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Original Article

The effect of vascular endothelial growth factor (VEGF) on mouse condylar articular cartilage cultured in vitro

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Abstract: In this study, we explored the direct effect of vascular endothelial growth factor (VEGF) on temporomandibular joint osteoarthritis (TMJ-OA) by analyzing the transformation of mouse condylar cartilage treated in vitro with various concentrations of VEGF. Tissue samples from 126 condyles of four-week-old male C57 mice were randomly divided into 21 groups and treated with VEGF (0 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, or 2 μg/mL). Furthermore, the samples were treated at different time points (1 d, 2 d, 4 d, and 7 d) and stained with hematoxylin and eosin (HE) and Safranin O and Fast Green stains to observe their morphology. The Mankin score was used to evaluate changes to the condylar cartilage tissues, and immunohistochemistry was performed to observe the expressions of VEGF receptor 2 (VEGFR2), matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 13 (MMP13), and tumor necrosis factor-related activation-induced cytokine (TRANCE). An HE staining analysis revealed that the experimental groups treated with VEGF exhibited the destruction of their condylar cartilage and a proliferation of their hypertrophic cells, in comparison to the control group. Safranin O and Fast Green staining showed that the experimental groups had decreased levels of proteoglycan and degenerative changes in their condylar cartilage. The Mankin score of the samples increased with increasing concentration and treatment time of VEGF, and the differences between the groups were statistically significant ($P < 0.05$). Immunohistochemistry demonstrated that the expression levels of VEGFR2, MMP9, MMP13, and TRANCE significantly increased in the experimental groups, in comparison to those in the control group, suggesting that VEGF promoted TMJ-OA in mice in vitro.

Keywords: Condylar articular cartilage, temporomandibular joint osteoarthritis, VEGF, in vitro culture

Introduction

Temporomandibular joint osteoarthritis (TMJ-OA) is a common clinical disease of unknown etiology; its pathogenesis has been a field of interest for many researchers. Vascular endothelial growth factor (VEGF) is a powerful angiogenic factor expressed during articular cartilage growth and re-expressed in osteoarthritis in normal, osteoarthritic, and osteoporotic osteoblasts [1-3]. Recently, the association of VEGF and TMJ-OA has become a focus of research. Hamilton *et al*. [4] determined that VEGF levels in the body correlated with osteoarthritis. Shen Pei *et al*. [5] demonstrated that the intraarticular injection of VEGF in the TMJ of mice induced osteoarthritis. In contrast, Walsh *et al*. [6] studied the relationship between VEGF and TMJ-OA by evaluating the effect of VEGF-induced angiogenesis, but there was minimal impact on TMJ-OA. Many scholars have investigated the possible relationship between VEGF and TMJ-OA through various animal experiments, but there are no *in vivo* studies that demonstrate a direct effect of VEGF on TMJ-OA. Although some groups have conducted studies using in vitro cell experiments, many argue that this method does not accurately reflect the development of TMJ-OA and structural changes in cartilage. In previous studies on the mechanism of VEGF on TMJ-OA, most scholars started from VEGF's angiogenesis and studied its relationship with TMJ-OA. However, there are few studies on the direct impact of VEGF on TMJ-OA.

On the basis of previous studies, this research group believes that it is necessary to find an

Table 1. Modified Mankin scoring system to evaluate the degree of osteoarthritis in articular cartilage

Mankin Evaluation Criteria	Score
① Peripheral Red O staining	
a. Normal	0
b. Mild strengthen	1
c. Severe strengthen	2
② Background Red O staining	
a. Normal	0
b. Mild increase or decrease	1
c. Severe increase or decrease	2
d. Non-staining	3
③ Cartilage cells arranged	
a. Normal	0
b. Cell aggregation	1
c. Cells decreased	2
④ Structure of cartilage	
a. Normal	0
b. Surface of fibrosis	1
c. Fibrosis over the surface	2
d. Lack	3

experimental method that can not only exclude the influence of systemic factors, but also evaluate the degree of osteoarthritis through changes in cartilage structure to study the direct influence of VEGF on TMJ-OA. In this study, the effect of VEGF on TMJ OA was excluded by the experimental group through the in vitro culture of the mouse condyle. The morphology of mouse condylar articular cartilage is observed at different time points with different concentrations of VEGF. The expressions of VEGF receptor 2 (VEGFR2), matrix metallopeptidase 9 (MMP9), matrix metallopeptidase 13 (MMP13), and tumor necrosis factor-related activation-induced cytokine (TRANCE), and cartilage degeneration in treated samples were analyzed to determine whether VEGF directly modulated the pathogenesis of TMJ-OA. In this study, we aimed to explore the direct effect of VEGF on TMJ-OA, and our findings can provide the basis for further study of the molecular mechanism of VEGF self-secretion axis induced TMJ-OA.

