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Review Article

Effects of hedgehog pathway genes on the response to tensile force and inflammatory cytokines in rat condylar cartilage cells

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Abstract: Objective: To explore the effect of different tensile strain stimulations on the proliferation, inflammatory factor expression and components of the Hedgehog pathway in rat mandibular condylar chondrocytes. Methods: Cultured rat mandibular condylar cartilage cells were used for this study. Different tensile strains were applied to the chondrocytes for 4 h using a Flexcell-4000TM strain unit. The tensile strain amplitudes were 0, 3, and 15%, at a frequency of 0.5 Hz. Cell proliferation was measured using the CCK-8 method. The mRNA expression levels of PCNA, caspase-3, COL II, IL-1β, TNF-α, Ihh, Ptc, and Smo were quantified via real-time PCR. Results: Compared with the control group, chondrocytes subjected to 3% tensile strain showed higher proliferation rates and had higher mRNA levels of PCNA, COL II, Ihh, Ptc and Smo. At a 15% tensile strain, the mRNA levels of caspase-3, IL-1β and TNF-α were up-regulated, but the mRNA levels of PCNA and Smo were down-regulated. Compared with those subjected to 3% tensile strain, chondrocytes subjected to 15% tensile strain showed a lower proliferation rate, down-regulated mRNA levels of PCNA, COL II, Ihh and Smo and up-regulated mRNA levels of IL-1β and caspase-3. Conclusions: The proliferation of chondrocytes and the expression of inflammatory factors may change with different levels of tensile strain. We show that a 15% tensile strain induces the expression of apoptotic and inflammatory factors, and a 3% tensile strain increases chondrocyte proliferation and cartilage matrix synthesis. Moreover, Hedgehog pathway signaling molecules are sensitive to tensile strain and were found to participate in the regulation of the mechanical stimulation of chondrocytes.

Keywords: Chondrocyte, tensile strains, proliferation, apoptosis, inflammatory factors, hedgehog pathway

Introduction

The temporomandibular joint (TMJ) is a linkage joint for the oral and maxillofacial region, and it is the body's most complex and most sophisticated weight-bearing joint. The TMJ has the ability to undergo lifelong alterations, and it is important for the maintenance of oral cavity function [1, 2].

Condylar cartilage bears and transfers the biomechanical load generated when the lower jaw moves, and it also undergoes adaptive remodeling [3, 4] thus influencing the direction and amount of mandibular growth, which regulates its length and height [5]. Skeletal Class II malocclusion is a common malocclusion, and its risk factors include a variety of coordination disorders and different factors relating to the teeth, dental arch, jaw and various muscles [6]. Previous research has [7-9] shown that functional appliances can promote the stretching of chewing muscles and that TMJ muscles can change their size and orientation, thus, contributing to the adaptability of hard and soft tissues in rebuilding a functional balance and promoting sudden alterations of the condyles. However, different types and intensities of mechanical loading have different effects on condylar cartilage, and the mechanism by which chondrocytes convert mechanical load into cellular biochemical reactions is unclear.

The Hedgehog (Hh) family of proteins includes Indian hedgehog (Ihh), Sonic hedgehog (Shh) and Desert hedgehog (Dhh) [10]. Two receptors

on the target cell membrane regulate Hh signaling [11, 12], namely Smoothened (Smo) and Patched (Ptc).

An oncogene encodes Smo, which is composed of 7 transmembrane single-peptide chains and is a necessary receptor for Hh signaling [13]. Ptc is encoded by a tumor suppressor gene, and it is a 12-channel transmembrane protein. Ptc plays a negative regulatory role in the Hh signaling pathway.

A previous study [14] has shown that the regulatory effects of stress on osteoblasts may be related to the Hh pathway, but there are few studies on the regulation of chondrocyte signaling in the TMJ.

Therefore, in this study, we cultured rat mandibular condylar chondrocytes (MCCs) and observed the effects of different tension forces on condylar cartilage cell proliferation, apoptosis, inflammatory cytokine expression, and Hh pathway genes. Furthermore, we explored the effect of tension on TMJ condylar cartilage and remodeling mechanisms to provide theoretical support for a clinical application of traction to the lower mandible and the treatment of condylar fractures.

Materials and methods

Animal models

Two-week-old male and female Sprague-Dawley (SD) rats were used for this study (provided by the Center of Jiamusi University).

