

Original Article

Therapeutic effect of low-intensity pulsed ultrasound on temporomandibular joint injury induced by chronic sleep deprivation in rats

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Abstract: Low-intensity pulsed ultrasound (LIPUS) treatment is an emerging physical therapy for treating bone, nerve, and muscle disorders. However, there have been no reports on the effectiveness of LIPUS for the treatment of temporomandibular joint injury, and the mechanisms of LIPUS remain unclear. The purpose of this study was to examine the therapeutic effects of LIPUS on temporomandibular joint injury in rats subjected to chronic sleep deprivation (CSD). In this study, after 2 weeks of chronic sleep deprivation in rats, the condylar cartilage exhibited rough surfaces, with a disorganized arrangement and partial sloughing of collagen fibers, decreased proliferation of chondrocytes, increased osteoclast activity in the calcified cartilage layer, and increased ratios of MMP-3/TIMP-1 and RANKL/OPG expression. After 4 weeks of LIPUS intervention in rats, the condylar cartilage displayed prominent reductions in these pathological changes, including noticeable repair of the injured cartilage structure, increased chondrocyte proliferation, a reduced number of osteoclasts, and marked reductions in the expression ratios of MMP-3/TIMP-1 and RANKL/OPG. These results demonstrated that LIPUS can effectively inhibit CSD-induced injury to condylar cartilage in rats. The therapeutic mechanism of LIPUS may involve promoting the repair function of chondrocytes and reducing the expression ratios of MMP-3/TIMP-1 and RANKL/OPG in condylar tissue, thus inhibiting the cleavage activity of MMP-3 on the condylar cartilage matrix and inhibiting osteoclast activation.

Keywords: Low-intensity pulsed ultrasound (LIPUS), chronic sleep deprivation (CSD), temporomandibular joint disorders (TMDs), condylar cartilage

Introduction

Temporomandibular joint disorders (TMDs) are common and frequent functional disorders occurring in the oral and maxillofacial regions, and the etiology of TMDs is complex and remains controversial [1]. In addition to occlusion, mental factors are one of the most important causes of TMDs [2, 3]. The course of TMDs is complicated and often characterized by functional abnormalities. In severe cases, the structure of joints may become disordered, or destruction may occur, but inflammatory damage and degenerative changes in the condylar tissue usually present throughout the disease course [4]. Reversible and conservative treatments targeting the cause of the injury are cur-

rently the main clinical therapies for TMDs [5], while direct treatment methods are still lacking. As a result, local TMD symptoms are not quickly or effectively alleviated. Therefore, the search for local therapies to directly control TMD pathological impairments and promote the repair of cartilage tissue has become an urgent problem in TMD treatment.

Low-intensity pulsed ultrasound (LIPUS) is an emerging physical therapy method that uses ultrasound to treat bone, nerve, and muscle diseases. LIPUS uses pulsed ultrasound with an intensity less than 100 mW/cm² to directly treat the affected area and produces various biological effects through mechanical actions, such as acoustic waves or acoustic microfluid-

ics, providing significant therapeutic effects in healing bone fracture, nerve damage and skeletal muscle injury [6-9]. LIPUS has good directionality, which allows ultrasonic wave concentration and transmission of a small amount of energy to the tissue, thus preventing thermal damage caused by excessive heat generated at the treatment site [10]. Consequently, LIPUS is a safe and reliable noninvasive clinical therapy method. In recent years, there have been reports of the positive effects of LIPUS in the treatment of osteoarthritis [11, 12], but the underlying mechanism is not fully understood. In addition, there are no reports on the application or effects of LIPUS for the treatment of TMDs.

Chronic sleep deprivation (CSD) is a relatively validated method for establishing temporomandibular joint injury in animal models using stress factors [13]. Compared with other modeling methods, CSD is highly efficient and has few interfering factors [14]. In this study, we established a model of temporomandibular joint injury in Wistar rats using the CSD method, and LIPUS was used to treat the pathological manifestations observed in the condylar cartilage. The behavioral phenomena, changes in condylar cartilage structure, and expression ratios of MMP-3/TIMP-1 and RANKL/OPG in the cartilage before and after CSD and LIPUS intervention were comprehensively observed. We aimed to clarify the therapeutic effect of LIPUS on the inflammatory damage to and degenerative changes in the condylar cartilage tissue of CSD rats, to explore the molecular mechanism of LIPUS for the treatment of condylar cartilage injury, to provide a theoretical basis for an in-depth study of TMD prevention and management, and to provide an experimental basis for the application of LIPUS technology in stomatology.

Materials and methods

Experimental animals and grouping

Prior approval from the Animal Care and Use Committee of Beijing Stomatological Hospital was obtained in accordance with international guidelines for care in animal research. The protocol (permit number: KQYY-201610-001) was approved by the Committee on the Ethics of Animal Experiments of Beijing Stomatological Hospital. All surgeries were performed under isoflurane gas anesthesia, and all efforts were made to minimize animal suffering.

Forty male 8-week-old Wistar rats (weighing 200 ± 10 g) were purchased from the Sipeifu experimental animal center (Beijing, China). The animals were acclimated to laboratory conditions for one week, housed in cages at $20 \pm 3^\circ\text{C}$ under a 12-hour light-dark cycle and given free access to food and water.

Forty Wistar rats were randomly divided into four groups: a blank control group (BC group), a chronic sleep deprivation group (CSD group), a LIPUS-treated group (LIPUS group), and a non-treated control group (NC group), with ten animals in each group. The left condyle of each rat was used for histomorphological and immunohistochemical observation, and the right condyle was used for molecular biology experiments.

CSD animal model

CSD was induced by a multiplatform method. As reported in a previous study by our research group [15], the rats were placed inside a sleep deprivation water tank containing 15 small circular platforms, each with a diameter of 6.5 cm. The tank was filled with water until the water level reached approximately 1 cm below the surface of the platform. The rats were free to move from one platform to another. When a rat reached the rapid-eye-movement (REM) sleep stage, it was awakened by its head touching the water. This cycle occurred repeatedly, thus inducing sleep deprivation.

In the BC group, a grid floor was placed on the platforms inside the water tank. The rats were able to sleep on the grid without falling into the water, but their tails were able to touch the water, thus providing environmental control. Rats in the CSD, LIPUS and NC groups were deprived of REM sleep for 22 h every day for 2 weeks and were allowed to sleep for 2 h as a buffer. After 2 weeks, the rats in the BC and CSD groups were sacrificed, and specimens were collected. The rats in the LIPUS and NC groups then underwent the next stage of intervention.

