Psychological Stress Alters Extracellular Matrix Metabolism in Mandibular Condylar Cartilage

Qiang LI[1]#, Fei HUANG[2]#, Jia LIU[3], Yin Hua ZHAO[1], Min ZHANG[1], Yong Jin CHEN[1]

Objective: *To explore the effect of long-term stress on the temporomandibular joint (TMJ) condyle and its possible underlying mechanism.*

Methods: *A 12-week, chronic unpredictable mild stress (CUMS) model was used to induce long-term psychological stress in rats. Rats were randomly divided into control group (CONT), chronic unpredictable mild stress group (CUMS) and chronic unpredictable mild stress with fluoxetine treatment group (CUMS + DT) (n = 30 per group). A 5 mg/kg dose of fluoxetine was intraperitoneally injected daily 0.5 h before stress. A sucrose preference test, plasma corticosterone test and open-field test were performed to verify the feasibility of the CUMS model. Histopathology was used to observe the pathological changes of condyle. The expression levels of inflammatory cytokines, matrix metalloproteases (MMPs) and extracellular matrix (ECM) were measured by real-time polymerase chain reaction, western blotting and immunohistochemistry.*

Results: *At 8 and 12 weeks after exposure to CUMS, the rats showed higher plasma corticosterone than the control rats. Additionally, for the open-field test, the rats exposed to CUMS spent more time in the centre zone and moved a shorter distance than the control and drug treatment rats. In addition, pathological changes in the condylar cartilage occurred in the 8-week CUMS subgroup and were more obvious in the 12-week CUMS subgroup. The CUMS caused an increase in the secretion of inflammatory cytokines, imbalanced expression of MMPs and tissue inhibitor of metalloproteinase-1 and accelerated degradation of ECM in condylar cartilage in a time-dependent manner.*

Conclusion: *Osteoarthritis-like lesions can be caused by long-term CUMS in the mandibular condyles, which suggests that the imbalance in chondrocyte-secreted regulatory factors within the cartilage of the TMJ may play an important role in cartilage injury induced by psychological stress.*

Key words: *chronic unpredictable mild stress, condylar cartilage, temporomandibular disorder (TMD), extracellular matrix, matrix metalloproteinase (MMP), tissue inhibitor of metalloproteinase-1 (TIMP)*

Chin J Dent Res 2017;20(3):125–135; doi: 10.3290/j.cjdr.a38767

1 State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi International Joint Research Center for Oral Diseases, Department of General Dentistry & Emergency, School of Stomatology, The Fourth Military Medical University, Xi'an , Shaanxi Province, P.R. China.

2 Department of Stomatology, PLA Navy General Hospital, Beijing, P.R. China.

3 State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi Clinical Research Center for Oral Diseases, Department of Orthodontics, School of Stomatology, The Fourth Military Medical University, Xi'an, Shaanxi Province, P.R. China.

# These authors contributed equally to the work.

Corresponding authors: Dr Min ZHANG, State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi International Joint Research Center for Oral Diseases, Department of General Dentistry & Emergency, School of Stomatology, The Fourth Military Medical University, Changle West Road 145, Xi'an 710032, Shaanxi Province, China. Tel: 86 29 84776488; Fax: 86 29 84776488. Email: zhangmin@fmmu.edu.cn.

Dr Yong Jin CHEN, State Key Laboratory of Military Stomatology and National Clinical Research Center for Oral Diseases and Shaanxi International Joint Research Center for Oral Diseases, Department of General Dentistry & Emergency, School of Stomatology, The Fourth Military Medical University, Changle West Road 145, Xi'an 710032, Shaanxi Province, China. Tel: 86 29 84776488; Fax: 86 29 84776488. Email: cyj1229@fmmu.edu.cn

This work was supported by the National Nature Science Foundation of China (No.81371188, 81271167, 81500851), Open Project of State Key Laboratory of Military Stomatology (No. 2014KA02) and the Natural Science Foundation of Shaanxi Province (No. 2016JQ8013).

Psychological stress is the consequence of failure of an organism, human or animal, to respond appropriately to emotional or physical threats[1]. Failure to cope with multiple stressors, which can generate psychological stress, impairs homeostasis and ultimately compromises organismal health[2]. Temporomandibular disorder (TMD) is a common disease of the stomatognathic system and is a collection of conditions affecting the bony structure and muscular tissue of that system[3]. Its occurrence and development can be attributed to a variety of factors[4]; thus, TMD is complex and difficult to treat. With the transformation of the medical model from a biomedical model to a biopsychosocial model, the influence of psychological stress on TMD onset and progression has gained widespread interest in recent years[5,6]. Patients suffering from TMD often exhibit a significant stress level, and the anti-stress strategy helps improve TMD-related clinical symptoms[7]. However, the mechanism underlying the adverse effects of psychological stress on the temporomandibular joint (TMJ) is not yet known.

The mandibular condylar cartilage covers the surface of the TMJ. It consists of cellular components and an extracellular matrix (ECM), the main components of which are aggrecan and collagen[8]. Normally, ECM synthesis and degradation are well regulated, but degradation of the cartilage matrix accompanies joint diseases. Matrix metalloproteinases (MMPs) are always involved in cartilaginous pathological processes[9]. Among the MMPs, MMP-3 degrades the major components of the ECM, including proteoglycan and type II collagen[10], while MMP-9 digests denatured collagens, type IV collagen, and aggrecan[11,12]. The activities of both MMP-3 and MMP-9 can be balanced by tissue inhibitor of metalloproteinase-1 (TIMP-1), which is considered the most common tissue inhibitor of metalloproteinases[12,13]. Once the activities of MMPs and TIMP-1 are imbalanced, collagens and aggrecan can be directly cleaved, leading to cartilage damage[14,15].

In our previous studies, pathological changes to the TMJ in stressed rats were confirmed as being accompanied by high expression of pro-inflammatory cytokines[16]. However, the effects of long-term psychological stress on the levels of MMPs and metabolism of ECM in the condylar cartilage of rats are still poorly understood. Therefore, in this study, we used chronic unpredictable mild stress (CUMS) to establish an animal model of psychological stress, and we evaluated the expression of ECM and MMPs in the condylar cartilage of stressed rats to explore the effects of long-term CUMS on condylar cartilage.

Materials and methods

Animals and grouping

Our study used 90 male Sprague-Dawley rats (8 weeks old, and weighing between 210 and 230 g) were obtained from the Animal Center of the Fourth Military Medical University. They were randomly divided into control group (CONT), chronic unpredictable mild stress group (CUMS) and chronic unpredictable mild stress with drug treatment group (CUMS + DT) (n = 30 per group). In the CUMS+DT group, fluoxetine (Eli Lilly and Company, Suzhou, China) was dissolved in saline (0.9% NaCl) and intraperitoneally injected daily with a 5 mg/kg dose half an hour before stress[17]. Each group was divided into an 8-week subgroup and a 12-week subgroup, according to the duration of stress (n = 15 per subgroup). All rats were housed in a temperature-controlled room at (24 ± 1°C) with a 12-hour light/dark cycle (light on from 08:00am to 20:00pm) and food and water available *ad libitum*. Before the beginning of the experiment, the animals were acclimatised to laboratory conditions for 1 week.