Reagents

High-glucose DMEM (Gibco, USA), fetal calf serum (Gibco), trypsin, (Hyclone, USA), collagenase II (Sigma, USA), penicillin/streptomycin (Invitrogen, USA), phosphate-buffered saline (PBS, self-distribution), Flexcell 4000TM strain units (Flexcell Corp. USA), COL II antibody (Abcam, UK), FITC-labeled antibody (Santa Cruz, USA), TRIzol reagent (Gibco), a reverse transcription kit (TaKaRa, Dalian) and a real-time quantitative PCR kit (TaKaRa) were used in this study.

Methods

MCC primary cultures were obtained from two-week-old SD rats under sterile conditions by dissecting out the rats' mandibular condylar cartilage. The fibrous tissue was separated, and the condylar surface was removed after clipping the condylar cartilage. The tissue was cut into pieces (approximately 1 mm^3 in size) with ophthalmic scissors, transferred to a centrifuge tube and digested using 0.25% trypsin at 37°C for 30 min and 0.2% collagenase II at 37°C for 4 h. The cells were collected via filtration through a 200-μm mesh and stained with 0.25% Trypan blue. The viable cell rate was greater than 90% when the cells were cultured in an incubator with DMEM containing 10% FBS at 37°C and 5% CO_2. The medium was changed every other day until 90% confluence was obtained. The trypsinization and passaging of the cultures were referred to as the P1 generation of cells.

For MCC identification, P1 chondrocytes were seeded in 24-well plates and cultured for 3 days. A light microscope was used to observe the growth rate. The cells were fixed using 4% paraformaldehyde and stained using Toluidine blue and type II collagen immunofluorescence.

P1 chondrocytes were seeded in Flexcell force plates, and after 80% confluency was obtained, the culture medium was replaced with DMEM lacking FBS for 24 h to synchronize the cells. The medium was replaced with DMEM containing 10% FBS, and 0 (control group), 3% (low strength) and 15% (high intensity) tensile strains were applied at 0.5 Hz for 4 h. Each group was placed in three vice-holes.

For the cell proliferation assay, each group of cells was cultured for 48 h, and 10 μl of the CCK-8 solution was added. After a 4-h incubation, the absorbance was measured at 450 nm (A450). This assay was repeated three times and results were averaged for the statistical analysis.

For RNA extraction and reverse transcription, cells were collected, and total RNA was isolated using TRIzol. Total RNA was then reverse transcribed into cDNA and stored at -80°C.

Real-time PCR using cDNA as a template and a two-step method (denaturation at 95°C for 30 s and 42 cycles of 60°C for 34 s, 95°C for 15 s, 60°C for 60 s, and 95°C for 15 s) was performed to detect changes in gene expression levels of a cell proliferation factor (PCNA), an

Table 1. Real-time PCR primer sequences

Primer name	Name	Sequence information (5'-3')
GAPDH	Upstream primer	TCAGCGTCTCTGCTGTCACTCA
	Downstream primer	CGTTCATGCTCCAGCCGTTA
PCNA	Upstream primer	CTTGGAATCCCAGAACAGG
	Downstream primer	AGACAGTGGAGTGGCTTTT
Caspase-3	Upstream primer	TGTCATCTCGCTCTGGTAGC
	Downstream primer	AAATGACCCCTTCATCACCA
COL II	Upstream primer	CCTGGGACTCCTGGACTT
	Downstream primer	GTACCACCAGTTGGTTGTCTTTGA
Ihh	Upstream primer	TCAGCGATGTGCTCATTT
	Downstream primer	CCTCGTGAGAGGAGCATAGG
Ptc	Upstream primer	TGCTTGCCACTAAGCTTCG
	Downstream primer	TCCTAATCTCTCTGCCTGCACC
Smo	Upstream primer	AACGGTACCGTGCTGG
	Downstream primer	CATCATGGGAGACAGTGTGGC

apoptosis factor (caspase-3), a component of the chondrocyte matrix (COL II), inflammatory cytokines (IL-1β and TNF-α) and Hedgehog signaling-pathway molecules (Ihh, Ptc and Smo). The primer sequences are shown in **Table 1**.

Statistical analysis

All experiments were repeated three times, and the data are presented as the means ± standard deviation (x (-) ± s). Analyses were performed using a one-way ANOVA in SPSS 13 software. Differences were considered statistically significant at $P < 0.05$.

Results

Identification of rat condylar cartilage cells

Inverted microscopy showed that the original condylar cartilage cells were in good condition. The cell body was round and polygonal, similar to a "paving stone" (**Figure 1A**). Toluidine blue staining showed blue nuclei and cytoplasm consistent with the characteristics of chondrocytes (**Figure 1B**), which indicates that the cells had a stable phenotype.