LIPUS intervention

After developing the animal model, the rats in the LIPUS and NC groups were transferred to normal cages. The rats in the LIPUS group began receiving LIPUS treatments on the bilateral temporomandibular joint the following day. An Osteotron IV ultrasonic therapy device (ITO

Therapeutic effect of LIPUS on TMJ injury

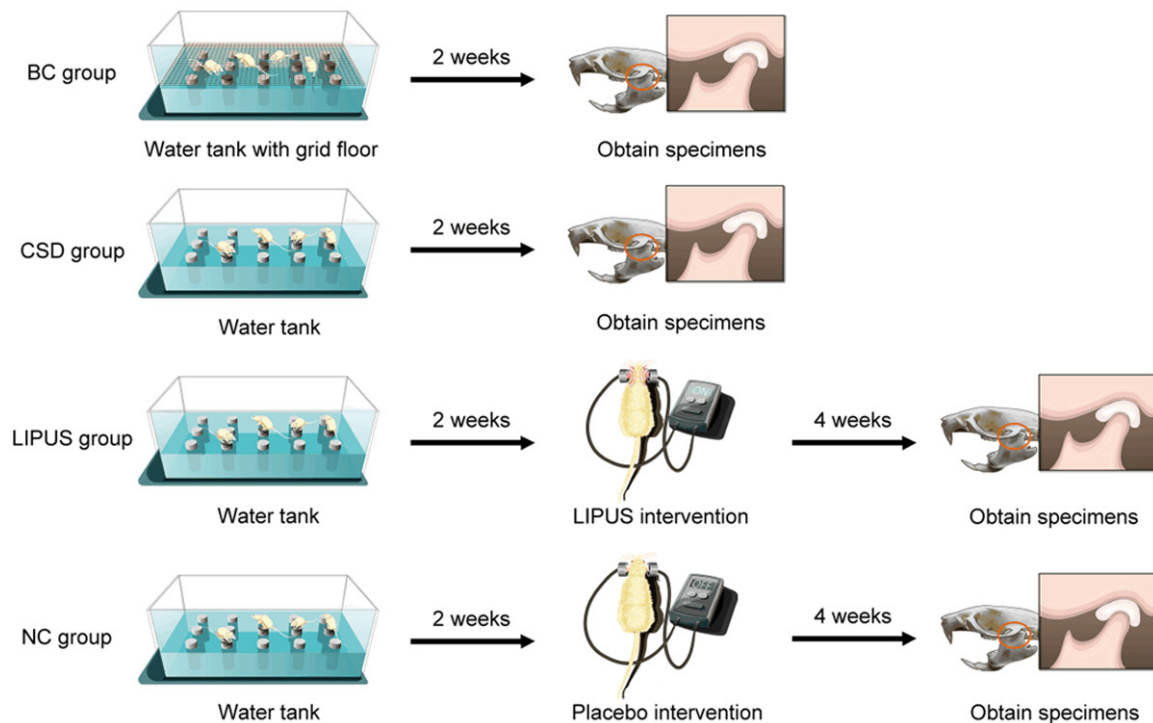


Figure 1. Diagram of the grouping method and interventions for the experimental animals. Forty Wistar rats were divided into four groups: a blank control (BC) group, a chronic sleep deprivation (CSD) group, a LIPUS-treated (LIPUS) group, and a non-treated control (NC) group. Rats in the CSD, LIPUS and NC groups were deprived of rapid-eye-movement sleep while placed in a water tank for 2 weeks. The rats in the BC group were able to sleep on the grid floor placed on the platforms inside the water tank. After 2 weeks, rats in the BC and CSD groups were sacrificed to obtain specimens. The rats in the LIPUS group began receiving the LIPUS intervention on the temporomandibular joint, and the NC group received a placebo intervention with no power. After 4 weeks, the rats in the LIPUS and NC groups were sacrificed to obtain specimens.

Ultrashort Wave Co. Ltd., Tokyo, Japan) was used to deliver ultrasound therapy at an ultrasonic intensity of 45 mW/cm^2 , an ultrasonic frequency of 1.0 MHz , a pulse width of $200 \mu\text{s}$, and a frequency of 1 kHz . The LIPUS probe was applied with a coupling agent and placed close to the skin surface of the rat condyle for 20 min. The treatment lasted for 4 weeks and was performed 5 times per week (once daily Monday through Friday). The non-treated control group received a placebo intervention in the same manner with no power. After 4 weeks, the rats in the LIPUS and NC groups were sacrificed, and specimens were obtained.

The details of the grouping method and the time points at which the rats were sacrificed are presented in **Figure 1**.

Behavioral testing

The activity of the rats in each group was determined by an open-field experiment, and the

degree of temporomandibular joint pain was indirectly evaluated by assessing the anxiety-like behavior of the rats. Each rat was placed in an opaque open field with a length, width and height of $100 \text{ cm} \times 100 \text{ cm} \times 50 \text{ cm}$. The bottom of the black-colored open field was divided into 25 squares of equal area. The behavior of the rats was recorded simultaneously by two researchers for 5 min. Horizontal activity was scored as follows: 1 point was scored for an animal running across 1 grid or 1 point for every 20 cm that an animal ran along a line. Vertical activity was scored as follows: 1 point was scored if both forelegs left the floor, regardless of how long the rat stood.

Detection of ACTH and CORT concentrations in rat serum

Before sacrificing the rats in each group, 2 ml of venous blood was collected from the tail vein of each rat. The blood sample was centrifuged at 3000 g for 10 min at 4°C . Approximately 0.5

ml of the supernatant was collected and stored at -80°C. Concentrations of adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) in the rat serum were measured using enzyme-linked immunosorbent assay (ELISA) kits (Elabscience, Wuhan, China) following the manufacturer's protocol.

Hematoxylin-eosin staining

After sacrifice, left condylar specimens were obtained and fixed in 10% paraformaldehyde, decalcified with 10% ethylenediaminetetraacetic acid (EDTA) for 6 weeks and embedded in paraffin wax. Three 5-µm sagittal plane sections were randomly selected from each sample for hematoxylin-eosin (HE) staining. Two researchers recorded the scores of various pathological changes in the condyle of the four groups according to the modified Mankin score method [16] in a blinded fashion. The Mankin score has a maximum of 16 points and a minimum of 4 points. The scores were then analyzed and compared.