Materials and methods

Mice and other reagents

Samples of condylar articular cartilage were obtained from 4-week-old male C57 mice (Shanghai Key Laboratory of Stomatology & Shanghai Research Institute of Stomatology) and cultured in vitro. Recombinant human VEGF 165 (Pepro Tech, Rocky Hill, USA) was used to treat the cartilage samples. For immunohistochemistry, we used antibodies for VEGFR2 (CST #2472, 1:50, Cell Signaling, Danvers, USA), MMP9 (ab38898; 1:50, Abcam, Cambridge, UK), MMP13 (ab39012, 1:50, Abcam, Cambridge, UK), and TRANCE (CST #5312, 1:50, Cell Signaling, Danvers, USA).

Isolation of condylar cartilage

The mice were sacrificed via cervical dislocation in sterile conditions to isolate bilateral condyles, and polybutylene succinate was used to prevent condylar adhesion around the soft tissue. Tissue samples from 126 condyles were randomly divided into 21 groups, and each group contained six condyles. A single group was selected as the control group, denoted as "Con0", while the remaining 20 groups were transferred to petri dishes (35 mm) and divided into five treatment groups of VEGF concentration (0 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, and 2 μg/mL). Each treatment group was further subdivided into four different treatment time points (1 d, 2 d, 4 d, and 7 d). Therefore, a total of six mouse condyle samples represented each treatment concentration at each time point.

Culturing and sampling of condyles

To study the effect of sex steroid hormones on bone growth, Maor *et al*. [7] established an isolated organ culture system of the mandibular condyle, derived from 3.5-to-5.5-week-old male and female mice. Following this previously published approach, six samples of condyles were distributed evenly in a cell culture dish for each group. The culture medium was composed of Minimum Essential Medium (MEM) containing fetal bovine serum, ascorbic acid, and antibiotics. The culture medium was added to each dish, and then VEGF was added to the remaining culture medium. Additional culture medium, containing VEGF, was added to each treatment group according to the respective treatment concentrations (100 ng/mL, 500 ng/mL, 1 μg/mL, and 2 μg/mL) via serial dilution. The cells were incubated at 37°C and 5% CO_2. The culture medium was changed once every two days. The plates were fixed at various time points (1 d, 2 d, 4 d, and 7 d), representing

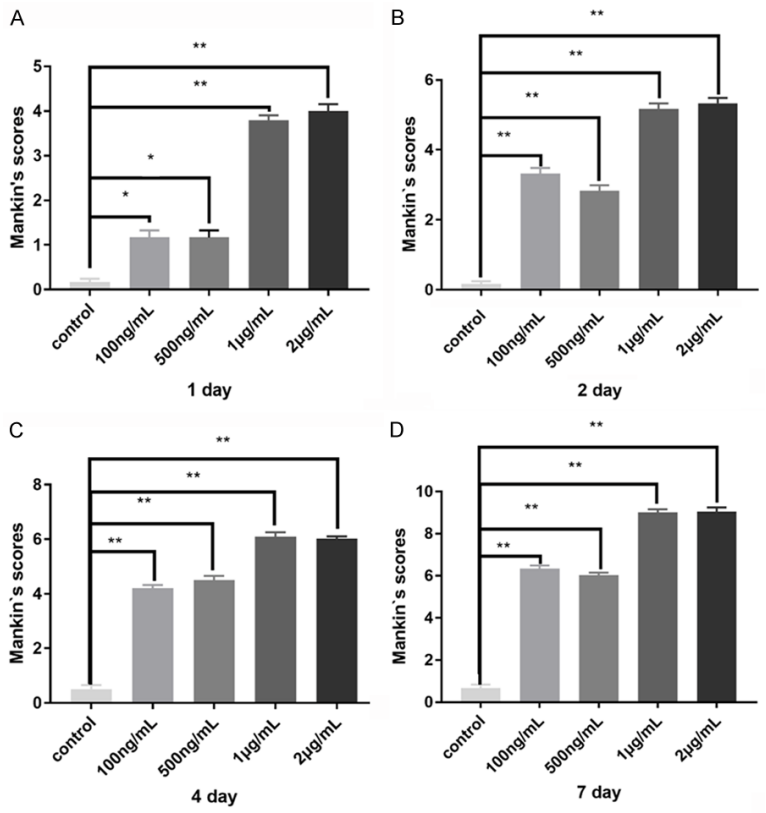

Figure 1. A-D. Mankin scores of condylar articular cartilage at various VEGF concentrations and treatment times. (**P < 0.01, *P < 0.05).

treatment times per treatment group, with paraformaldehyde (4%). The condylar group denoted as "Con0" was fixed immediately after culturing.