Effects of different tensions on the proliferation of condylar cartilage cells

There were no significant differences in the titers of cells exposed to each level of loading tensile strain before cell loading or 4 h after cell loading. After culturing for 48 h, the A450 value of the 3% strain group was higher than that of the control group ($P < 0.05$), and the A450 value of the 15% strain group was lower than that of the control ($P < 0.05$) and 3% strain groups ($P < 0.01$). Thus, cell proliferation in the 3% strain group was enhanced, while that in the 15% strain group was decreased (**Figure 2**).

Real-time PCR

Different tensions applied to condylar cartilage cells had different effects on cell proliferation and matrix expression after 4 h. In the 3% strain group, rat condylar cartilage cells had significantly higher PCNA mRNA expression levels ($P < 0.01$) than the control group, and the 15% tensile strain group had lower PCNA mRNA expression levels than the control ($P < 0.05$) and 3% strain groups ($P < 0.01$). Caspase-3 mRNA expression levels were higher in the 15% strain group than in the 3% strain group ($P < 0.01$). COL II mRNA expression levels in the 3% strain group were significantly higher than in the 15% strain group ($P < 0.01$) (**Figure 3**).

Different levels of tension had different effects on the expression of inflammatory factors after 4 h. In the 15% strain group, IL-1β mRNA expression levels were higher than those in the control and 3% strain groups ($P < 0.05$), and TNF-α mRNA expression levels were higher than levels in the control group in both groups experiencing strain ($P < 0.05$) (**Figure 4**).

Different intensities of strain on the condylar chondrocytes had different effects on Hh pathway-related gene expression after 4 h. For the 3% strain group, Ihh and Smo mRNA expression levels were higher than those of the 15% strain group ($P < 0.05$ and $P < 0.01$), and Ptc mRNA expression levels were higher in both the 3% and 15% strain groups than in the control group ($P < 0.05$). Moreover, the mRNA expression level of Smo was lower than that of the control group in the 15% strain group ($P < 0.05$), as was the expression level of Ihh (< 15%) (**Figure 5**).

Discussion

Chondrocytes are central to various metabolic activities in articular cartilage. Chondrocytes

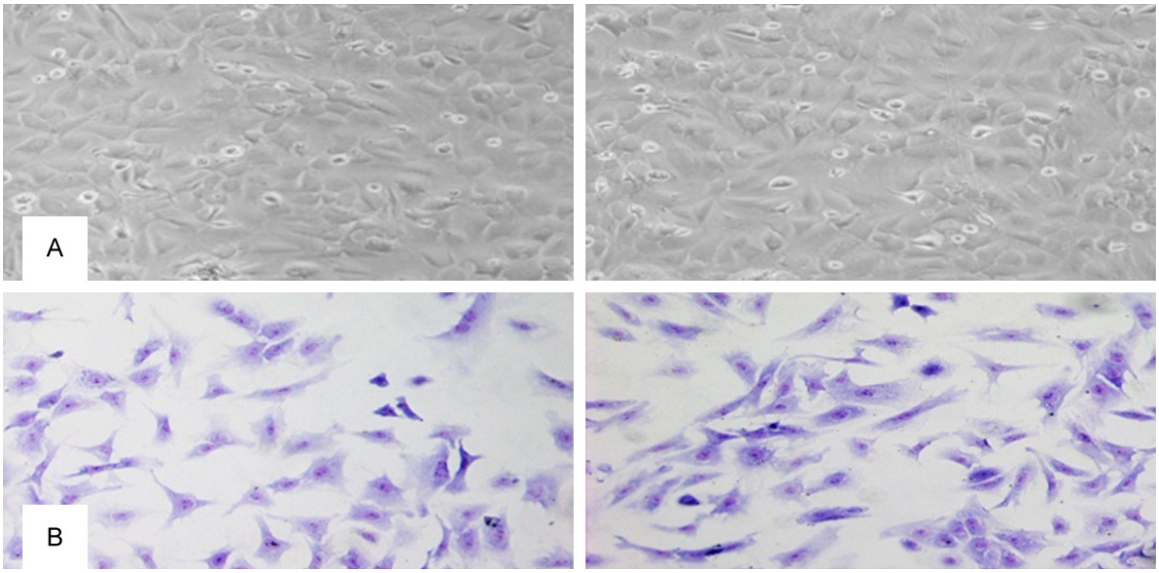

Figure 1. Identification of rat condylar chondrocytes (100×). A. Cell morphology observed under an inverted microscope. B. Toluidine blue staining.