Tartrate-resistant acidic phosphatase staining

The left condylar specimen sections described above were routinely deparaffinized and hydrated in water, and osteoclastic activity was detected using a tartrate-resistant acidic phosphatase (TRAP) kit (Sigma, St. Louis, USA). The procedures were carried out in strict accordance with the instructions provided by the manufacturer. Osteoclast staining was observed under an optical microscope (Leica, Germany), and the number of TRAP-positive cells was counted.

Immunofluorescence detection of PCNA expression

The left condylar specimen sections described above were routinely deparaffinized and hydrated in water, followed by antigen retrieval in 0.01 M sodium citrate in a 95°C water bath. After inactivating endogenous peroxidase using 3% H₂O₂, the specimens were blocked in goat serum and incubated with the proliferating cell nuclear antigen (PCNA) primary antibody (ABclonal, Wuhan, China) overnight at 4°C at a dilution of 1:200. The specimens were then incubated with a rhodamine red fluorescent secondary antibody (ABclonal, Wuhan, China). Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) solution (ABclonal, Wuhan, China).

Images were collected using a fluorescence microscope (Leica, Germany), and the results were processed by Image-Pro Plus 6.0 to calculate the average optical density (AOD) of PCNA in each section.

Immunohistochemical detection of MMP-3/TIMP-1 and RANKL/OPG expression ratios

The left condylar specimens were sectioned for immunohistochemical staining. Primary antibody treatment was conducted as described above in the immunofluorescence step, and the antibody dilution ratios were as follows: MMP-3, 1:500; TIMP-1, 1:1000; OPG, 1:500; and RANKL, 1:200 (Abcam, Cambridge, England). The samples were incubated with a biotinylated secondary antibody (ZSGB-BIO, Beijing, China) followed by diaminobenzidine (DAB) chromogenic substrate; nuclei were counterstained with hematoxylin. The samples were dehydrated with an ethanol gradient, clarified in xylene, and mounted in neutral resin.

Staining was observed and images were acquired using an optical microscope (Leica, Germany). The detection results were processed to calculate the AOD of the positive staining in each section by Image-Pro Plus 6.0. We calculated the expression ratios of MMP-3/TIMP-1 and RANKL/OPG and evaluated matrix destruction and bone metabolism damage.

RT-qPCR detection of mRNA expression levels of MMP-3/TIMP-1 and RANKL/OPG

Immediately after sacrifice, the right condylar cartilage samples were obtained and frozen in liquid nitrogen. After the addition of TRIzol solution, the tissue samples were fully ground with a glass grinder. Total RNA was extracted using an RNeasy Mini Kit (Qiagen, MD, USA). Then, 1 µg of total RNA was used for reverse transcription with a PrimeScript RT Reagent Kit and gDNA Eraser (Takara, Shiga, Japan). Real-time PCR was accomplished using a SYBR Premix Ex Taq II kit (Takara, Shiga, Japan). The primer sequences for the genes encoding MMP-3, TIMP-1, RANKL and OPG were designed previously (Sangon Biotech, Shanghai, China) (Table 1). The 2^{-ΔΔCt} method was used to calculate the mRNA expression levels.

Table 1. Primer sequences used for RT-qPCR

Gene	Primer Sequence (5' to 3')
MMP-3	Forward: TGGACCAGGGACCAATGGA Reverse: GGCCAAGTTCATGAGCAGCA
TIMP-1	Forward: ACAGGTTTCCGGTTCGCCTAC Reverse: CTGCAGGCAGTGATGTGCAA
RANKL	Forward: ACTTTCGAGCGCAGATGGAT Reverse: AGTCGAGTCCTGCAAACCTG
OPG	Forward: TGGAAATAGATGTCACCCTGTGC Reverse: TTTGGTCCCAGGCAAACCTGT
β -actin	Forward: ATGTGGATCAGCAAGCAGGA Reverse: GGTGTAAACGCAGCTCAGTAA

Statistical analysis

SPSS version 21.0 software was used for the statistical analysis. The data were expressed as the mean \pm standard deviation. The experimental data of the four different groups were analyzed using one-way analysis of variance (ANOVA). The level of significance was defined according to two *P*-values (*: $P < 0.05$, **: $P < 0.01$).

Results

Behavioral scores in the open-field experiments

Compared with the rats in the BC group, the sleep-deprived rats showed obvious anxiety-like behavioral manifestations, such as increases in irregular activity, speed of movement, and confrontational behavior, and their horizontal and vertical activity scores were also significantly increased ($P < 0.05$). The horizontal and vertical activity scores of the rats in the LIPUS and NC groups were significantly lower than those of rats in the CSD group ($P < 0.05$). There was no significant difference between these two groups or between either of these groups and the BC group (**Figure 2A, 2B**).

Comparison of serum ACTH and CORT concentrations

The ELISA results showed that serum ACTH and CORT concentrations were significantly higher in the CSD group than in the BC group (ACTH $P < 0.01$, CORT $P < 0.05$). After 4 weeks of intervention, the LIPUS and NC groups showed decreased serum ACTH and CORT concentrations, and except for the ACTH concentration

between the CSD and LIPUS groups, all remaining indexes showed statistically significant differences ($P < 0.05$) (**Figure 2C, 2D**).

Histological results and Mankin scores

The HE staining showed no abnormalities in the articular cartilage in the BC group; the surface of the fibrocartilage was smooth and flat, and the deep layer was clear (**Figure 3A**). Compared with the BC group, the surface of the articular cartilage in the CSD group was rough, some fibers were detached and remained free in the joint space, the chondrocytes in the proliferative layer were irregularly arranged, the tide mark was not clear in some samples, and the trabecular bone was disordered (**Figure 3B**). The pathological changes were significantly alleviated in the LIPUS group compared with the CSD group (**Figure 3C**). However, the surface of the fibrocartilage in the NC group was rough and rugged, and the cellular repair ability of the proliferating zone was insufficient. Furthermore, some samples exhibited small areas with hyalinization, and the pathological changes did not significantly improve (**Figure 3D**).

The Mankin scores were analyzed for each group (**Figure 3E**). The total score of the CSD group was significantly higher than that of the BC group ($P < 0.01$), and the total score of the LIPUS group was lower than that of the CSD group ($P < 0.05$). The total score of the NC group was similar to that of the CSD group, and the total score of the LIPUS group was lower than that of the NC group ($P < 0.05$).

