or four weeks (approximately 40% of all cartilage cells) due to the thinning of the cartilage layer and the decrease in the number of chondrocytes (**Figure 5**).

Discussion

ADD of the TMJ is the most common type of TMD and is associated with TMJ OA [7]. Although many studies have described the

pathogenesis of ADD in humans, the availability of human cadaver and human specimens has limited investigation of the pathogenesis of ADD. The relationship between disc displacement and pain, mandibular dysfunction, OA, and growth disturbances remains unclear [8]. Several animal models [9-11] have been introduced to study the pathological progress and cartilage and bone changes of the TMJ. In our previous research, we have successfully created an ADD rabbit model without opening the joint capsule, and degenerative and OA changes can be observed after ADD [9].

TNF-α is a pleiotropic cytokine that triggers cell proliferation, cell death, and inflammation [12]. In general, TNF-α is a major proinflammatory mediator with an additional capacity to induce apoptosis. Previous studies have shown that in antigen-induced arthritis, TNF-α can be secreted by synovial and cartilage cells and targets the cartilage extracellular matrix, and it has been shown to be related to cartilage degeneration [13-15]. Some researchers have reported that the TNF-α level is increased in the synovial fluid of TMJs of patients with OA, and differences in TNF-α levels have been shown at different stages of internal derangement [16]. TNF-α is thought to play an important role in the pathogenesis of OA. However, whether this pre-inflammatory factor is involved in disc displacement situation is still known. Most animal models have used surgical or chemical manipulation to disrupt the TMJ through disc perforation or intra-articular injection of inflammatory agents into the joint [17, 18]. This study is the first to determine the expression TNF-α in cartilage during changes due to condylar stress after ADD in vivo.

In this study, TNF-α exhibits a functional duality as it is involved in both tissue regeneration/expansion and destruction. Typical levels of expression of TNF-α in the normal TMJ cartilage were observed in this study, which indicate that TNF-α may be involved in the physiological metabolism of normal chondrocytes. However, in a pathological state (for example, ADD), the expression of TNF-α is altered, which indicates that TNF-α may be involved in the degeneration of cartilage. In fact, overexpression of TNF-α alone in rodents is sufficient to trigger destructive arthritis with synovial inflammation, cartilage damage, and bone destruction [19].

Moreover, it should be noted that pre-hypertrophic cells (in the outer hypertrophic zone immediately behind the proliferating zone) express high levels of TNF-α during ADD, which indicates that this layer of chondrocytes is sensitive to mechanical stress and may secrete cytokines to regulate the chondrocytes [20]. Traditionally, cartilage breakdown is thought to initially occur at the articular surface in OA, and the synovium may produce proteases and cytokines that accelerate the progression of this disease [21]. However, in our study, we have shown that the expression of TNF-α in cartilage is also changed relative to normal controls, which indicates that cartilage degeneration may also begin directly after changes occur in the chondrocytes.

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Disclosure of conflict of interest

None.

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