Psychological stress induces alterations in temporomandibular joint ultrastructure in a rat model of temporomandibular disorder

Gaoyi Wu, MD, a,b,# Lei Chen, DDS, c,# Guoxiong Zhu, a Yucheng Su, DDS, d Yongjin Chen, MD, e## Jing Sun, MD, e and Yanliang Wang, MD, b,* Ji’nan, Xi’an, Beijing, and Wenzhou, China

JINAN GENERAL MILITARY HOSPITAL, THE 261ST HOSPITAL OF PLA, JINAN STOMATOLOGICAL HOSPITAL, FOURTH MILITARY MEDICAL UNIVERSITY, AND THE WENZHOU MEDICAL COLLEGE

Objective. The objective of this study was to investigate the effects of psychological stress on temporomandibular disorder (TMD).

Study design. A communication box was used to induce psychological stress (PS) in rats. Then, the ultrastructure of temporomandibular was observed using scanning electron microscopy. Interleukin-1 (IL-1) and IL-6 were measured with reverse transcription polymerase chain reaction.

Results. The PS group showed evidence of ultrastructural changes in the condyle and articular disk after stimulation, i.e., incomplete gelatinlike material was observed on the condyle after 1 week of PS, wider waves on the articular disk and exposed condylar collagen were observed after 3 weeks of PS, and cracks were apparent on the surface of the condyle. The expression of IL-1 and IL-6 in the condyle cartilage significantly increased after exposure to psychological stress.

Conclusions. These results indicate that psychological stress induces ultrastructure alterations in the temporomandibular joint and plays an important role in TMD. (Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112:e106-e112)

Temporomandibular disorder (TMD), a functional disorder of the temporomandibular joint that is characterized by temporomandibular joint sounds, impaired movement of the mandible, limitation in mouth opening, preauricular pain, facial pain, jaw tenderness on function, and headaches, is an important area of investigation in dentistry. The etiology of TMD is now considered to be multifactorial, and psychological stress has been regarded as an important factor in the etiology and maintenance of TMD.1,2

Most studies of psychological stress on TMD are epidemiology reports, clinical case studies, or questionnaire surveys; well-controlled experiments have seldom been reported.3,5

One reason for this lack of data is that it has been difficult to establish research models of psychological stress that correlate with TMD. Moreover, there are many factors involved in the etiology of TMD, part of them are difficult to analyze quantitatively.3 Researchers have shown that psychological stress can lead to symptoms, such as abnormal mandibular movements, pain, and facial muscle fatigue.7 It was reported that psychological stress caused mitochondrial injuries and hyperemia of the masticatory muscle capillaries in rats. Furthermore, such stress can result in dramatic alterations in the energy metabolism of the masticatory muscles as reported in a recent study.8

In this study, we hypothesized that psychological stress could increase the serum concentration of cortisol and adrenocorticotropic hormone (ACTH), alter the ultrastructure of the temporomandibular joint (TMJ), and induce inflammation of the TMJ. With an emotional stress paradigm, which used intraspecies emotional communication within a communication box,3,9,10 we introduced experimental correlates of psychological stress,
such as anxiety and depression, in rats. We then tested our hypothesis by examining the TMJ of the animals that experienced psychological stress. Additionally, we included a negative control group, in which rats were administered the antianxiety drug diazepam before being exposed to psychological stress. This study sought to assess alterations in the serum concentration of cortisol and ACTH, the TMJ ultrastructure, and in the expression levels of interleukin-1 (IL-1) and IL-6 in the condyle cartilage of the TMJ in rats following exposure to psychological stress.