Histological staining

The mouse condylar cartilage samples were fixed for 24 h and then rinsed in water overnight. The samples were decalcified with EDTA (10%) at 4°C, and then dehydrated with increasing concentrations of n-butyl alcohol and embedded with paraffin. Cross sections of 4 μm were obtained from each tissue specimen. Conventional Red O and Fast Green compound staining and HE staining were conducted. The morphologies of the mouse condylar cartilage samples were observed under a microscope. According to the manufacturer's instructions (Sigma 387-A, St Louis, MO), a standard, 3-step, avidin-biotin complex immunohistochemical staining protocol was used. For immunohistochemistry, we used antibodies for VEGFR2 (CST #2472, 1:50, Cell Signaling, Danvers, USA), MMP9 (ab38898; 1:50, Abcam, Cambridge, UK), MMP13 (ab39012, 1:50, Abcam, Cambridge, UK), and TRANCE (CST #5312, 1:50, Cell Signaling, Danvers, USA). So Immunohistochemical staining was used to visualize the expression of VEGFR2, MMP9, MMP13, and TRANCE in the samples.

Mankin scoring system

Red O and Fast Green compound staining was used to evaluate the degree and severity of osteoarthritis in the mouse condylar samples according to Mankin's criteria (**Table 1**).

Statistical analysis

SPSS 23.0 software was used for the statistical analysis. The Kruskal-Wallis method was used for all paired comparisons and to inspect the differences between multiple sets of data. For all bilateral analyses, P < 0.05 was considered to be statistically significant.

Results

Morphology of the mouse condylar articular cartilage cultured in vitro

We used HE staining to classify the structure of the mouse condylar cartilage into four categories: the fibrous layer, the hyperplastic layer, the layer of mast cells, and the cartilage (**Figure 2**). Over various time points, the condylar cartilage samples that were not treated with VEGF exhibited no significant changes in the condylar cartilage layer thickness, the number of cells, or the cartilage surface, compared with the control group. This validated that *in vitro* culturing had no influence on condylar cartilage morphology. The addition of VEGF to the condylar cartilage samples resulted in morphological changes, such as fibrous layer thinning, fat layer thickening, and an increased number of mast cells (**Figure 2**). Moreover, the increased concentration and treatment time of VEGF resulted in increasingly degenerative changes to the cartilage.

Red O and Fast Green compound staining showed that the condyle's tinting of the control