CUMS protocol

CUMS was adapted from the procedure described by Zhao et al[18]. Rats were subjected to seven different kinds of stressors – food deprivation (12 h), water deprivation (12 h), damp sawdust (24 h), restraint stress (1 h), 4°C cold water immersion (5 min), 45°C hot water immersion (5 min) and inversion of the light/dark cycle. One random stressor was applied daily, and the same stressor appeared only once per week to avoid stress habituation in rats. All procedures and care administered to the animals were approved by the ethics of animal research of the Fourth Military Medical University (Xi'an, China), and performed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Sample preparation

At the end of the eighth and 12th weeks of CUMS, all rats were given the behavioural test. Then, the rats were anaesthetised with intraperitoneal injections of sodium pentobarbital (50 mg/kg bodyweight). Blood samples were obtained from retinal veins for centrifugation (4°C, 12,000 rpm for 15 min) and stored at -80°C until the hormonal assays were performed. All TMJs were carefully removed. For eight rats, the left condyles were used for hematoxylin and eosin (H&E) staining, while the right condyles were used for immunohistochemical staining.

The bilateral condylar cartilages of the remaining seven animals were removed and preserved at -80°C for real-time polymerase chain reaction (PCR) and western blotting.

Open field test

All animals underwent the open-field test to evaluate behavioural changes, as previously described[19]. The total distance moved and the time spent in the centre were quantified using the automated video tracking system.

Sucrose preference test

The depressive-like phenotype was assayed using the sucrose preference test for anhedonia-like reactivity, as previously described[17]. The test was performed immediately after 8 weeks and 12 weeks of CUMS. The volumes of sucrose solution and water consumed over 24 h were recorded. Sucrose preference, which indicates anhedonia, was defined as the ratio of the volume of sucrose consumed to the total volume of sucrose and water consumed.

Plasma corticosterone (CORT) level

Plasma CORT was measured using rat ELISA Kits (Shanghai Westang Bio-Tech Co, Shanghai, China) according to the manufacturer's instructions.

Histological staining

For histological observation, serial sections (5 μm) were cut along the sagittal plane of the condyle and stained with H&E. The condylar cartilage in each region was divided into the fibrous layer, proliferative layer, hypertrophic layer and endochondral ossification layer[14,15].

Enzyme linked immunosorbent assay (ELISA)

The expressions of inflammatory cytokines in the temporomandibular joint were measured by ELISA. Condylar cartilages were homogenised in 500 mL of a PBS solution containing and treated with RPMI lysis buffer with proteinase inhibitor at 4°C for 10 min. The lysates were centrifuged at 11,000 g for 10 min, and the supernatants (diluted 1:2 in the same PBS solution) were used to evaluate the protein levels of IL-1β, IL-6 and TNF-α with ELISA kits (Endogen, USA) following the manufacturer's instructions. Total protein assay was performed using the bicinchoninic acid assay method (Pierce, Waltham, MA, USA). Cytokine concentrations were reported per mg of total protein. The absorbance was measured at 450 nm. These experiments were repeated three times.

Immunohistochemistry

Immumohistochemical staining was performed to observe the immunoactivity of ECM, MMPs and TIMP-1. The sections (5 μm) were each deparaffinised in xylenes twice for 30 min, hydrated gradually through graded series of alcohols (100% ethanol twice for 5 min each, then 95%, 85%, 75% ethanol for 5 min), and then-rinsed in distilled water for 1 min. Deparaffinised sections were treated with 3% H_2O_2 for 10 min at 37°C to quench the endogenous peroxidase activity and then rinsed in 0.01% PBS three times for 5 min apiece. Then the slides were digested with pepsin (Boster Bioengineering Limited Company, Wuhan, China) for 30 min at 37°C to unmask the antigen and rinsed (0.01% PBS, 5 min × 3). To retrieve the antigen, epitope retrieval solution I (Boster Bioengineering) was applied. Then the slides were incubated with normal serum for 1.5 h at 37°C to block non-specific binding of antibodies as follows: Aggrecan (Abcam, Cambridge, UK); Collegan II (Abcam); MMP-3 (Abcam); MMP-9 (Abcam); TIMP-1 (Abcam); Tublin (Santa Cruz Biotechnology, Dallas, TX, USA).

Real-time PCR

Gene expressions of Aggrecan, Collegan II, MMP-3, MMP-9 and TIMP-1 were detected by real-time PCR. The condylar cartilages were carefully peeled off from the condyle and immersed in liquid nitrogen for 10 min. They were then broken into pieces with a masher. Total RNA was extracted using Trizol reagent (Ambion, Austin, TX, USA) according to standard procedures and reversed transcribed with PrimeScript®RT reagent kit (Takara Biotechnology, Liaoning Province, China). Quantitative real-time PCR was performed in triplicates in Applied Biosystems 7500 Real-time PCR system and analysed using ABI 7500 software. Each experiment was performed three times and the mean values were derived. The amount of target cDNA, relative to β-actin, was calculated using the formula $2^{-\Delta\Delta Ct}$. Primers for target genes are listed in Table 1.

Western blotting

The condylar cartilages were ground by mortar and pestle and lysed using RIPA buffer (50 mM Tris • HCl pH

Table 1 Real-time PCR primers used in the experiments.

Target	Primer sequence (5'-3')	Bp	GenBank Acc
β-actin	AGGCCAACCGTGAAAAGATG ACCAGAGGCATACAGGGACAA	100	NM_031144
Aggrecan	TCCGCTGGTCTGATGGACAC CCAGATCATCACTACGCAGTCCTC	101	NM_022190
Collagen II	CTGGTGGAGCAGCAAGAGC GTGGACAGTAGACGGAGGAAAG	144	NM_012929
MMP-3	TCCCAGGAAAATAGCTGAGAACTT GAACCCAAATGCTTCAAAGACA	73	NM_133523
MMP-9	CCACCGAGCTATCCACTCAT GTCCGGTTTCAGCATGTTTT	159	NM_031055
TIMP-1	ACAGGTTTCCGGTTCGCCTAC CTGCAGGCAGTGATGTGCAA	134	NM_053819

7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS). 50 mg of total protein extracts were size fractionated by SDS/PAGE and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were then incubated with primary antibodies that recognise rat aggrecan (1:5000, Abcam), Collagen II (1:500, Abcam), MMP-3 (1:500, Abcam), MMP-9 (1:5000, Abcam), TIMP-1 (1:200, Abcam); Tublin (Santa Cruz Biotechnology) overnight at 24°C. Then membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:2000, ZhongShan Goldenbridge Biotechnology Co, Beijing, China) for 1 h at room temperature. Finally, the membranes were washed again and visualised using an enhanced chemiluminescence kit (Millipore, Billerica, MA, USA). The densitometric analysis of the protein band was performed using Bio-Rad Quantity One software (Bio-Rad, Hercules, PA, USA). All samples were run in duplicate on separate gels, and HSP70 content was expressed relative to Tublin in arbitrary units.