**MATERIAL AND METHODS**

The animal stress model used in this study was referenced by Rosales et al, Yamamoto et al, and Funada et al. A total of 120 male, Wistar, albino rats were included in the study. The animals weighed between 140 and 160 g and were approximately 35 days old. The rats were housed in 80 × 45 × 40-cm cages in a temperature-controlled room at 24°C under a 12-hour light/dark cycle. They were given free access to food and water. Before the experiments, the rats were housed in a communication box for 1 hour a day for 5 days to allow them to acclimatize to the box. The rats were then randomly divided into the control (CON), foot-shocked (FS), psychological stress (PS), and psychological stress plus diazepam (PS+DI) groups. Each group included 30 rats. The durations of psychological stimulation were 1 week (1 wk), 3 weeks (3 wk), and 5 weeks (5 wk). Each group was divided into 3 subgroups (1 wk, 3 wk, and 5 wk). The FS, PS, and PS+DI rats were housed in 1 communication box during the procedure, as later in this article. The FS rats received an electric foot shock, the PS rats were subjected to psychological stress, and the PS+DI rats were given a subcutaneous injection of diazepam 30 minutes before being subjected to the psychological stress. The rats in the control group were housed in a second communication box under the same conditions, but they were not subjected to the electric foot shock or psychological stress. The study was performed twice. Thus, 30 animals were included per group, with 10 animals in each of the 3 subgroups.

After 1, 3, and 5 weeks of stimulation, blood was drawn from the ophthalmic artery of the 10 rats in the control, PS, and PS+DI groups. Serum samples were prepared to measure the serum stress indexes. Then, the 10 rats in each group were randomly divided into 2 subgroups, with 5 rats in each group. The first 5 rats in each subgroup were immediately killed with an intraperitoneal injection of an overdose of thiamylal sodium, and their bilateral TMJs were removed. The other 5 rats were housed in another communication box under the same conditions as the control group to observe their recovery. The recovery period was as long as the stimulation period. Thus, the recovery period was evaluated at 1 wk, 3 wk, and 5 wk. Following the recovery period, the 5 rats were killed to extract the TMJ samples. All of the TMJ samples were obtained according to guidelines established by the University Internal Review Board for the Use of Mouse Subjects. The experimental procedures were reviewed and approved by the Ethics Committee of the Fourth Military Medical University. The articular disk and condyle were dissected for ultrastructural observations using electron microscopy. Additionally, RNA was extracted from the condyle cartilage to investigate the expression of IL-1 and IL-6 using reverse transcription polymerase chain reaction (RT-PCR).

**Animal model for psychological stress**

The communication box was selected as the psychological stress apparatus in this study. The box consisted of 16 compartments that were each 16 × 16 cm and were separated by transparent plastic boards with several small holes. The boards prevented the animals from making physical contact with each other, but allowed them to receive cues, such as visual, auditory, and olfactory sensations, from the neighboring animals. Each compartment was equipped with a grid floor of stainless steel rods, which were 5 mm in diameter and placed at intervals of 0.3 cm. A 48-V electric generator, which was made by the Department of Biomedical Engineering of the Fourth Military Medical University, was connected to the grid floor to produce an electric current and generate an electric foot shock every 2 seconds. The grid floors of the non–foot-shock compartments were covered with plastic plates to prevent electric foot shock, and these non–foot-shock compartments were used for the PS and PS+DI rats (Fig. 1).

The stress stimulation within the communication box began 7 days after the electrode installation. Before the day of stress stimulation, the PS, PS+DI, and FS rats

![Fig. 1. Diagram representing the communication box. FS, foot-shocked; PS, psychological stress; PS+DI, psychological stress plus diazepam injection.](image-url)
were individually confined in compartments in the communication box for 1 hour per day without any electric foot shock for 1 week. During this period, the rats were allowed to acclimate to the surroundings. During the stress stimulation period, the electric foot shock was introduced to the FS rats (stress senders) for 0.5 hour a day at a fixed time (9:00-9:30 AM). At 8:30 AM each day, the PS+DI rats were given an injection of diazepam (1 mg/kg). The PS and PS+DI rats (stress responders) were confined in the non–foot-shock compartments and were simultaneously exposed to the emotional cues from the neighboring FS rats, such as shrieks, the smell of urine or feces, and jumping responses. Consequently, the PS and PS+DI rats were assumed to be in a state of fear or anxiety. The purpose of this study was to investigate the effects of purely psychological stress upon the TMJ. Because the FS group experienced physical stress and was only used to induce psychological stress in the neighboring PS and PS+DI rats, the FS group was not included in the following experimental evaluations (Table I).