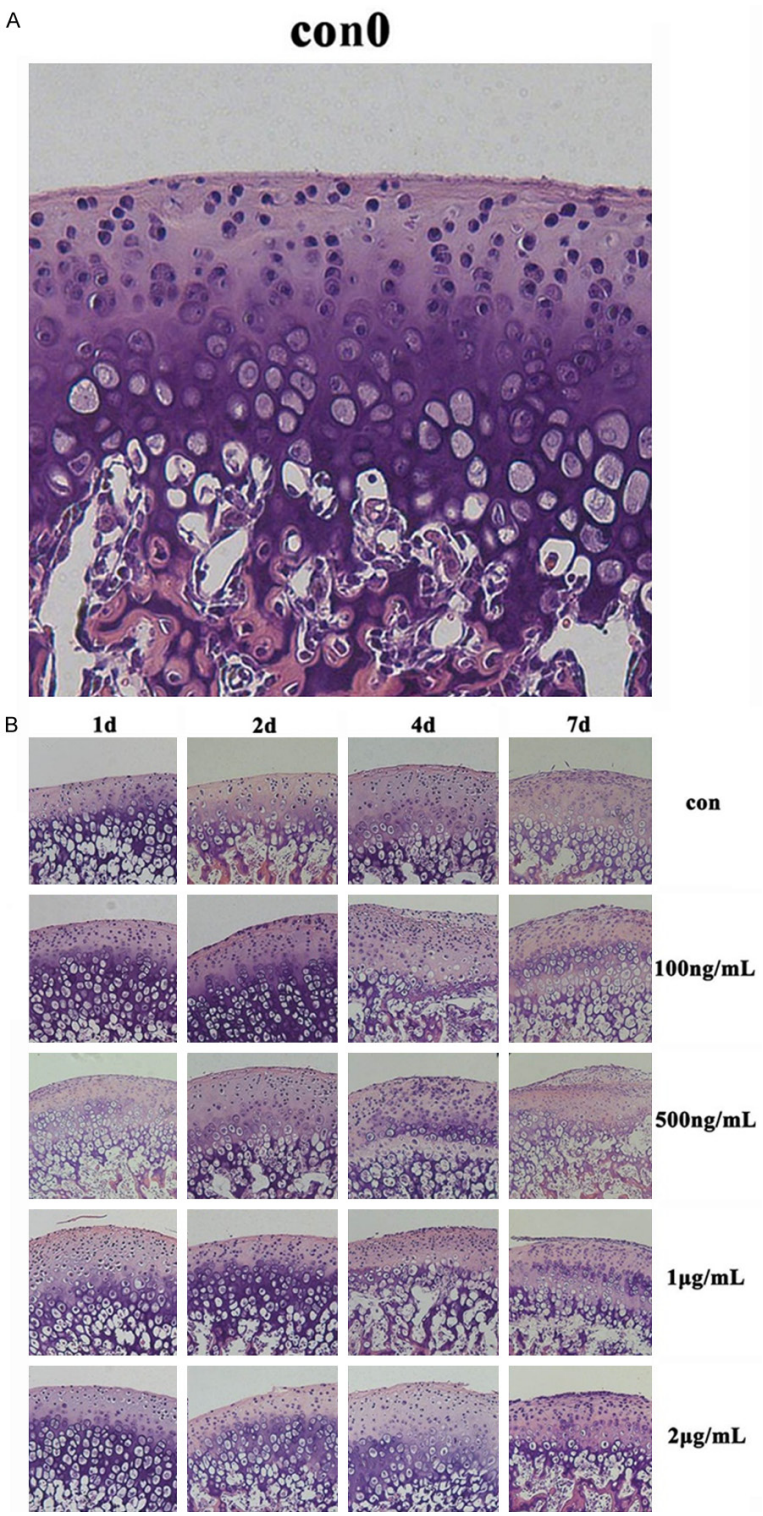

Figure 2. A, B. HE staining of mouse condylar articular cartilage.

group is more obvious, especially in the deep cartilage, from the enrichment of protein polysaccharides (**Figure 3**). Over the various time points, the condylar cartilage samples within the control group exhibited no significant changes. However, increasing VEGF concentration and treatment time resulted in an irregular arrangement of cartilage cells and a cell-free zone that appeared to be fibrous hyperplasia (**Figure 3**). These H-E staining and Red O and Fast Green compound staining results demonstrated that VEGF treatment resulted in structural abnormalities in mouse condylar cartilage cultured in vitro.

Mankin score of osteoarthritis in condylar cartilage samples

The Mankin scores of each treatment group are illustrated in **Figure 1**. We observed that overall, the Mankin scores of the samples significantly increased as the VEGF concentration and treatment time increased. Multiple pairwise comparisons revealed that the relationship between the Mankin score and the VEGF concentration and treatment time was statistically significant ($P < 0.05$).

However, the differences in the Mankin scores between the treatment groups of 100 ng/mL and 500 ng/mL and the treatment groups of 1 μg/mL and 2 μg/mL, respectively, showed no statistical significance. The Mankin scores of the samples within the same VEGF concentration group increased as treatment time increased, which was determined to be statistically significant ($P < 0.05$). Multiple pairwise comparisons revealed that the relationship between the Mankin scores and the treatment time was statistically significant. In fact, the treatment time significantly increased the Mankin scores more than VEGF concentration alone. The control group, denot-