Statistical analysis

All data of the control group, CUMS group and CUMS + DT group were analysed by one-way analysis of variance (ANOVA) with SPSS 13.0 software (SPSS institute, Chicago, IL, USA). The SNK-q test was then used to calculate any differences between the two subgroups. Data were expressed as means ± standard deviation (SD). $P < 0.05$ indicates statistical significance for all analyses.

Results

Psychological stress assessment

In the open field test, the total distance moved in the CUMS group was shorter than that in the CONT and CUMS + DT groups (week 8, both $P < 0.05$; week 12, both $P < 0.05$) (Fig 1a), while the time spent in the centre zone in the CUMS group was longer than that in the other two groups at each time point (week 8, both $P < 0.05$; week 12, both $P < 0.05$) (Fig 1b). No significant differences were observed between the CONT and CUMS + DT groups at either time point (week 8, both $P > 0.05$; week 12, both $P > 0.05$) (Fig1 a and b). Additionally, the CUMS group had an increased plasma CORT level compared with the CONT and CUMS + DT groups (week 8, both $P > 0.05$; week 12, both $P > 0.05$) (Fig 1c). Furthermore, as shown in Figure 1d, after stress the sucrose preference of the CUMS group was reduced significantly compared with that of the CONT group (week 8, $P < 0.05$; week 12, $P < 0.05$), and this reduction was restored by supplementation with fluoxetine in the CUMS + DT group (week 8, $P > 0.05$; week 12, $P > 0.05$). These results confirmed that the CUMS applied to rats in this study induced long-term psychological stress; this ensures that follow-up studies will be conducted.

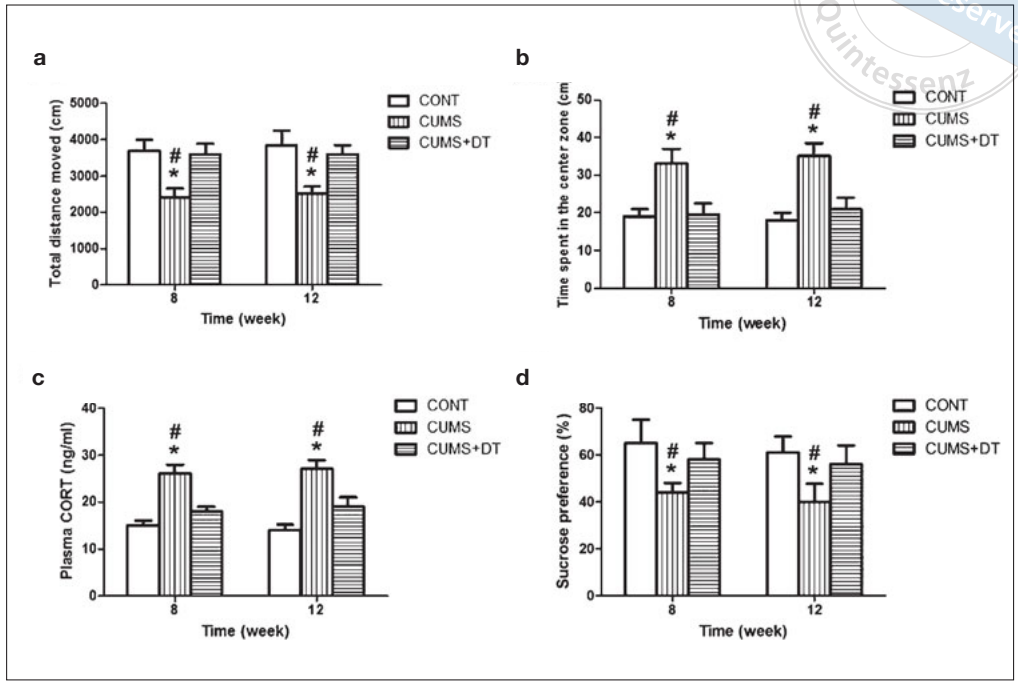

Fig 1 Evaluation of CUMS model. a) Total distance moved; b) Time spent in the centre zone; c) Plasma CORT; d) Sucrose preference. All data are means ± SD * $P < 0.05$, vs matched CONT subgroup; # $P < 0.05$, vs matched CUMS + DT subgroup. CONT, control group; CUMS, chronic unpredictable mild stress group; CUMS + DT, chronic unpredictable mild stress with drug (fluoxetine) treatment group.

Histological changes in condylar cartilage

The H&E staining results showed that the surface of the condylar cartilage in the CONT group was smooth and intact with clearly recognised fibrous, proliferative, hypertrophic and endochondral ossification layers, and the ECM was distributed evenly, predominantly in the proliferative and hypertrophic layers (Figs 2a and d). However, in the CUMS group, lesions appeared in the central and posterior parts of the mandibular condylar cartilage after long-term psychological stress. These lesions were characterised by disarrangement of cellular disposition and damaged continuity in the proliferative and hypertrophic layers of the mandibular condylar cartilage in five out of eight joints examined in the 8-week CUMS subgroup (Fig 2b). In addition to the above path-

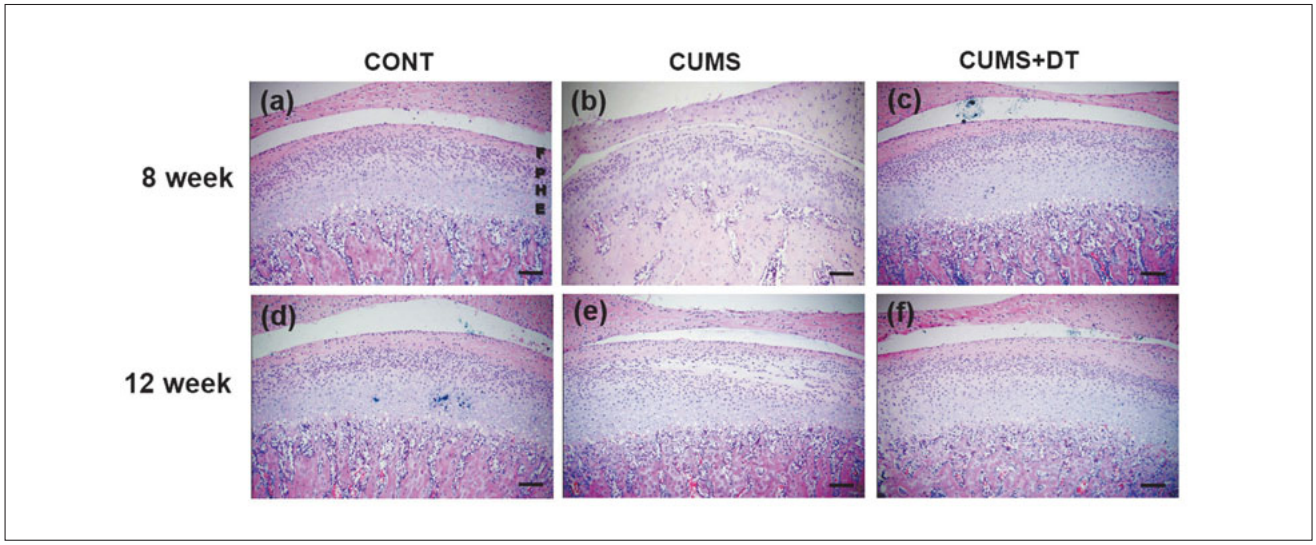

Fig 2 CUMS induced pathological lesions in condylar cartilage morphology. a) Sagittal section of condyle from the 8-week CONT subgroup; b) Sagittal section of condyle from the 8-week CUMS subgroup; c) Sagittal section of condyle from the 8-week CUMS + DT subgroup; d) Sagittal section of condyle from the 12-week CONT subgroup; e) Sagittal section of condyle from the 12-week CUMS subgroup; f) Sagittal section of condyle from the 12-week CUMS + DT subgroup. F, fibrous layer; P, proliferative layer; H, hypertrophic layer; E, endochondral ossification layer. Bar = 200 μm.