Rat condylar osteoclast staining results

Various numbers of osteoclasts were found in the condylar calcified cartilage of the rats in all four groups. Significantly more osteoclasts were found in the CSD group than in the BC group (**Figure 4A, 4B**), while the LIPUS group exhibited significantly fewer osteoclasts than the CSD group did (**Figure 4C**). The number of osteoclasts in the NC group was not significantly reduced compared with the CSD group (**Figure 4D**). Microscopic counting and quantitative analysis showed that the sleep deprivation intervention significantly increased osteoclastic activity in the condylar cartilage, and the number of osteoclasts also significantly increased ($P < 0.01$). LIPUS treatment effectively

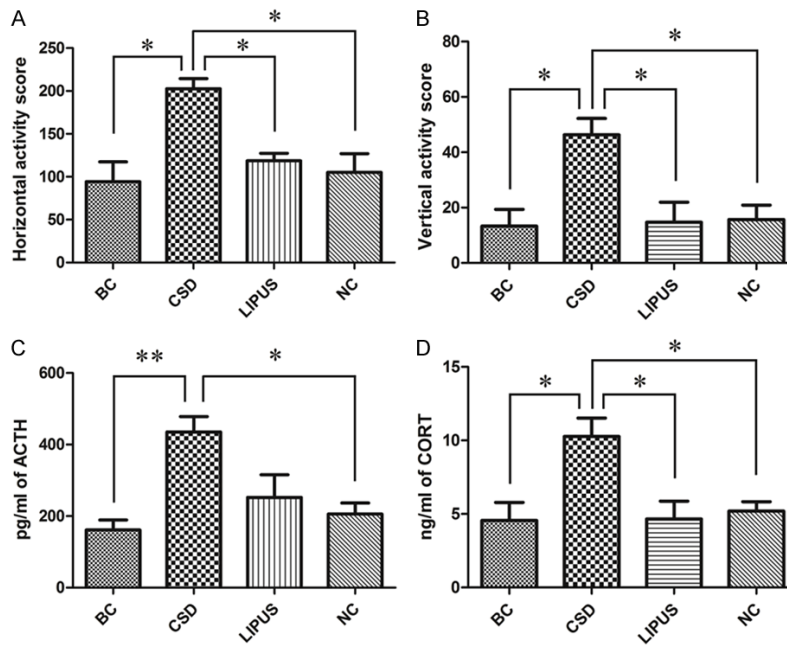


Figure 2. Results of the behavioral test and serum levels of ACTH and CORT in the rats of the four groups. The rats in the CSD group displayed significantly higher scores for horizontal and vertical activities than did the rats in the BC group. After 4 weeks of intervention, the rats in the LIPUS and NC groups displayed apparent reductions in activity scores (A, B). The serum levels of ACTH and CORT in the CSD group were significantly higher than those in the BC group but were decreased in the LIPUS and NC groups (C, D). The results are expressed as the mean \pm standard deviation. *, $P < 0.05$; **, $P < 0.01$.

morphological abnormalities, with the structural stratification shown in figure (A). The cartilage surface of the CSD group was rough, with some sloughing fibers (arrowhead), and the arrangement of cartilage cells was disorganized (B). Compared with the CSD group, the LIPUS group manifested prominent reductions in the pathological changes, including a smooth and intact cartilage surface and a normal arrangement of cartilage cells (C). The cartilage surface of the NC group was rugged and even concave (arrowhead) (D). Scale, 50 μ m; magnification, 20 \times . The Mankin scores were significantly higher in the CSD group than in the BC group. A significant decrease in the score was observed in the LIPUS group, and the score of the NC group was similar to that of the CSD group (E). The results are expressed as the mean \pm standard deviation. *, $P < 0.05$; **, $P < 0.01$.

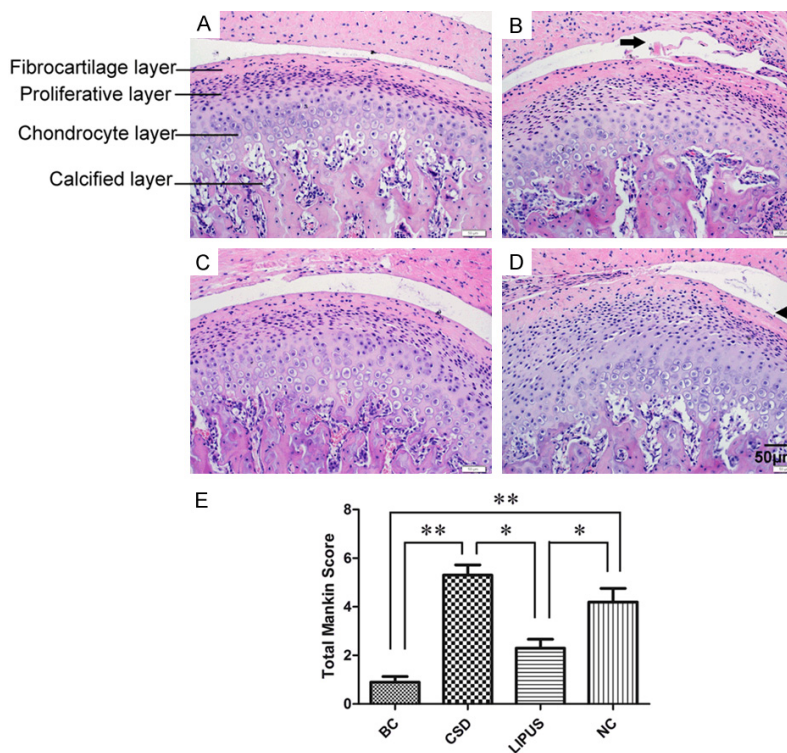


Figure 3. HE staining and pathological analysis of the condylar cartilage in the rats of the four groups. The condylar cartilage of the BC group showed no

rescued this pathological change and reduced the number of osteoclasts ($P < 0.05$) (Figure 4E).