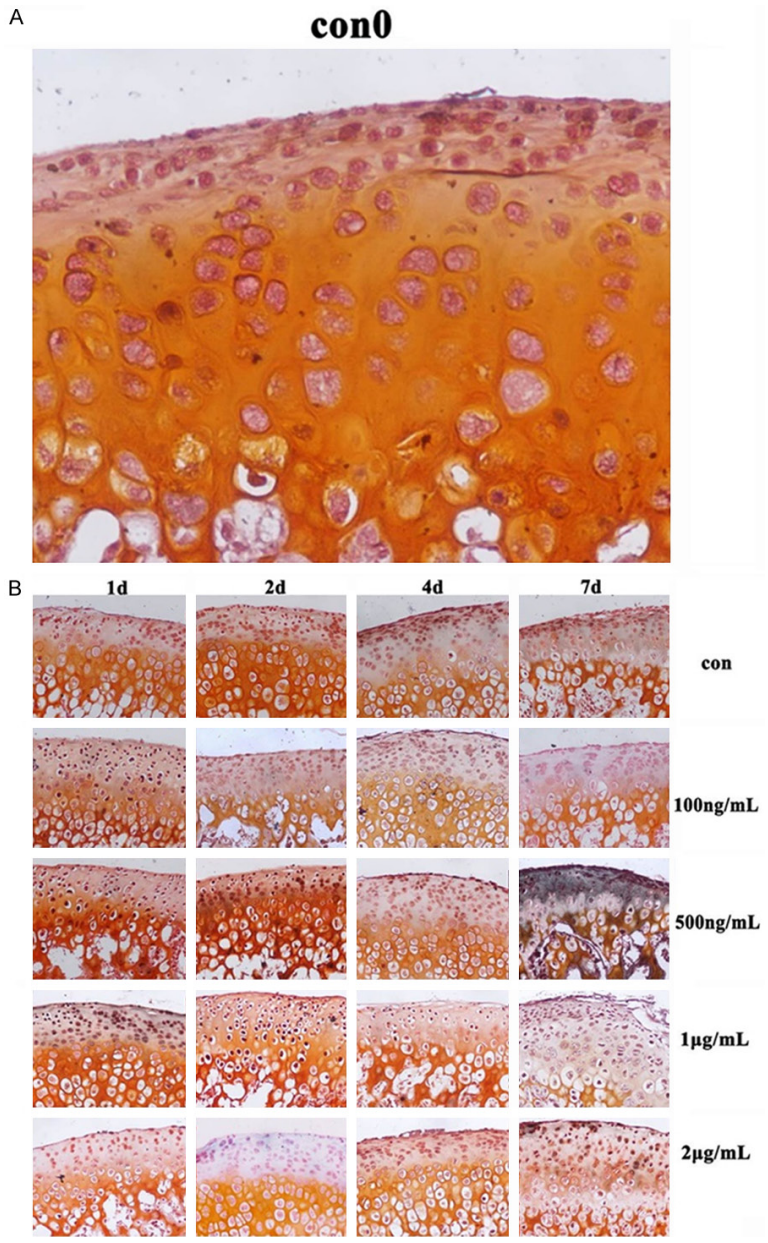

Figure 3. A, B. Safranin O and Fast Green compound staining of mouse condylar articular cartilage.

ed as Con0, did not exhibit any statistically significant changes in Mankin scores over the various time points ($P > 0.05$). This validated that culturing condylar articular cartilage in vitro did not affect the Mankin score.

Immunohistochemistry of mouse condylar articular cartilage cultured in vitro

We performed an immunohistochemical analysis to examine the expression of VEGFR2, MMP9, MMP13, and TRANCE in mouse condylar articular cartilage. The expressions of VEGFR2 and MMP13 were distributed throughout all four structural layers of cartilage previously mentioned (**Figures 4**, **6**). The expression of MMP9 was primarily localized in the mast cells in condylar cartilage (**Figure 5**). The expression of TRANCE was mainly distributed in the hypertrophic and proliferative layers of condylar cartilage (**Figure 7**). The expression levels of VEGFR2, MMP9, MMP13, and TRANCE in the cartilage samples significantly increased with VEGF treatment compared to the control group (**Figures 4B**, **5B**, **6B**, **7B**) ($P < 0.001$).

Discussion

TMJ-OA is a common clinical disease with diverse etiologies, and its pathogenesis is still unclear. Research on its molecular mechanisms has been a hot spot among domestic and foreign scholars. At present, the clinical diagnosis of TMJ-OA is often based on its imaging examination. When changes can be observed from the imaging data, the disease has to be treated surgically and is difficult to cure. Therefore, it is important and clinically significant to improve our understanding of the factors that influence the diagnosis of TMJ-OA lesions and that can be used for early diagnosis. TMJ-OA has vast similarities to osteoarthritis, and its pathology is strongly connected to abnormal cartilage degeneration and regeneration [8]. Histopathologically, fibrosis of cartilage surface, loss of matrix proteoglycans, destruction of collagen matrix, and hyperplasia of synovial tissue comprise the early stages of TMJ-OA [9, 10]. Previous studies confirmed that the abnormal activation of estrogen and the Wnt5A signaling pathways

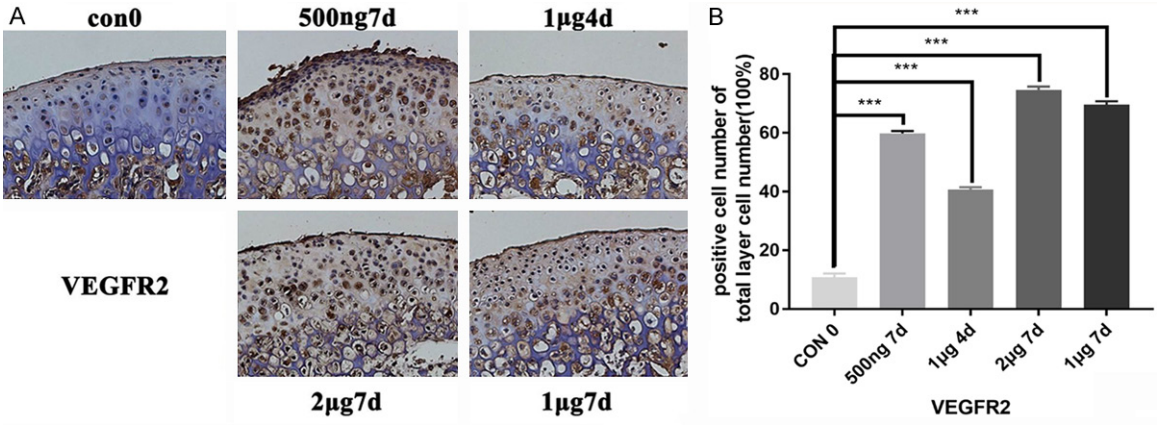

Figure 4. A. Expression of VEGFR2 measured by immunohistochemistry. B. Positive cell number of total layer cell number in the different groups (100%). (***$P < 0.001$).