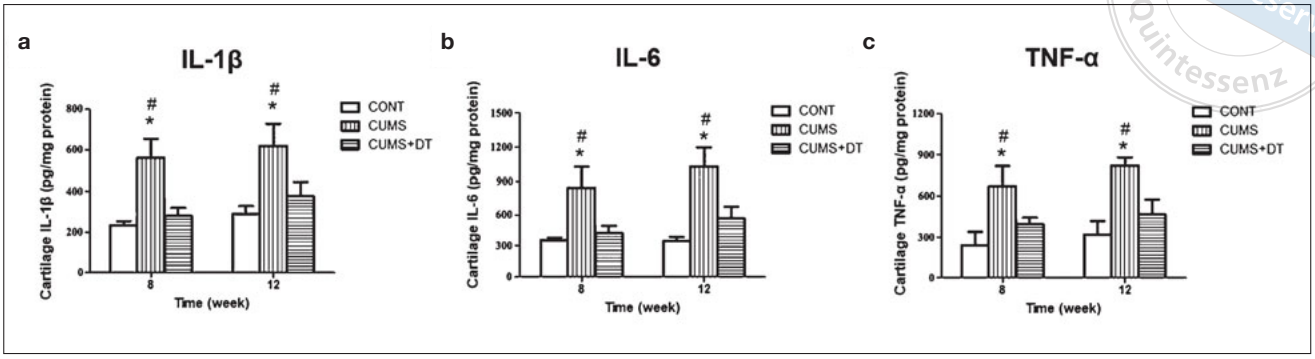

Fig 3 Expressions of inflammatory cytokines in condylar cartilage. a) The level of IL-1β; b) The level of IL-6; c) The level of TNF-α. * $P < 0.05$, vs matched CONT subgroup; # $P < 0.05$, vs matched CUMS + DT subgroup. CONT, control group; CUMS, chronic unpredictable mild stress group; CUMS + DT, chronic unpredictable mild stress with drug (fluoxetine) treatment group.

ological changes, severe cell loss in the proliferative and hypertrophic layers was observed in six out of eight joints in the 12-week CUMS subgroup (Fig 2e). There were no obvious pathological alterations in the condylar cartilage of the CUMS + DT group (Figs 2c and f).

The expressions of inflammatory cytokines

As shown in Figure 3, psychological stress significantly increased the contents of IL-1β, IL-6 and TNF-α in the condylar cartilage not only in the 8-week but also the 12-week CUMS subgroups when comparing with the ones that in the time-matched controls (all $P < 0.05$). After administrating the fluoxetine, the abnormal expressions of the inflammatory cytokines at either time point reversed to the control level (all $P > 0.05$).

Protein levels of aggrecan and collagen II

In the CONT group, the aggrecan immunoreactivity was distributed evenly in the ECM of the proliferative and hypertrophic cartilage layers, and the collagen II immu-

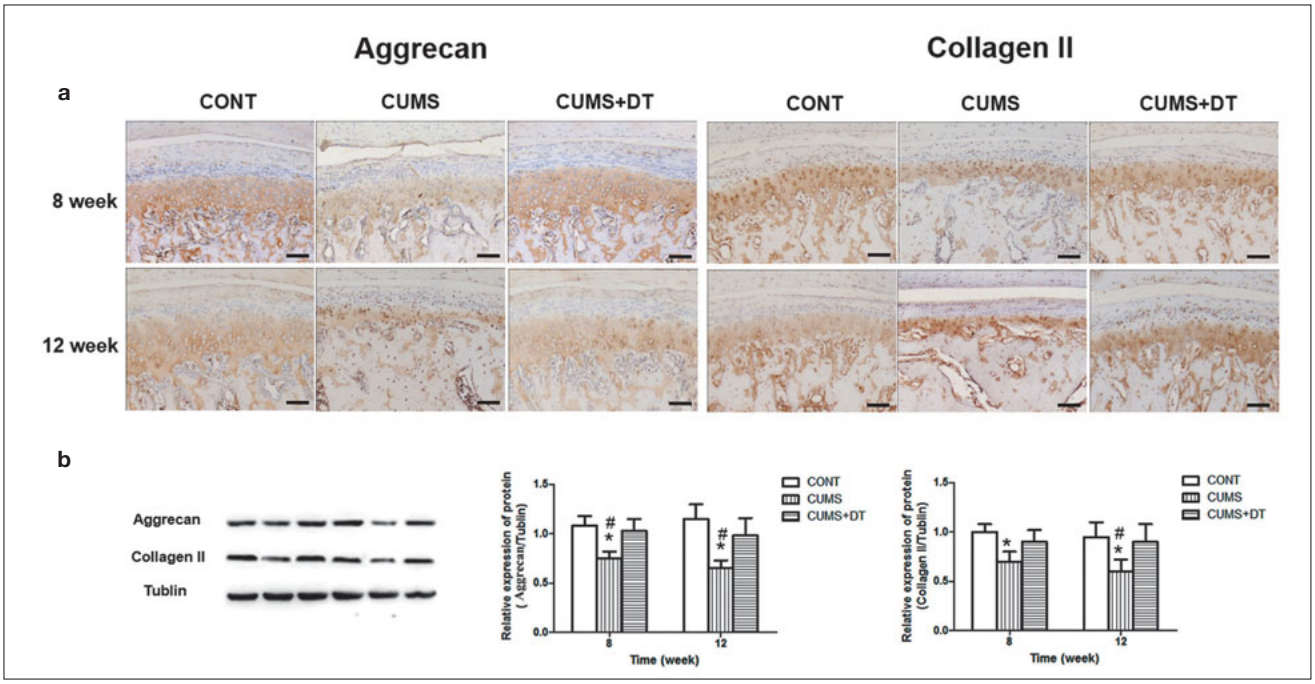

Fig 4 Protein levels of aggrecan and collagen II in cartilaginous ECM. a) Immunohistochemical staining of aggrecan and collagen II; b) Western blotting analysis of aggrecan and collagen II. * $P < 0.05$, vs matched CONT subgroup; # $P < 0.05$, vs matched CUMS + DT subgroup. CONT, control group; CUMS, chronic unpredictable mild stress group; CUMS + DT, chronic unpredictable mild stress with drug (fluoxetine) treatment group. Bar = 200 μm.

noreactivity was distributed in the ECM of the hypertrophic cartilage layer. However, psychological stress resulted in weaker aggrecan and collagen II immunoreactivity in the ECM of the CUMS group. The above changes were reversed by drug treatment (Fig 4a). In addition, the results of western blotting for aggrecan and collagen II are shown in Figure 4b. Significantly lower expression levels of aggrecan and collagen II were found in the CUMS group than in the CONT (aggrecan, both $P < 0.05$ at weeks 8 and 12; collagen II, both $P < 0.05$ at weeks 8 and 12) and CONT + DT groups (aggrecan, both $P < 0.05$ at weeks 8 and 12; collagen II, both $p < 0.05$ at weeks 8 and 12), with no differences between the CUMS subgroups (aggrecan, $P > 0.05$; collagen II, $P > 0.05$) (Fig 4b). Aggrecan and collagen II levels were not significantly different between the CONT and CONT + DT groups (aggrecan, both $P > 0.05$ at weeks 8 and 12; collagen II, both $P > 0.05$ at weeks 8 and 12) as shown in Figure 4b.