PCNA expression in the condylar cartilage

Positive red fluorescence of PCNA was localized in the nuclei of the chondrocytes, mainly in the area of the chondrocyte layer near the proliferative layer, while nuclear DAPI counterstaining showed blue fluorescence (Figure 5A). PCNA fluorescence was attenuated in the CSD group compared with the BC group ($P < 0.05$), whereas the fluorescence was significantly enhanced after LIPUS intervention. The AODs of PCNA were significantly different between the CSD and LIPUS groups ($P < 0.01$), and the LIPUS group had signifi-

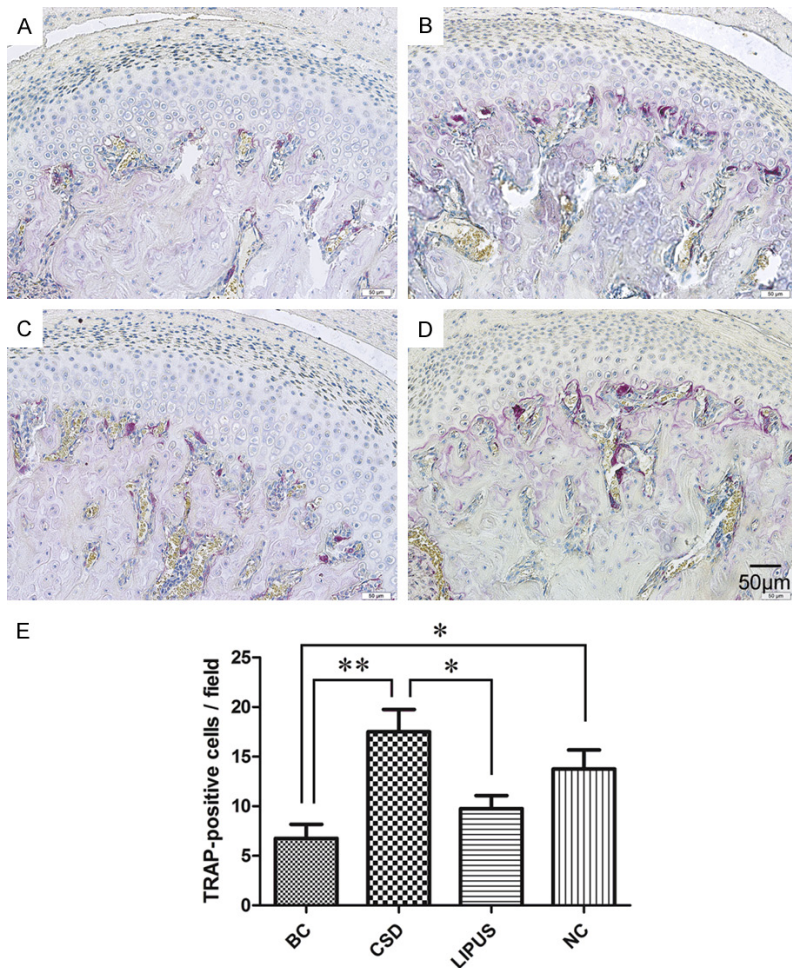


Figure 4. TRAP staining and osteoclast count in the condylar cartilage in the rats of the four groups. In the BC group, there were few osteoclasts present in the calcified cartilage layer (A). In the CSD group, more osteoclasts with a darker color were apparent (B). Compared with the CSD group, the LIPUS group displayed a marked reduction in osteoclastic activity (C), whereas the NC group was similar to the CSD group (D). Scale, 50 μ m; magnification, 20 \times . The results of the TRAP-positive cell count showed a significantly higher osteoclast number in the CSD group than in the BC group and a significant decrease in the LIPUS group (E). The data are reported as the mean \pm standard deviation. *, $P < 0.05$; **, $P < 0.01$.

cantly higher values than the NC group ($P < 0.05$) (Figure 5B).

Immunohistochemical staining of MMP-3, TIMP-1, RANKL, and OPG in the condylar cartilage

MMP-3-, TIMP-1-, OPG- and RANKL-immunopositive cells were mainly located in the chondrocyte layer. MMP-3, TIMP-1, OPG and RANKL staining was observed in the cytoplasm and extracellular matrix as brownish yellow staining. The staining intensity was classified as strongly positive, positive, weakly positive, or negative (Figure 6A).

Compared with the BC group, MMP-3 staining was strongly positive in the CSD group, weakly positive in the LIPUS group, and positive in the NC group. The AODs of MMP-3 were significantly different among the groups ($P < 0.05$; $P < 0.01$ between the BC and CSD groups) (Figure 6B). TIMP-1 staining was weakly positive in the CSD and NC groups and positive in the LIPUS group. Although there was no statistically significant difference in TIMP-1 expression between the groups (Figure 6C), the ratio of MMP-3/TIMP-1 was significantly higher in the CSD group than in the BC group ($P < 0.05$). This ratio was significantly reduced after LIPUS treatment ($P < 0.05$) and was lower than that in the NC group (Figure 6D).

Compared with the BC group, RANKL staining was positive in the CSD and NC groups and weakly positive in the LIPUS group. The AODs of RANKL were significantly different between the BC and CSD groups ($P < 0.01$) and between the CSD and LIPUS groups ($P < 0.05$) (Figure 6E). OPG expression was weakly positive in the CSD and NC

groups and positive in the LIPUS group. A significant difference in OPG expression was observed between the BC and LIPUS groups ($P < 0.05$) (Figure 6F). The ratio of RANKL/OPG was significantly higher in the CSD group than in the BC group ($P < 0.05$) and was significantly decreased in the LIPUS group ($P < 0.05$) (Figure 6G).

Comparison of MMP-3/TIMP-1 and RANKL/OPG mRNA expression ratios in the condylar cartilage

The mRNA expression levels of MMP-3 and RANKL were significantly higher in the CSD