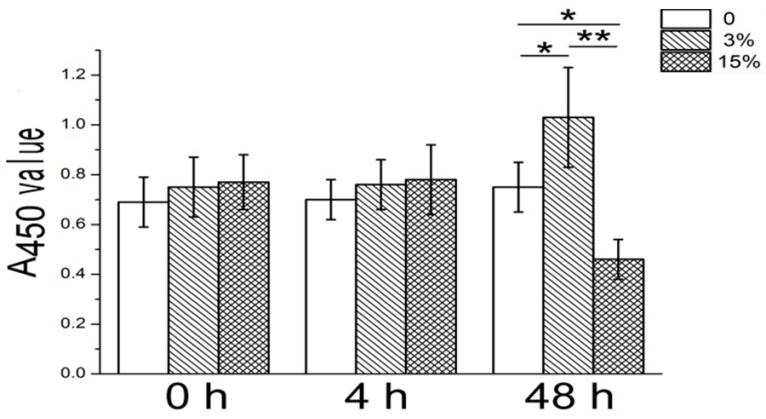

Figure 2. Cell proliferation activity induced by tensile strains after 48 h as measured using the CCK-8 assay.

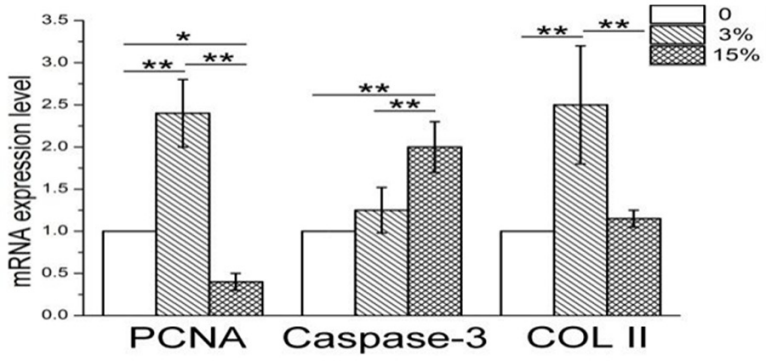

Figure 3. Gene expression levels of PCNA, caspase-3 and COL II after the application of 0, 3, and 15% tensile strains. *P < 0.05, **P < 0.01.

synthesize the extracellular matrix and also regulate the metabolism of the substrate. As cartilage cells can grow dynamically, they are a type of mechanically sensitive cell. Physiological mechanical stimulation is conducive to the growth, differentiation and metabolism of chondrocytes. In contrast, strong or weak mechanical stimulation can inhibit the growth of cartilage cells [15], affecting the balance of the synthesis and degradation of the extracellular matrix, which results in articular cartilage degeneration.

Under normal physiological conditions, cartilage is subjected to various types of mechanical stimuli, such as tension, shear stress and pressure [16]. Kelly et al. [17] studied a static elastic model of fast loading of articular cartilage and found that the cartilage around the cartilage compression zone of the joint is mainly affected by tensile

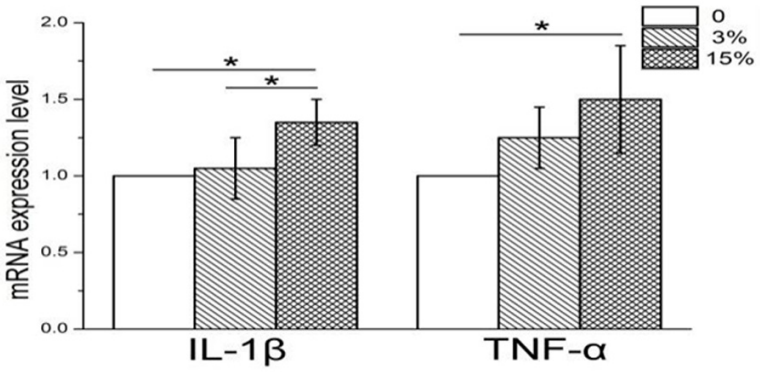

Figure 4. Gene expression levels of IL-1β and TNF-α after 0, 3, and 15% tensile strains. *P < 0.05.

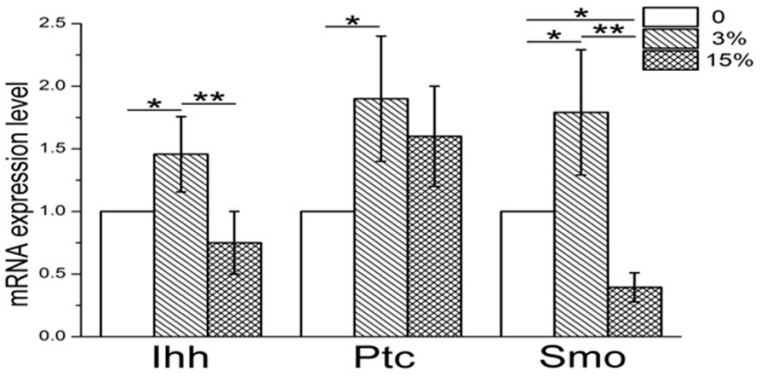

Figure 5. Gene expression levels of Ihh, Ptc and Smo after 0, 3, and 15% tensile strains.*P < 0.05, **P < 0.01.