### Serum assay
The serum concentrations of cortisol and ACTH, and the stress indexes were measured using radioimmuno logical analysis kits (Northern Bioengineering Institute, Beijing, China) according to the protocols provided by the manufacturer.

### Ultrastructure
The condyle and articular disk were dissected and cut into 1-mm pieces and then fixed with 4% glutaraldehyde and 1% osmic acid. The samples were embedded in Epon812, sectioned with an LKBV ultramicrotome (LKB, Bromma, Sweden), and stained with uranyl acetate and lead citrate. The ultrastructures of the muscles were then observed with a transmission electron microscope (JEM-100SX, JEOL Company, Japan).

### Expression level of IL-1 and IL-6
The TMJ specimens were dissected and fast frozen with liquid nitrogen. Then, RNA was extracted from the condyle cartilage to investigate the expression of IL-1 and IL-6 using RT-PCR with β-actin as an internal control.

### Statistical analysis
Experimental data were analyzed by 1-way analysis of variance (ANOVA) across the control group; 1 wk, 3 wk, and 5 wk PS groups; and the 1 wk, 3 wk, and 5 wk PS+DI groups using SPSS, version 11.0 (SPSS Co., Chicago, IL). The Student-Newman-Keuls-q test was then used to calculate any differences between the 2 groups. A P value less than .05 was considered statistically significant.

### RESULTS
To confirm that the experimental rats were in a state of stress, we first analyzed the serum levels of cortisol and ACTH in the rats. As shown in Table II, the concentrations of cortisol in the PS group after 1 wk, 3 wk, and 5 wk of psychological stimulation were significantly higher than the concentrations measured in the control group and the PS+DI group. Similar changes were observed in ACTH levels. As shown in Table III, the serum ACTH concentrations in the PS group after 1 wk, 3 wk, and 5 wk of psychological stimulation were significantly higher than the serum ACTH concentrations measured in the control group, indicating that the rats in the PS group were anxious. In addition, both the cortisol and ACTH values in the PS+DI group were not significantly different from those of the control group (P > .05). Therefore, we could assume that diazepam antagonized the psychological stress in these rats and made them less anxious.
Next, we examined the ultrastructures of the TMJ from the control group, and from the PS and PS+DI groups after 1 wk, 3 wk, and 5 wk of psychological stimulation, and after 1 wk, 3 wk, and 5 wk of recovery from the psychological stimulation. As shown in Fig. 2, the ultrastructure of the condyle and articular disk appeared different in the PS group from in the control and PS+DI groups. In the 1-wk PS rats, some of the synovial membrane broke off from the surface of the articular disk. There was incomplete gelatinlike material on the condyle, and some gelatinlike material gathered on the fibrous chord. The superficial collagen fiber of the condyle was exposed, and the uniform distribution of collagen fiber was disturbed. In the 3-wk PS rats, the collagen fibers appeared to have wider waves and worn strips that changed their size on the articular disk. There was exposure of the deep condylar collagen, and cracks appeared on the surface of the condyle. The pathologic changes of the TMJ began to reverse at 5 weeks, and tissue repair was observable around the altered areas. In the PI+DI rats, there were no obvious changes on the surface of the condylar and articular disk after 1 to 5 weeks of psychological stimulation.

During the recovery period in the PS rats, the bundles of collagen fibers on the articular disk appeared rippled. A small part of synovial membrane was still broken off from the surface of the articular disk in the 1-wk recovery rats, and the surface collagen fibers on the condyle were less disorganized. In the 3-wk recovery rats, the condition of the articular disk and condyle was observed to be improving slowly, and the collagen fibers on both the articular disk and the condyle were less disorganized than in the 1-wk recovery rats. The scanning electron microscopic (SEM) image of the 5-wk recovery rats was similar to that of the 3-wk recovery rats (Fig. 3).