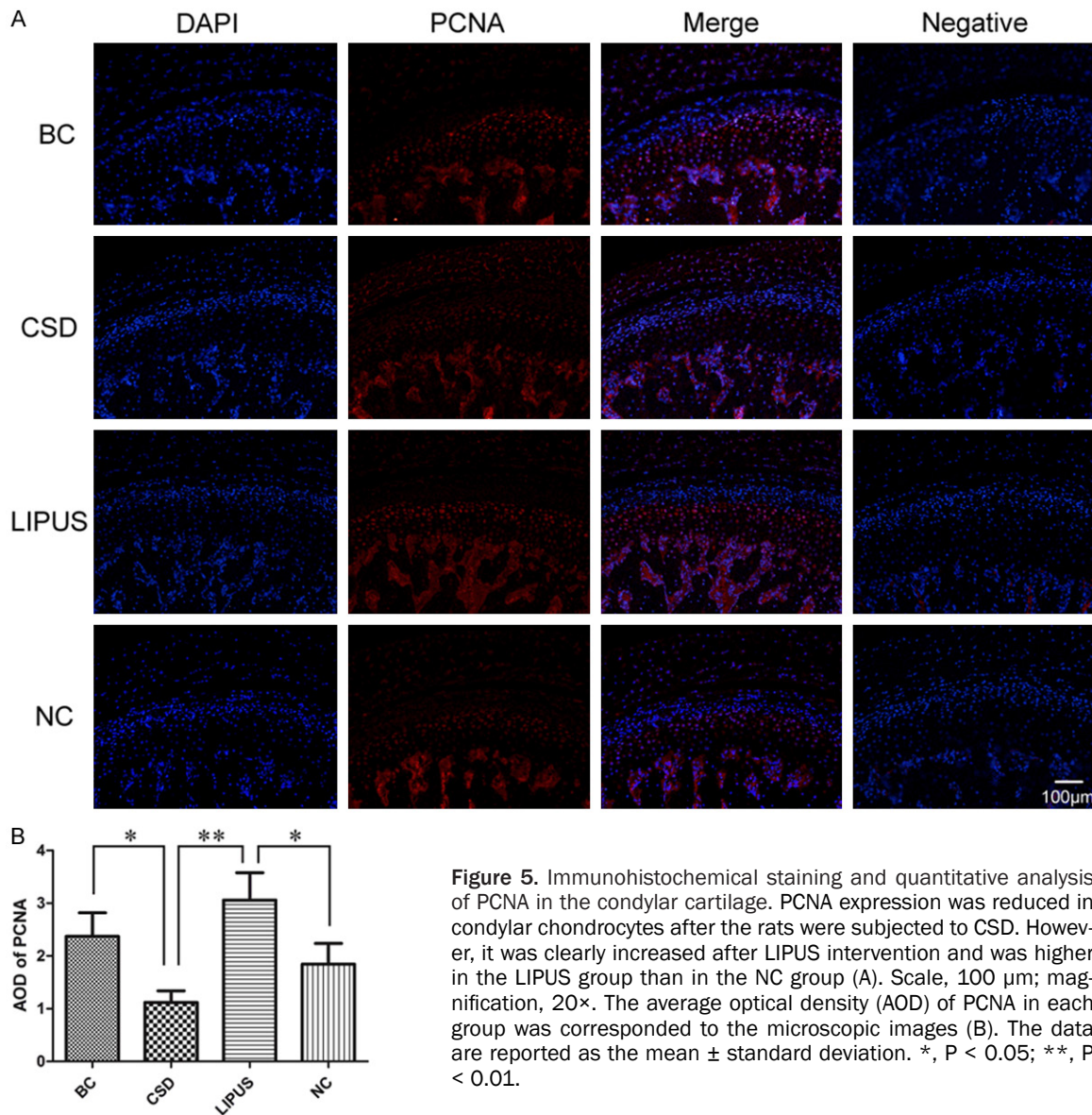


Figure 5. Immunohistochemical staining and quantitative analysis of PCNA in the condylar cartilage. PCNA expression was reduced in condylar chondrocytes after the rats were subjected to CSD. However, it was clearly increased after LIPUS intervention and was higher in the LIPUS group than in the NC group (A). Scale, 100 μ m; magnification, 20 \times . The average optical density (AOD) of PCNA in each group was corresponded to the microscopic images (B). The data are reported as the mean \pm standard deviation. *, P < 0.05; **, P < 0.01.

group than in the BC group (MMP-3, P < 0.05; RANKL, P < 0.01) (**Figure 7A, 7D**); in contrast, the expression levels of TIMP-1 and OPG were slightly increased after sleep deprivation, although the differences were not statistically significant (**Figure 7B, 7E**). The mRNA expression levels of MMP-3 and RANKL in the condylar tissue were significantly decreased after LIPUS intervention compared with the expression levels in the CSD group (P < 0.05) (**Figure 7A, 7D**), and the expression level of OPG was significantly increased after LIPUS intervention (P < 0.05) (**Figure 7E**). CSD significantly increased the ratio of MMP-3/TIMP-1 mRNA expression in rat condylar tissue (P < 0.05), as well as the ratio of RANKL/OPG. LIPUS intervention

resulted in a significant reduction in the MMP-3/TIMP-1 and RANKL/OPG ratios in condylar tissue (P < 0.05) (**Figure 7C, 7F**).

Discussion

The temporomandibular joint is an important structure for the growth and function of craniofacial organs. TMDs have become common syndromes given the increasing pressure experienced in workplaces and poor quality of rest [17]. Due to the complicated etiology and pathology of TMDs, effective and noninvasive treatments for the pathological changes that occur as a result of TMDs are currently lacking [18].