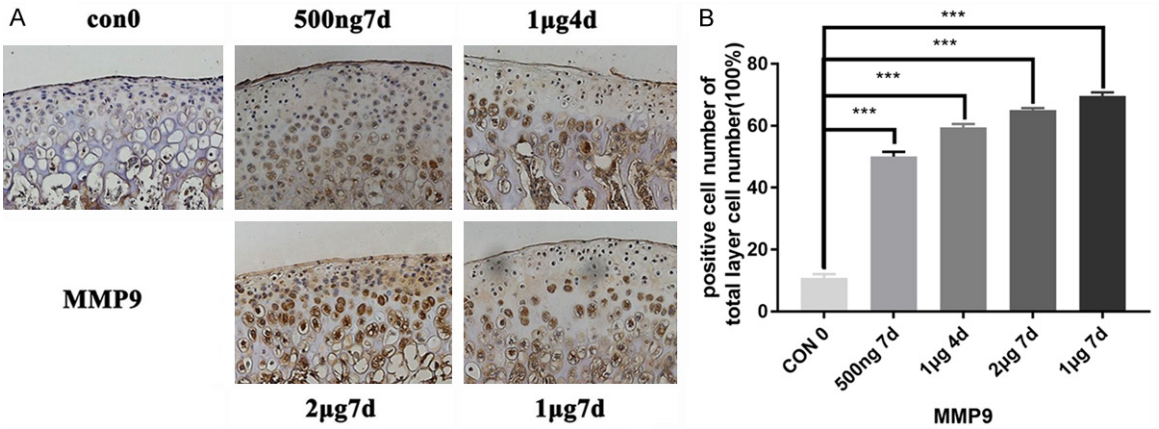

Figure 5. A. Expression of MMP9 measured by immunohistochemistry. B. Positive cell number of total layer cell number in the different groups (100%). (***$P < 0.001$).

induced TMJ-OA occurrence and development [11, 12]. Zhang *et al*. [13] observed in animals that synovial tissue increases the expression of VEGFR2, as scored by immunohistochemistry, following synovial inflammation, which subsides through the regeneration of TMJ-OA. Therefore, VEGFR2 may have some effect on the induction of TMJ-OA. In recent years, the relationship between VEGF and TMJ-OA development has become a focus in the field of TMJ-OA research.

The present study on mouse condylar articular cartilage cultured in vitro demonstrated that VEGF has a significant effect on TMJ-OA. Cultured condylar cartilage samples were scored according to the widely used and reliable Mankin scoring system for osteoarthritis. Our results confirmed that in vitro culturing had no significant effect on the degree of condylar bone arthritis, as determined by the Mankin score, in condylar articular cartilage samples. However, we demonstrated that there was a relationship between samples treated with VEGF and an increased pathology of osteoarthritis. A previous study conducted on animal models by Tibesku *et al*. [14] supports the results of our current study. Studies have reported that VEGF targets synovial articular cartilage and causes cartilage degeneration, progressive degradation of matrix components, and cell apoptosis [15]. In previous studies in vivo, an increased expression of matrix metallopeptidases in articular cartilage was observed, causing cartilage matrix degradation and, consequently, promoting the expression of VEGF to induce angiogenesis [16-18]. These studies demonstrate that an increased expres-

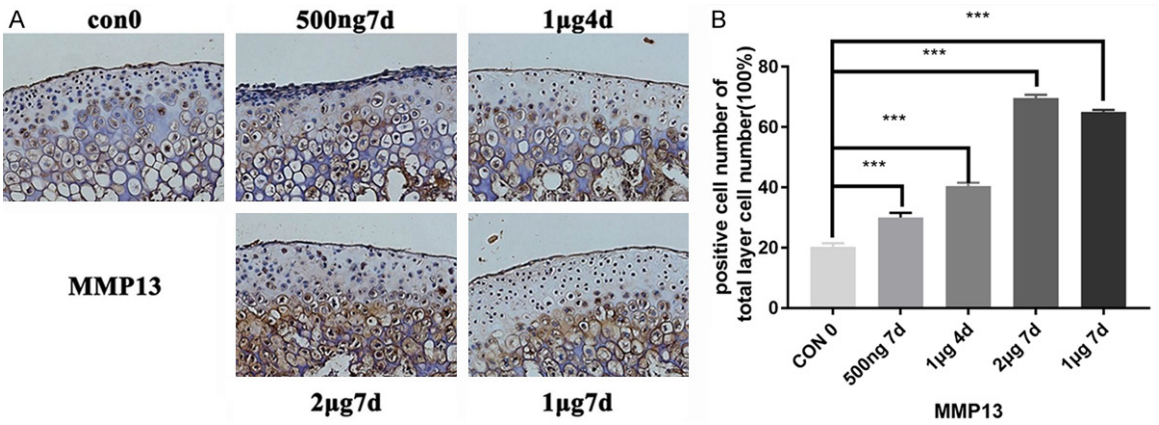

Figure 6. A. Expression of MMP13 measured by immunohistochemistry. B. Positive cell number of total layer cell number in the different groups (100%). (***$P < 0.001$).