stress during the fast loading of the articular cartilage. Xuejun et al. [18] studied mandibular condylar protrusive force by performing a three-dimensional finite element analysis (finite essence model, FEM), and they found that the upper region was mainly responsible for the performance of the joint following condylar tensile stimulation. The present study investigated the effect of tension on condylar cartilage cells. The Flexcell 4000TM mechanical signal loading system can be applied to cultured cells in a dynamic, stable and controllable manner. Here, we used low-frequency stimulation (0.5 Hz) [19] and tensile strains of 0% (control group), 3% (low intensity) and 15% (high intensity) to simulate the physiological environment, and we examined the changes that occurred in cartilage cells within 4 h.

After 4 h, cartilage cell proliferation in the 3% strain group was significantly higher than that in the control and 15% strain groups. Moreover, mRNA levels of the cartilage proliferating cell nuclear antigen (PCNA) were significantly higher in the 3% strain group than in the control group. Thus, stimulation with low-intensity tensile stress can induce condylar cartilage cell proliferation. Moreover, the cell proliferation capacity of the 15% strain group was lower than that of the control group, and the PCNA mRNA expression levels in this group were also lower than those in the control and 3% strain groups. Caspase-3 mRNA expression levels were higher in the 15% strain group than in the control and 3% strain groups, indicating that high-strength tensile stress can inhibit the proliferation of chondrocytes and promote apoptosis. The mRNA expression level of COL II was significantly higher in the 3% strain group than in the control and 15% strain groups, which indicate that low-intensity tensile stimulation promotes the synthesis of the extracellular matrix in cartilage. In the 15% strain group, IL-1β mRNA expression levels were higher than those in the control and 3% strain groups, and TNF-α mRNA expression levels were higher than those in the control group. These results indicate that high-strength tensile stress induces inflammation in cartilage cells and that low-intensity mechanical stimulation does not significantly evoke inflammation.

Tang et al. [20] found that tension can stimulate the expression of a member of the Hh gene family, Ihh, and promote cell proliferation in the mandibular condyle. Our experimental results show that after 4 h, Ihh mRNA expression levels were higher in the 3% strain group than in the control and 15% strain groups. Moreover, the Ptc and Smo mRNA expression levels were higher in the 3% strain group than in the control and 15% strain groups. These data show that the expression of Ptc, Smo and Ihh correlates with the intensity of mechanical stimulation.

The expression of Ihh may promote the proliferation of chondrocytes, but the Hh signaling pathway is inhibited by changes in PCNA and caspase-3 expression.

In conclusion, stimulation from moderate tension can promote the proliferation of chondrocytes. The synthesis of the extracellular matrix plays an active role in the remodeling of condylar cartilage, but it can inhibit the proliferation of chondrocytes, promote apoptosis and induce inflammatory reactions. In this process, the Hh signaling pathway may play a role in regulating the proliferation of chondrocytes, further affecting the synthesis of the extracellular matrix. To provide a theoretical basis for the clinical treatment of mandibular condylar fractures, conservative treatments using mandibular traction, and the treatment of mandibular deformities, further studies should be performed on the mechanical control and mechanical signal transduction of condylar cartilage.

Conclusions

Different intensities of tensile strain have different effects on cell proliferation and the expression inflammatory factors in rat condylar chondrocytes.

Subjecting cells to 15% tensile strain induces apoptosis and increases the expression of inflammatory cytokines. To promote cell proliferation and matrix synthesis, a 3% tensile strain should be used.

Hedgehog signaling pathway-related genes are sensitive to stretch stimulation and may be involved in the regulation of cartilage cells via mechanical stimulation.

Disclosure of conflict of interest

None.

Abbreviations

RT-PCR, Reserve Transcription-Polymerase Chain Reaction; RPM, Revolutions Per Minute; FBS, Fetal Bovine Serum; DMEM, Dulbecco's Modified Eagle's Medium; PBS, Phosphate Buffered Saline; DNase I, Deoxyribonuclease I; SD, Sprague-Dawley; PCNA, Proliferating Cell Nuclear Antigen; PCR, Polymerase Chain Reaction; COL II, Collagen II; TMJ, Temporomandibular Joint; MCC, Mandibular Condylar Chondrocyte.

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