Therapeutic effect of LIPUS on TMJ injury

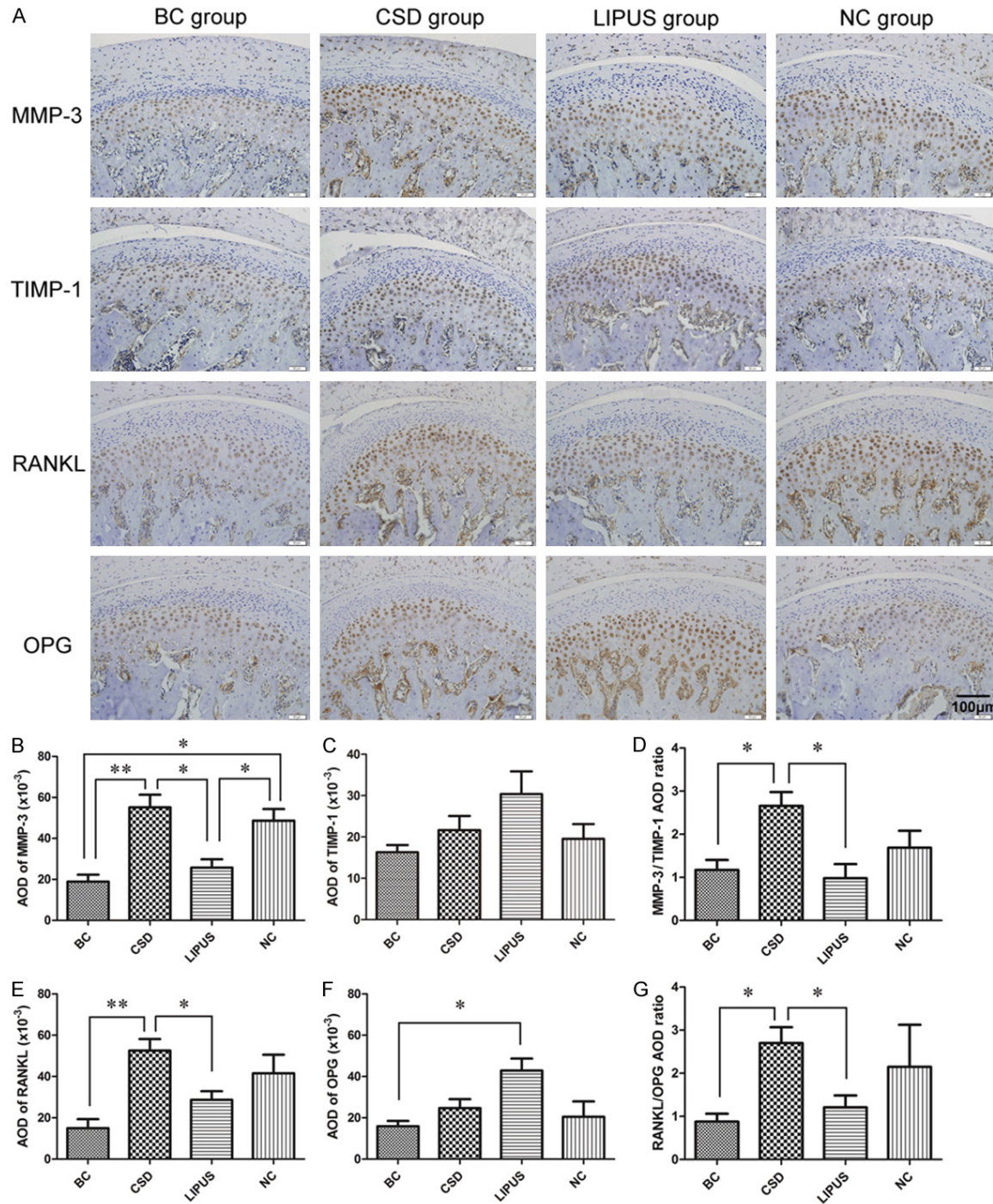


Figure 6. Immunohistochemical staining and quantitative analysis of MMP-3/TIMP-1 and RANKL/OPG in the condylar cartilage. After CSD, the expression levels of both MMP-3 and RANKL were significantly increased. In addition, they were clearly decreased after the subsequent LIPUS intervention, which was followed by increases in the expression levels of both TIMP-1 and OPG (A). Scale, 100 μ m; magnification, 20 \times . The average optical density (AOD) of MMP-3 (B), TIMP-1 (C), RANKL (E) and OPG (F) in each group was corresponded to the microscopic images. The expression ratios of both MMP-3/TIMP-1 and RANKL/OPG were significantly higher in the CSD group than in the BC group and were clearly decreased after subsequent LIPUS intervention (D, G). The results are expressed as the mean \pm standard deviation. *, $P < 0.05$; **, $P < 0.01$.

Ultrasound therapy is a type of physical intervention that uses ultrasound to promote the

recovery of injured tissue. Ultrasound therapy was first reported in 1942, when an Austrian

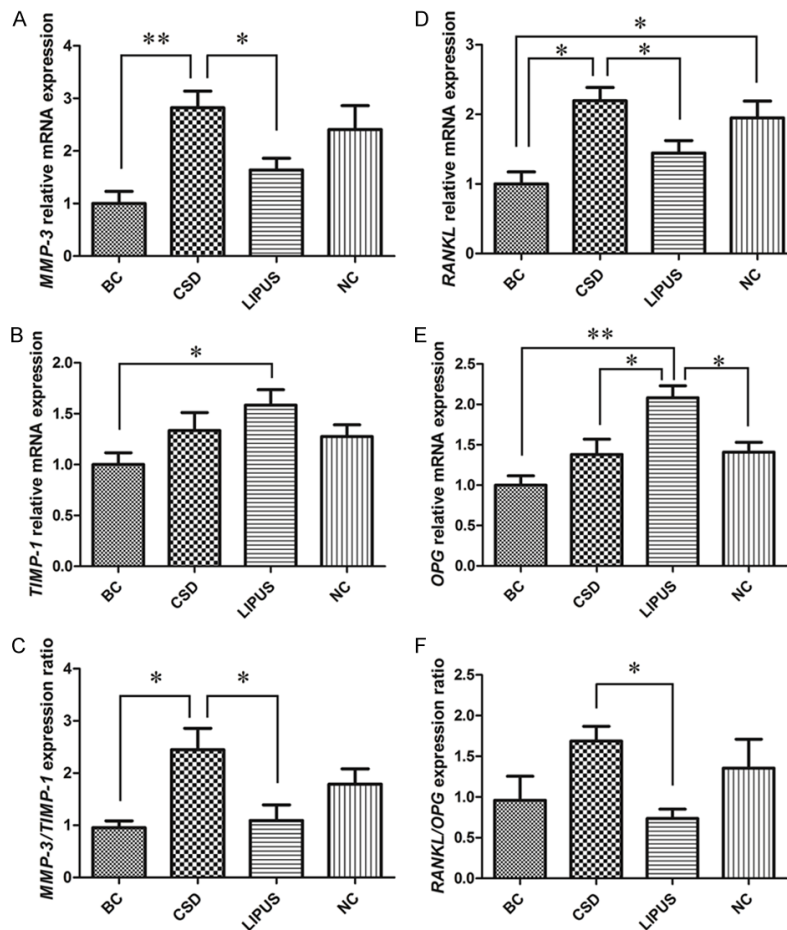


Figure 7. Comparison of the MMP-3/TIMP-1 and RANKL/OPG mRNA expression ratios in condylar tissue. After CSD, the mRNA expression levels of both MMP-3 and RANKL were significantly increased but were clearly decreased after the LIPUS intervention (A, D). OPG mRNA expression was markedly increased in the LIPUS group (E), and TIMP-1 mRNA expression was slightly elevated (B). The mRNA expression ratios of both MMP-3/TIMP-1 and RANKL/OPG were significantly higher in the CSD group than in the BC group and were markedly decreased after the subsequent LIPUS intervention (C, F). The data are reported as the mean \pm standard deviation. *, $P < 0.05$; **, $P < 0.01$.

doctor, Dussik [19], applied it for the first time to diagnose and treat craniocerebral diseases, pioneering a new field of ultrasound medicine. In recent years, LIPUS has gradually become an important treatment modality for bone and cartilage damage [20, 21]. Because LIPUS employs an elastic mechanical wave with an intensity of less than 100 mW/cm^2 , biological tissues conduct the waves with weak thermal effects, resulting in changes in the biological stress of the local microenvironment and in cytokine expression. These changes promote tissue healing and bone remodeling without causing tissue damage or degeneration [22]. Given the abovementioned characteristics of LIPUS, we

sought to apply it for the treatment of temporomandibular joint degenerative changes and inflammatory damage. Changes in histomorphology, osteoclast activity, chondrocyte proliferation ability, and MMP-3/TIMP-1 and RANKL/OPG expression ratios were observed in rat condylar cartilage following LIPUS treatment. These findings further clarify the therapeutic effect of LIPUS on the temporomandibular joint and its possible molecular mechanisms.