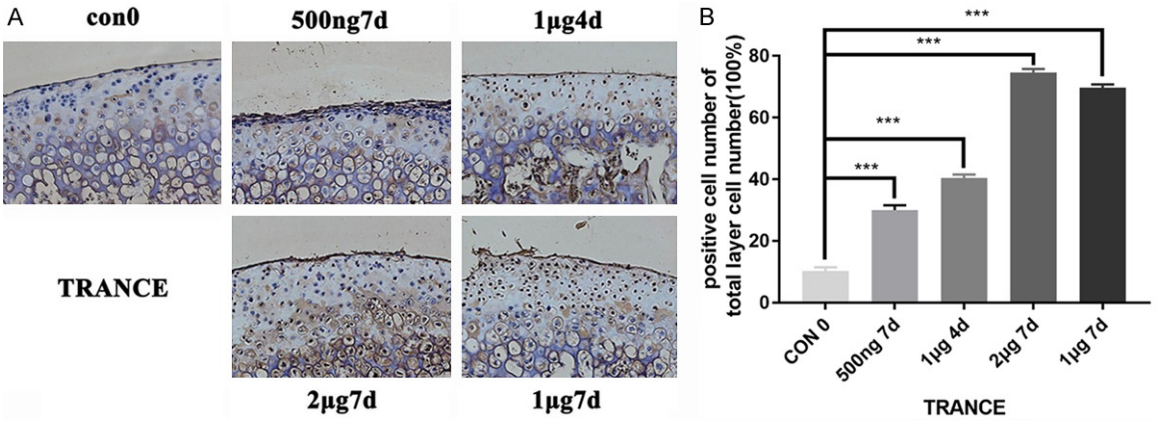

Figure 7. A. Expression of TRANCE measured by immunohistochemistry. B. Positive cell number of total layer cell number in the different groups (100%). (***$P < 0.001$).

sionofVEGFandangiogenesissubsequentlyincreased the production of matrix metallopeptidases, resulting in a cycle of degradation and regeneration of the cartilage matrix. Over time, this led to a progressive degeneration of cartilage. In this study, we eliminated the effect of MMP on VEGF by limiting our experiments to in vitro culture. Meanwhile, our in vitro study demonstrated that the expression of MMP9 and MMP13 increased. This is consistent with Kim's and Suri's research [19, 20]. To exclude the effects of these inflammatory factors on cartilage in our study, we added experimental TRANCE dye to culture soft cells with VEGF, inducing the apoptosis of chondrocytes in vitro.

In the current study, it was essential to maximize the advantages of using an in vitro system to study the direct effect of VEGF on TMJ-OA. In comparison to the experimental methods of the in vivo animal studies previously conducted, our in vitro system effectively eliminated the effects of other risk factors of TMJ-OA, allowing us to explore VEGF as a single factor of the disease. Furthermore, we were able to monitor cartilage structural changes and evaluate the severity of osteoarthritis in mouse condylar cartilage samples using our in vitro system. We determined that increasing VEGF concentration and treatment time affected the severity of TMJ-OA, as determined by the Mankin score. We reported that samples in the following treatment groups, 100 ng/mL for 7 d, 500 ng/mL for 7 d, 1 μg/mL for 7 d and 2 μg/mL for 7 d, were representative of the positive correlation between VEGF treatment and TMJ-OA. We conducted an immunohistochemical analysis using these hallmarks as a basis. These results may serve to improve the efficiency of future experiments.

However, this study had a few limitations. The sample size we used in the study was sufficient for statistical evaluation, but increasing the sample size would increase the precision and clarity of the results. We only analyzed the expression of three different proteins, MMP9, MMP13, and TRANCE, as molecular markers of TMJ-OA. There are many other proteins related to osteoarthritis that should be investigated to increase our understanding of the pathology of TMJ-OA. In conclusion, our results demonstrated that VEGF directly affected the development and severity of TMJ-OA in mouse condylar articular cartilage, supporting the existence of a relationship between the VEGF autocrine axis and TMJ-OA. However, the mechanism of action of VEGF and its related signaling pathways in the pathogenesis of TMJ-OA needs to be elucidated in future work.

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Disclosure of conflict of interest

None.