Protein levels of MMP-3, MMP-9 and TIMP-1

In the CONT group, the cells that were immunoreactive for MMPs were mainly located in the proliferative and hypertrophic cartilage layers. After psychological stress, MMP immunoreactivity was stronger in the 8-week and 12-week CUMS subgroups than in the time-matched CONT and CUMS + DT subgroups. However, the application of fluoxetine weakened MMP immunoreactivity. Stronger TIMP immunoreactivity was only observed in the 12-week CUMS subgroup (Fig 5a). Additionally, the western blotting results showed that CUMS significantly increased the expression of MMP-3 and MMP-9 at all experimental time points (MMP-3, both $P < 0.05$ at week 8s and 12; MMP-9, both $P < 0.05$ at weeks 8 and 12) and the expression of TIMP-1 at the 12-week time point ($P > 0.05$). None of these changes were observed in the CUMS + DT group (MMP-9, both $P > 0.05$ at weeks 8 and 12; TIMP-1, both $P < 0.05$ at weeks 8 and 12), except for the increase in MMP-3 (both $P > 0.05$ at weeks 8 and 12) (Fig 5b).

mRNA expressions of Aggrecan, Collagen II, MMP-3, MMP-9 and TIMP-1

The mRNA levels of aggrecan and collagen II in the condylar cartilage decreased in the 8-week and 12-week CUMS subgroups compared with the time-matched controls (aggrecan, both $P < 0.05$ at weeks 8 and 12; collagen II, both $P < 0.05$ at weeks 8 and 12) (Figs 6a and b), but no significant differences in those parameters were observed between the CONT and CUMS + DT groups at either time point (aggrecan, both $P > 0.05$ at weeks 8 and 12; collagen II, both $P > 0.05$ at weeks 8 and 12) (Figs 6a and b). In addition, the mRNA levels of MMP-3 and MMP-9 were significantly increased at week 8 and week 12 and the mRNA level of TIMP-1 was significantly increased at week 12 in the CUMS group compared to the CONT and CUMS + DT groups (vs CONT: MMP-3: both $P < 0.05$ at weeks 8 and 12; MMP-9: both $P < 0.05$ at weeks 8 and 12; TIMP-1: $P < 0.05$ at week 12; vs CUMS + DT: MMP-3: both

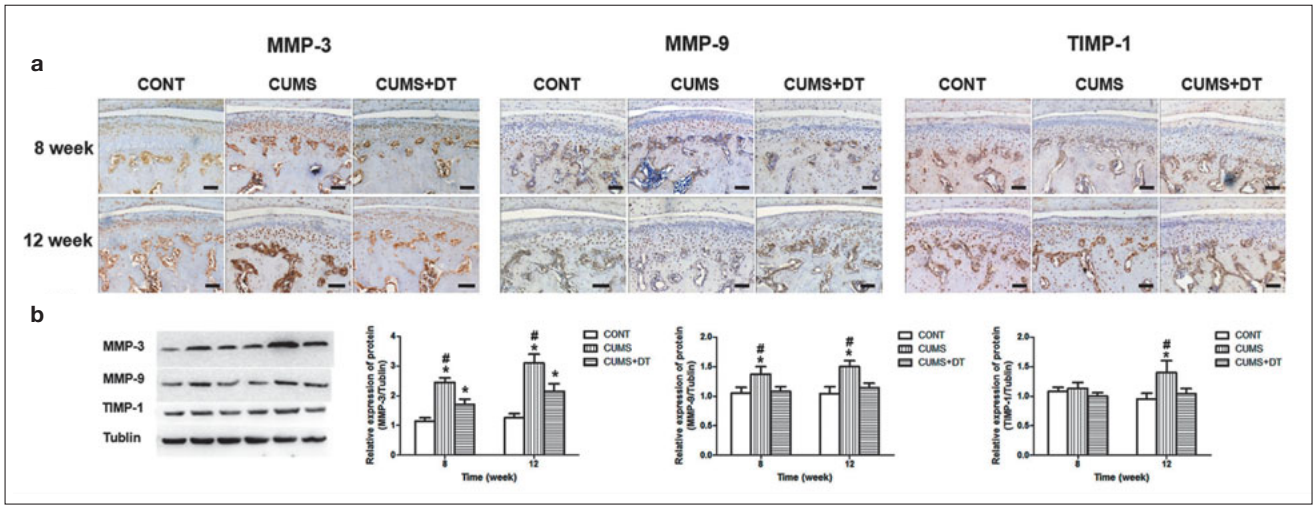

Fig 5 Protein levels of MMP-3, MMP-9 and TIMP-1 in cartilaginous ECM. a) Immunohistochemical staining of MMP-3, MMP-9 and TIMP-1; b) Western blotting analysis of MMP-3, MMP-9 and TIMP-1. * $P < 0.05$, vs matched CONT subgroup; # $P < 0.05$, vs matched CUMS + DT subgroup. CONT, control group; CUMS, chronic unpredictable mild stress group; CUMS + DT, chronic unpredictable mild stress with drug (fluoxetine) treatment group. Bar = 200 μm.