CSD is a validated method used to establish temporomandibular joint injury in rats [13, 14]. In the present study, obvious behavioral abnormalities and significant anxiety were observed in rats after CSD intervention, and the levels of ACTH and CORT in the serum of these rats increased significantly. The rats in the CSD group showed varying degrees of pathological changes in temporomandibular joint cartilage. The surface of the articular cartilage was rough, and some fibers were even detached, confirming that the temporomandibular joint injury model was successfully established.

Condylar cartilage can be divided into four layers: the fibrocartilage layer, the proliferative layer, the chondrocyte layer and the calcified layer [23]. The first three layers are also called the noncalcified cartilage layer; the boundary between the chondrocyte layer and the calcified cartilage layer is the basophilic tidemark. The calcified cartilage layer and the subchondral bone plate form a functional unit known as the osteochondral junction [24]. The chondrocytes in the condylar tissue structure are highly differentiated, and their main functions are the

synthesis and secretion of cartilage matrix [25]. Abnormalities in the structure and function of condylar cartilage are self-limiting to some extent. However, the results of the current study showed that mature chondrocytes have limited repair and reconstruction abilities. The rats in the NC group that received a placebo intervention showed no obvious amelioration of the pathological changes induced by CSD. A cytological study by Naito et al. [26] revealed that LIPUS is beneficial for stabilizing the chondrocyte phenotype, promoting proliferation, and positively affecting the formation of the cartilage matrix. In this study, after LIPUS treatment, the pathological changes of the temporomandibular joint in rats were alleviated to varying degrees. LIPUS stimulation reduced cartilage surface roughness, fiber loss and condylar cell disorders, significantly reduced the number of osteoclasts in the calcified cartilage, and enhanced chondrocyte proliferation. Therefore, we preliminarily conclude that LIPUS can effectively promote the repair of temporomandibular joint cartilage and prevent articular cartilage degeneration.

In addition, previous studies revealed that rats with CSD not only had temporomandibular joint injury but also showed abnormalities in the MMP/TIMP and RANKL/OPG systems in condylar chondrocytes [13, 15], which can exacerbate cartilage matrix breakdown and subchondral bone metabolism disorders. Whether LIPUS treatment influences these two systems to alleviate temporomandibular joint cartilage damage remains a key question.

MMP-3 is an important member of the matrilysin family and is now considered one of the most important proteases for inducing physiological and pathological degradation of the extracellular matrix in cartilage [27, 28]. MMP-3 can also be used to measure cartilage damage and predict the prognosis of patients with joint inflammation [29]. In pathological states, factors such as MMP-3 accelerate the degradation of type II collagen, a key component of the extracellular matrix of articular cartilage, and lead to destruction of the structure of the cartilage collagen network, thus causing degradation of the condylar cartilage matrix and inducing or aggravating temporomandibular joint injury [30]. TIMP-1 is a specific inhibitor of MMP-3 by binding and forming an irreversible noncovalent bond complex [31]. In this study,

the MMP-3/TIMP-1 ratio was used to indirectly reflect the state of damage and the inflammation level of the condylar cartilage. In normal organisms, MMP-3 and TIMP-1 are maintained in a dynamic balance [32], and MMP-3 does not exhibit lytic activity. Once this equilibrium is disturbed, the extracellular matrix of the cartilage can be excessively degraded, resulting in pathological changes in the articular cartilage [33].

In this study, MMP-3 expression in the condylar cartilage of the CSD group was significantly increased, and TIMP-1 expression was slightly increased, thus contributing to a higher ratio of MMP-3/TIMP-1. Our results confirm the hypothesis proposed above and suggest that the MMP-3/TIMP-1 imbalance plays an important role in the degenerative changes in and progressive destruction of the condylar cartilage. After 4 weeks of LIPUS intervention, condylar MMP-3 expression was significantly decreased, while TIMP-1 expression was increased. The treatment resulted in a significantly decreased MMP-3/TIMP-1 ratio in the condylar cartilage. Taken together with the results of HE staining, we conclude that LIPUS can effectively alleviate CSD-induced pathological injury in rat condylar cartilage and that the mechanism of cartilage protection is related to the reduced MMP-3/TIMP-1 ratio.

The OPG/RANKL/RANK system is important for the induction of osteoclast formation. RANKL is a necessary signaling protein for osteoclast growth and differentiation and can bind with its receptor, RANK, on the cell membrane of osteoclast precursor cells to induce maturation and inhibit osteoclast apoptosis [34]. OPG specifically inhibits osteoclast activity by competitively binding to RANKL and blocking the binding site for RANK [35]. Therefore, the ratio of RANKL/OPG may be key to determining osteoclast activity.

An imbalance in the RANKL/OPG ratio can lead to abnormal articular bone metabolism and the destruction of subchondral bone tissue, resulting in damage to the calcified cartilage layer adjacent to the tidemark [36]. In the present study, the RANKL/OPG ratio in rat condylar tissue significantly increased after CSD, and the TRAP staining results also showed that osteoclast activity near the tidemark was significantly activated. After 4 weeks of LIPUS interven-

tion, we observed a significant decrease in condylar RANKL expression, while OPG expression increased. The number of osteoclasts also significantly decreased according to the TRAP staining results, indicating that LIPUS has a good inhibitory effect on the activation of osteoclasts and can regulate condylar tissue repair.

In summary, the present results confirm that LIPUS can effectively inhibit inflammatory lesions and degenerative changes in condylar articular cartilage in an animal model of CSD. The therapeutic mechanism of LIPUS may involve promoting chondrocyte proliferation, reducing the ratios of MMP-3/TIMP-1 and RANKL/OPG in the condylar articular cartilage, inhibiting the cleavage activity of MMP-3 in the cartilage matrix, and inhibiting the damage to bone metabolism regulated by the abnormal expression of RANKL. This study provides a novel basis and guidance for the application of LIPUS in the treatment of TMDs in oral and maxillofacial surgery.

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Disclosure of conflict of interest

None.

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