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References

[1] Lingaraj K, Poh CK, Wang W. Vascular endothelial growth factor (VEGF) is expressed during articular cartilage growth and re-expressed in osteoarthritis. Ann Acad Med Singapore 2010; 39: 399-403.

[2] Chim SM, Tickner J, Chow ST, Kuek V, Guo B, Zhang G, Rosen V, Erber W, Xu J. Angiogenic factors in bone local environment. Cytokine Growth Factor Rev 2013; 24: 297-310.

[3] Corrado A, Neve A, Cantatore FP. Expression of vascular endothelial growth factor in normal, osteoarthritic and osteoporotic osteoblasts. Clin Exp Med 2013; 13: 81-84.

[4] Hamilton JL, Nagao M, Levine BR. Targeting VEGF and its receptors for the treatment of osteoarthritis and associated pain. J Bone Miner Res 2016; 31: 911-924.

[5] Shen P, Jiao ZX, Zheng JS. Injecting vascular endothelial growth factor into the temporomandibular joint induces osteoarthritis in mice. Sci Rep 2015; 5: 16244.

[6] Walsh DA, McWilliams DF, Turley MJ. Angiogenesis and nerve growth factor at the osteochondral junction in rheumatoid arthritis and osteoarthritis. Rheumatology (Oxford) 2010; 49: 1852-1861.

[7] Maor G, Segev Y, Phillip M. Testosterone stimulates insulin-like growth factor-I and insulin-like growth factor-I-receptor gene expression in the mandibular condyle-a model of endochondral ossification. Endocrinology 1999; 140: 1901-1910.

[8] Loeser RF, Goldring SR, Scanzello CR. Osteoarthritis: a disease of the joint as an organ. Arthritis Rheum 2012; 64: 1697-1707.

[9] Zhang S, Huang D, Liu X. Arthroscopic treatment for intra-articular adhesions of the temporomandibular joint. J Oral Maxillofac Surg 2011; 69: 2120-2127.

[10] Zhang S, Yang C, Cai X. Prevention and treatment for the rare complications of arthroscopic surgery in the temporomandibular joint. J Oral Maxillofac Surg 2011; 69: 347-353.

[11] Liu XM, Zhang SY, Yang C. Correlation between disc displacements and locations of disc perforation in the temporomandibular joint. Dentomaxillofac Radio 2010; 39: 149-156.

[12] Chen MJ, Yang C, Zhang SY. Use of coblation in temporamandibular joint arthroscopy to treat internal derangement. J Oral Maxillofac Surg 2011; 68: 2085-2091.

[13] Zhang S, Cao W, Wei K. Expression of VEGF-receptors in TMJ synovium of rabbits with experimentally induced internal derangement. Br J Oral Maxillofac Surg 2013; 51: 69-73.

[14] Tibesku CO, Daniilidis K, Skwara A. Expression of vascular endothelial growth factor on chondrocytes increases with osteoarthritis. Open Orthop J 2011; 5: 177-180.

[15] Schwartz HC. Pathophysiology and pharmacologic control of osseous mandibular condylar resorption. J Oral Maxillofac Surg 2013; 71: 4.

[16] Nisato RE, Hosseini G, Sirrenberg C, Butler GS, Crabbe T, Docherty AJ, Wiesner M, Murphy G, Overall CM, Goodman SL, Pepper MS. Dissecting the role of matrix metalloproteinases (MMP) and integrin alpha(v)beta3 in angiogenesis in vitro: absence of hemopexin C domain bioactivity, but membrane-type 1-MMP and alpha(v)beta3 are critical. Cancer Res 2005; 65: 9377-9387.

[17] Ichikawa Y, Ishikawa T, Momiyama N, Kamiyama M, Sakurada H, Matsuyama R, Hasegawa S, Chishima T, Hamaguchi Y, Fujii S, Saito S, Kubota K, Hasegawa S, Ike H, Oki S, Shimada H. Matrilysin (MMP-7) degrades VE-cadherin and accelerates accumulation of beta-catenin in the nucleus of human umbilical vein endothelial cells. Oncol Rep 2006; 15: 311-315.

[18] Bini A, Wu D, Schnuer J, Kudryk BJ. Characterization of stromelysin 1 (MMP-3), matrilysin (MMP-7), and membrane type 1 matrix metalloproteinase (MT1-MMP) derived fibrin(ogen) fragments D-Dimer and D-like monomer: NH2-Terminal sequences of late-stage digest fragments. Biochemistry 1999; 38: 13928-13936.

[19] Kim HR, Lee JH, Kim KW. The relationship between synovial fluid VEGF and serum leptin with ultrasonographic findings in knee osteoarthritis. Int J Rheum Dis 2016; 19: 233-240.

[20] Suri S, Walsh DA. Osteochondral alterations in osteoarthritis. Bone 2012; 51: 204.