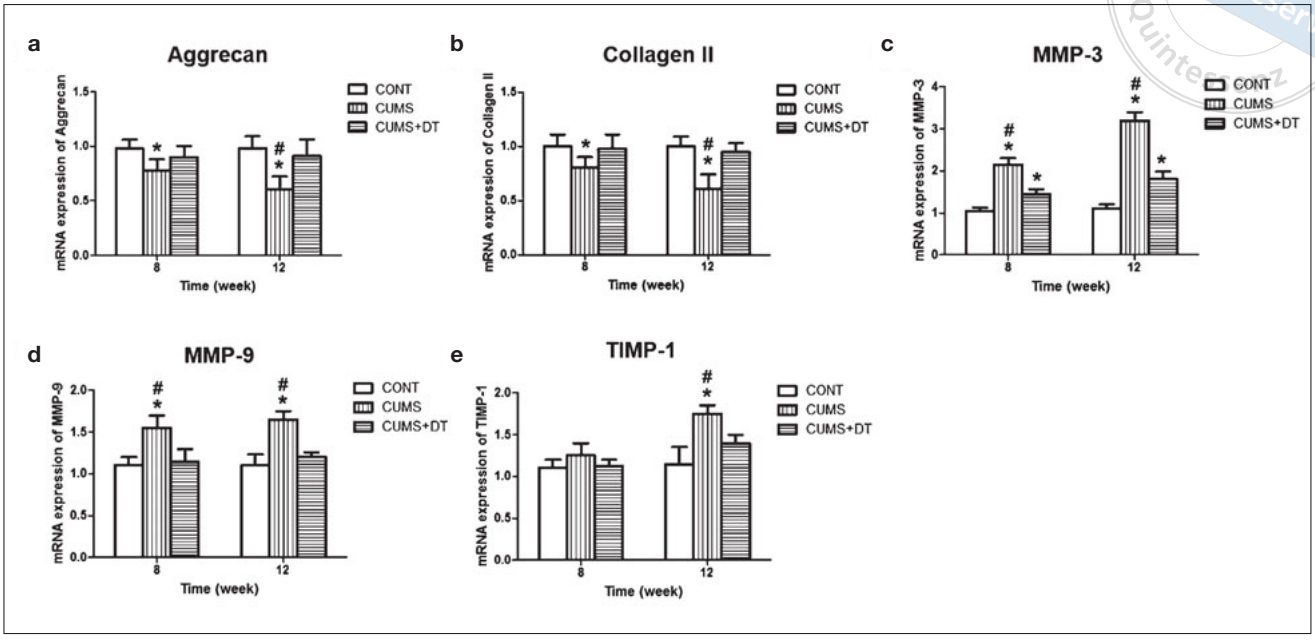

Fig 6 mRNA expression of aggrecan, collagen II, MMP-3, MMP-9 and TIMP-1 in cartilaginous ECM. a) mRNA expression of aggrecan; b) mRNA expression of collagen II; c) mRNA expression of MMP-3; d) mRNA expression of MMP-9; e) mRNA expression of TIMP-1. * $P < 0.05$, vs matched CONT subgroup; # $P < 0.05$, vs matched CUMS + DT subgroup. CONT, control group; CUMS, chronic unpredictable mild stress group; CUMS + DT, chronic unpredictable mild stress with drug (fluoxetine) treatment group.

$P < 0.05$ at weeks 8 and 12; MMP-9: both $P < 0.05$ at weeks 8 and 12; TIMP-1: $P < 0.05$ at week 12). The mRNA levels of MMP-9 and TIMP-1 were not altered in the CONT + DT group compared to the CONT group (MMP-9, both $P > 0.05$ at weeks 8 and 12; TIMP-1, both $P > 0.05$ at weeks 8 and 12) (Figs 6c to e), but the mRNA level of MMP-3 was higher in the CONT + DT group than in the CONT group (both $P < 0.05$ at weeks 8 and 12) (Fig 6c).

Discussion

In modern society, people are faced with multiple stimuli from all aspects of life and work, including enormous life pressures and fierce social competition. Long-term stimulation inevitably produces psychological stress in humans, which affects everyone on a daily basis. Although psychological stress in mammals triggers a rapidly organised response for survival, it can also result in a variety of behavioural disorders and can alter physiological functions[2]. For simulating the human depression situation, a series of rodent psychological stress models were established. For example, the restraint stress model by immobilising animals in snug body-size cages of wire mesh is regularly employed while studying behavioural changes and pathologic processes associated with human depressive disorders[20,21]. The phenomenon of altered locomotor activity, as well as physiological function, were also found in the rats, who experienced socially isolation[22], maternal deprivation[23] or tail suspension[24]. However, the stresses in the above-mentioned models are too single to prevent the animals' habituation if presented repeatedly[25]. In addition, they are not suitable to evaluate the efficiency of antidepressants for a long period of observation. CUMS consists of several unpredictable and mild stressors. It is widely used in studies to mimic chronic stresses in human daily life[26,27]. The major advantage of CUMS is the avoidance of animal adaptation to invariant stressors by employing various physical and psychological stressors in a predetermined manner. Also, the prolonged time course of the model was suitable for determining the effects of chronic drug treatments. Consequently, we established a psychological stress animal model using CUMS in this study. After 8 and 12 weeks of exposure to CUMS, the concentration of CORT was altered, indicating alterations in HPA axis function. Additionally, the observed behavioural alterations and development of anhedonia were similar to the symptoms of clinical depression. These results indicate the effectiveness of the CUMS paradigm for establishing a model of depression, which is one of the most common stress-related human psychological disorders; thus, CUMS closely mimics the stressors in human life.

Obvious pathological changes occurred in the condylar cartilage of rats exposed to psychological stress, and TMJ lesions appeared in a time-dependent manner, with more lesions and more severely damaged condyles in the 12-week CUMS subgroup than in the 8-week CUMS subgroup. These histological findings suggest that stress-induced TMD is a cumulative process. Although the condyle can compensate for the adverse effects of external chronic pessimal stimulation via autoregulation, TMJ damage is unavoidable if the intensity and duration of the stress exceeds the compensatory capacity of the condyle. In addition, the pathological changes were mainly observed in the proliferative and hypertrophic layers of the middle and posterior regions of the TMJ. This may be because these layers are susceptible to mechanical stress[28,29] and because the central and posterior parts of the TMJ are its main functional areas where most joint loading occurs[14,15].

Physiologically, mandibular condylar cartilage remodels throughout life to accommodate changes in mechanical loading during mandibular functions, such as occlusion and chewing. Excessive masticatory muscle activity inevitably results in chronic overuse of the TMJ, consequently leading to cartilage damage[30]. We have previously confirmed that stress causes masseter over-activity[31]. Other research also demonstrated that emotional stress induces brux-like activity in the masseter muscles of rats[32]. Therefore, the observed pathological changes in the condyle when rats are exposed to long-term psychological stress are logical.

The ECM is responsible for the unique biomechanical properties of articular cartilage. Progressive destruction of the ECM causes cartilage failure[33,34]. Proteoglycan and collagen are the essential components of articular ECM. The main proteoglycan of articular ECM is aggrecan, and it is crucial for maintaining the normal chondrocyte phenotype; in contrast, collagen has a role in resisting tensional forces[8]. When physiology is normal, the synthesis and degradation of the matrix proteins in the joint are in equilibrium. However, excessive degradation leads to progressive loss of matrix proteins and joint integrity[35]. Type II collagen and aggrecan are the two major targets of this degradation, and their loss substantially contributes to the occurrence and progression of TMD[14,36]. In the present study, the protein and mRNA levels of aggrecan and collagen II in the condylar cartilage of the 8-week and 12-week CUMS subgroups were decreased significantly compared with the time-matched CONT and CONT + DT subgroups. Similar findings of decreased aggrecan and collagen II levels were also reported in other animal models of condylar cartilage degeneration[14], indicating that long-term psychological stress increases collagenolytic activity and may increase the vulnerability of the condylar cartilage.

MMPs are classified into several subfamilies: collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), membrane-type MMPs, and other sub-families. They are considered destructive enzymes under certain pathogenic conditions of joints[37]. Many studies have documented the important roles of MMPs in the development of joint diseases. For example, Freemont et al[38] found no MMPs in normal knee cartilage. However, MMP-3 and MMP-9 expression was increased in the knees of an osteoarthritis model. Domestic researchers have also reported over-expression of MMP-3 and MMP-9 in the condyles of a TMJ osteoarthritis model, and this was accompanied by subchondral bone loss[14]. Physiologically, MMPs are secreted by chondrocytes following stimulation by proinflammatory cytokines[39]. We have noted the up-regulation of IL-1β, IL-6 and TNF-α in mandibular condyles after CUMS paradigm in this study. Our group previously confirmed similar observations in rats[16]. Therefore, the observation of higher MMP-3 and MMP-9 expression levels in the condylar cartilage of animals under psychological stress induced by CUMS seems reasonable. Moreover, in the current study, the increase in the expression of MMPs in the CUMS group was observed at the same time as ECM degradation. Combined with the observation of high expression of MMP-3 in the CUMS + DT group at both of the observed time points, we propose that MMPs are the key enzymes in the degradation of ECM in condylar cartilage[40] and that increased MMP-3 expression is a key factor of the present cartilage degradation model. It is commonly thought that MMP-3, which is secreted by fibroblasts, synovial cells and chondrocytes, is the most important protease for cartilage matrix degradation[10], as MMP-3 could degrade most components of the extracellular matrix such as proteoglycans, basement membrane, elastin, laminin, fibronectin, and collagen IV and X. Among the MMPs, MMP-3 could also activate MMPs-1, 8, and 9 to promote the pathological degradation of collagen[11]. MMPs are regulated by TIMPs. Because TIMPs directly regulate MMP activities, their expression in pathological conditions is considered to be as important as the expression of MMPs. A positive relationship between the intercartilaginous expression of TIMP-1 and stimulation intensity has been reported[41]. In this study, the TIMP-1 expression did not change in accordance with the increased MMPs at week 8. And the MMP-3

still exhibited higher expressed though the TIMP-1 restored to the control level after administration drug. This hinted to us the imbalance between MMPs and TIMP-1, which may play a role in the degradation of the condylar cartilage of our experimental animals. For verifying this presumption, the loss of type IV and type V collagens and aggrecan that are the substrates of MMP-3 and MMP-911[42], respectively, are expected in the future. In addition, we noted the significant TIMP-1 expression after 12 weeks of psychological stress. This may be due to the compensatory activity of TIMP-1 in response to the noxious stimulation of long-term psychological stress, as well as the local condylar protective response, both of which counteract the sustained increase in MMPs.

In the current study, we found that the altered exploratory behaviour and loss of responsiveness to pleasant stimuli caused by CUMS did not occur in the rats exposed to CUMS who were also treated with fluoxetine. There was less condylar cartilage degradation in the CUMS-DT group than in the CUMS groups at both time points. This anti-stress treatment also obviously reversed the abnormal ECM metabolism and increased MMP expression caused by CUMS. These findings further support the assumption that psychological stress exerts adverse influences on the TMJ. As a potent and selective inhibitor of neuronal serotonin (5-hydroxytryptamine) reuptake[43], fluoxetine may reduce the velocity of cortical spreading depression propagation[44], which results in obvious improvement of the depressive-like behaviour of animals. Moreover, it is well known that the position and movements of the jaw in mammals are controlled by the masticatory muscles, which are innervated by motor neurons located in the divisions of the trigeminal motor nucleus (MoV). Researchers have found that fluoxetine suppresses the MoV transmission and attenuates the enhancement of the electrical activity of the masseter muscle in stressed rats[45], proving the inhibitory effects of fluoxetine on masseter over-activity. By combining the results of previous reports and those of the current study, we propose that psychological stress induces oral parafunctional activity[31,33] giving rise to increased TMJ load and further condylar cartilage degeneration[30] and that these effects of psychological stress can be effectively alleviated by the antagonistic effects of fluoxetine.

Conclusion

In summary, the present study confirmed that psychological stress induced by CUMS results in obvious cartilage degradation in the TMJ accompanied by altered MMP metabolism and local loss of ECM. These findings indicate one possible pathological mechanism of TMD. Early antidepressant intervention therapy is beneficial for the treatment of psychogenic TMD.

Acknowledgements

We appreciate Dr Ya Juan ZHAO (Department of Stomatology, Hospital attached to Aeromedicine Institute of PLA, Beijing, China) for her great assistance with the writing and Dr Min CUI's (Department of General Dentistry, School of Stomatology), Xi'an Jiaotong University, Xi'an, China) great technical assistance.

Conflicts of interest

The authors reported no conflicts of interest related to this study.

Author contribution

Drs Qiang LI and Fei HUANG performed part of the experiments and wrote the manuscript; Dr Jia LIU performed some of the experimental studies and made the data collection; Dr Yin Hua ZHAO made the statistical analysis and revised the manuscript; Drs Min ZHANG and Yong Jin CHEN conceived and designed the study.

(Received May 31, 2017; accepted June 26, 2017)

References

1. Selye Hans. The stress of life. New York: McGraw-Hill, 1956.
2. Papaghiuc C, Constantin B, Mihalache C, Oprea V, Carja E. Effects of psychological stress on health [In Romanian]. Rev Med Chir Soc Med Nat Iasi 2005;109:705–708.
3. McNeill C, Mohl ND, Rugh JD, Tanaka TT. Temporomandibular disorders: diagnosis, management, education, and research. J Am Dent Assoc 1990;120:253,255,257 passim..
4. Huang GJ, LeResche L, Critchlow CW, Martin MD, Drangsholt MT. Risk factors for diagnostic subgroups of painful temporomandibular disorders (TMD). J Dent Res 2002;81:284–288.
5. Slade GD, Diatchenko L, Bhalang K, Sigurdsson A, Fillingim RB, Belfer I, et al. Influence of psychological factors on risk of temporomandibular disorders. J Dent Res 2007;86:1120–1125.
6. Fillingim RB, Ohrbach R, Greenspan JD, Knott C, Diatchenko L, Dubner R, et al. Psychological factors associated with development of TMD: the OPPERA prospective cohort study. J Pain 2013;14:T75–90.
7. Lajnert V, Franciskovic T, Grzic R, et al. Depression, somatization and anxiety in female patients with temporomandibular disorders (TMD). Coll Antropol 2010;34:1415–1419.
8. Kuroda S, Tanimoto K, Izawa T, Fujihara S, Koolstra JH, Tanaka E. Biomechanical and biochemical characteristics of the mandibular condylar cartilage. Osteoarthritis Cartilage 2009;17:1408–1415.
9. Sun HB. Mechanical loading, cartilage degradation, and arthritis. Ann N Y Acad Sci 2010;1211:37–50.

10. Billinghurst RC, Dahlberg L, Ionescu M, Reiner A, Bourne R, Rorabeck C, et al. Enhanced cleavage of type II collagen by collagenases in osteoarthritic articular cartilage. J Clin Invest 1997;99:1534–1545.
11. Lijnen HR. Matrix metalloproteinases and cellular fibrinolytic activity. Biochemistry (Mosc) 2002;67:92–98.
12. Fosang AJ, Neame PJ, Last K, Hardingham TE, Murphy G, Hamilton JA. The interglobular domain of cartilage aggrecan is cleaved by PUMP, gelatinases, and cathepsin B. J Biol Chem 1992;267: 19470–19474.
13. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 2003;92:827–839.
14. Wang GW, Wang MQ, Wang XJ, Yu SB, Liu XD, Jiao K. Changes in the expression of MMP-3, MMP-9, TIMP-1 and aggrecan in the condylar cartilage of rats induced by experimentally created disordered occlusion. Arch Oral Biol 2010;55:887–895.
15. Jiao K, Niu LN, Wang MQ, Dai J, Yu SB, Liu XD, et al. Subchondral bone loss following orthodontically induced cartilage degradation in the mandibular condyles of rats. Bone 2011;48:362–371.
16. Lv X, Li Q, Wu S, Sun J, Zhang M, Chen YJ. Psychological stress alters the ultrastructure and increases IL-1β and TNF-α in mandibular condylar cartilage. Braz J Med Biol Res 2012;45:968–976.
17. Cui M, Li Q, Zhang M, Zhao YJ, Huang F, Chen YJ. Long-term curcumin treatment antagonizes masseter muscle alterations induced by chronic unpredictable mild stress in rats. Arch Oral Biol 2014;59: 258–267.
18. Zhao YJ, Li Q, Cheng BX, Zhang M, Chen YJ. Psychological stress delays periodontitis healing in rats: the involvement of basic fibroblast growth factor. Mediators Inflamm 2012;2012:732902.
19. Li Q, Zhang M, Chen YJ, Wang YJ, Huang F, Liu J. Oxidative damage and HSP70 expression in masseter muscle induced by psychological stress in rats. Physiol Behav 2011;104:365–372.
20. Solomon MB, Furay AR, Jones K, Packard AE, Packard BA, Wulsin AC, Herman JP. Deletion of forebrain glucocorticoid receptors impairs neuroendocrine stress responses and induces depression-like behavior in males but not females. Neuroscience 2012;203:135–143.
21. Reznikov LR, Reagan LP, Fadel JR. Effects of acute and repeated restraint stress on GABA efflux in the rat basolateral and central amygdala. Brain Res 2009;1256:61–68.
22. Shigemi K, Tsuneyoshi Y, Yamada S, Kabuki Y, Hayamizu K, Denbow DM, et al. Oral administration of L-serine reduces the locomotor activity of socially isolated rats. Neurosci Lett 2010;468:75–79.
23. Wertheimer GS, Girardi CE, de Oliveira AS, Monteiro Longo B, Suchecki D. Maternal deprivation alters growth, food intake, and neuropeptide Y in the hypothalamus of adolescent male and female rats. Dev Psychobiol 2016;58:1066–1075.
24. Du F, Wang J, Gao Y, Wang H, Wang Q, Jiang S, et al. A hind limb disuse model inducing extensor digitorum longus atrophy in rats: tail suspension-immobilization. Aviat Space Environ Med 2011;82: 689–693.
25. Willner P. Chronic mild stress (CMS) revisited: consistency and behavioural-neurobiological concordance in the effects of CMS. Neuropsychobiology 2005;52:90–110.
26. Qiao H, An SC, Xu C, Ma XM. Role of proBDNF and BDNF in dendritic spine plasticity and depressive-like behaviors induced by an animal model of depression. Brain Res 2017;1663:29–37.
27. Li W, Zhu Y, Saud SM, Guo Q, Xi S, Jia B, et al. Electroacupuncture relieves depression-like symptoms in rats exposed to chronic unpredictable mild stress by activating ERK signaling pathway. Neurosci Lett 2017;642:43–50.
28. Lucchinetti E, Adams CS, Horton WE Jr, Torzilli PA. Cartilage viability after repetitive loading: a preliminary report. Osteoarthritis Cartilage 2002;10:71–81.
29. Costouros JG, Dang AC, Kim HT. Comparison of chondrocyte apoptosis in vivo and in vitro following acute osteochondral injury. J Orthop Res 2004;22:678–683.
30. Akhter R, Morita M, Esaki M, Nakamura K, Kanehira T. Development of temporomandibular disorder symptoms: a 3-year cohort study of university students. J Oral Rehabil 2011;38:395–403.
31. Song F, Li Q, Wan ZY, Zhao YJ, Huang F, Yang Q, et al. Lamotrigine reverses masseter overactivity caused by stress maybe via Glu suppression. Physiol Behav 2014;137:25–32.
32. Rosales VP, Ikeda K, Hizaki K, Naruo T, Nozoe S, Ito G. Emotional stress and brux-like activity of the masseter muscle in rats. Eur J Orthod 2002;24:107–117.
33. Poole AR, Kojima T, Yasuda T, Mwale F, Kobayashi M, Laverty S. Composition and structure of articular cartilage: a template for tissue repair. Clin Orthop Relat Res 2001;(391 Suppl):S26-33.
34. Huber M, Trattnig S, Lintner F. Anatomy, biochemistry, and physiology of articular cartilage. Invest Radiol 2000;35:573–580.
35. Poole AR. An introduction to the pathophysiology of osteoarthritis. Front Biosci 1999;4:D662–670.
36. Nishino T, Chang F, Ishii T, Yanai T, Mishima H, Ochiai N. Joint distraction and movement for repair of articular cartilage in a rabbit model with subsequent weight-bearing. J Bone Joint Surg Br 2010;92:1033–1040.
37. Birkedal-Hansen H, Moore WG, Bodden MK, Windsor LJ, Birkedal-Hansen B, DeCarlo A, et al. Matrix metalloproteinases: a review. Crit Rev Oral Biol Med 1993;4:197–250.
38. Freemont AJ, Hampson V, Tilman R, Goupille P, Taiwo Y, Hoyland JA. Gene expression of matrix metalloproteinases 1, 3, and 9 by chondrocytes in osteoarthritic human knee articular cartilage is zone and grade specific. Ann Rheum Dis 1997;56:542–549.
39. Fini EM, Cook JR, Mohan R, Brinckerhoff CE. Regulation of matrix metalloproteinase gene expression. In: Parks WC, Mecham RP, eds. Matrix Metalloproteinases, San Diego, CA: Academic Press. 1998.
40. Burrage PS, Mix KS, Brinckerhoff CE. Matrix metalloproteinases: role in arthritis. Front Biosci 2006;11:529–543.
41. Correro-Shahgaldian MR, Colombo V, Spencer ND, Weber FE, Imfeld T, Gallo LM. Coupling plowing of cartilage explants with gene expression in models for synovial joints. J Biomech 2011;44: 2472–2476.
42. Brown PD. Matrix metalloproteinase inhibitors. Angiogenesis 1998;1:142–154.
43. Wilde MI, Benfield P. Fluoxetine. A pharmacoeconomic review of its use in depression. Pharmacoeconomics 1998;13:543–561.
44. Mirelle Costa Monteiro H, Lima Barreto-Silva N, Elizabete Dos Santos G, de Santana Santos A, Séfora Bezerra Sousa M, Amâncio-Dos-Santos Â. Physical exercise versus fluoxetine: antagonistic effects on cortical spreading depression in Wistar rats. Eur J Pharmacol 2015;762:49–54.
45. Ribeiro-do-Valle LE, Metzler CW, Jacobs BL. Facilitation of masseter EMG and masseteric (jaw-closure) reflex by serotonin in behaving cats. Brain Res 1991;550:197–204.