The expression levels of IL-1 and IL-6 are shown in Tables IV and V. In the PS group, the expression levels of IL-1 and IL-6 were significantly higher than in the control group ($P < .05$). The expression levels of IL-1 and IL-6 in the 1-wk PS group were higher than at 3 wk and 5 wk. The expression of IL-1 and IL-6 in the 5-wk PS group had returned to the control group levels ($P > .05$). Although the expression of IL-1 and IL-6 in PS+DI group also increased compared with the control group, the increase was less substantial than that of the PS group ($P < .05$).

During the recovery period, the expression of IL-1 and IL-6 in the 1-wk PS and PS+DI groups had decreased significantly compared with the expression...
measured 1 week after stimulation; however, the expression values were still higher than those of the control group ($P < .05$). Moreover, the expression of IL-1 and IL-6 were higher in the PS group than the PS+DI group after 1 week of recovery ($P < .05$). After 3 weeks of recovery in the PS group, the expression of IL-1 began to decrease, but it was still higher than that of the control group ($P < .05$), and the expression of IL-6 was similar to the control group ($P > .05$). After 3 weeks of recovery, the expression levels of IL-1 and IL-6 in the PS+DI group were nearly normal compared with the control ($P > .05$). After 5 weeks of recovery, both the PS and PS+DI groups showed normal IL-1 and IL-6 expression levels compared with the controls ($P > .05$).

**DISCUSSION**

To our knowledge, this is the first study to report significant changes in TMJ ultrastructure and the expression of IL-1 and IL-6 in the TMJ of rats exposed to psychological stress. The communication box is a well-established method for introducing psychological stress to animals. In this paradigm, the animals that do not undergo physical stress are able to perceive the re-

Table IV. IL-1 mRNA levels in condylar cartilage

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PS</th>
<th>PS+DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 wk</td>
<td>0.453 ± 0.021</td>
<td>0.981 ± 0.024*</td>
<td>0.549 ± 0.014</td>
</tr>
<tr>
<td>3 wk</td>
<td>0.439 ± 0.028</td>
<td>0.746 ± 0.017*</td>
<td>0.498 ± 0.014</td>
</tr>
<tr>
<td>5 wk</td>
<td>0.454 ± 0.023</td>
<td>0.510 ± 0.016*</td>
<td>0.444 ± 0.022</td>
</tr>
</tbody>
</table>

IL, interleukin; PS, psychological stress; PS+DI, psychological stress plus diazepam injection.

* $P < .05$, significantly different from the control group. Data are represented as the ± s of $n = 10$, standard deviation; n, sample size.

Table V. IL-6 mRNA levels in condylar cartilage

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>PS</th>
<th>PS+DI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 wk</td>
<td>0.525 ± 0.028</td>
<td>0.820 ± 0.023*</td>
<td>0.599 ± 0.015</td>
</tr>
<tr>
<td>3 wk</td>
<td>0.515 ± 0.02</td>
<td>0.694 ± 0.019*</td>
<td>0.541 ± 0.015</td>
</tr>
<tr>
<td>5 wk</td>
<td>0.518 ± 0.022</td>
<td>0.579 ± 0.015*</td>
<td>0.487 ± 0.008</td>
</tr>
</tbody>
</table>

IL, interleukin; PS, psychological stress; PS+DI, psychological stress plus diazepam injection.

* $P < .05$, significantly different from the control group. Data are represented as the ± s of $n = 10$, standard deviation; n, sample size.

Fig. 3. Ultrastructure of the TMJ disk in the PS group, the control group, and PS+DI group (magnification ×1000).
sponses of neighboring animals that are exposed to physical stress, which is delivered through an electric foot shock. The intraspecies emotional communication then signals the nonstimulated rats to become anxious, which can increase the plasma level of stress hormones, such as corticosterone. In this article, we successfully simulated a psychologically stressful environment using the communication box as indicated by the increased levels of cortisol and ACTH after 1, 3, and 5 weeks of psychological stimulation.

Psychological stress has been shown to be accompanied by regional modification of the TMJ, which may be attributable to differential gene expression or accessibility to hormones in certain regions of the TMJ. In this study, we observed subtle but significant changes in the ultrastructure of the rat TMJ under psychological stress. The pathologic changes in the TMJ were observed under SEM, and included incomplete gelatinlike material on the condyle, exposure and disorganization of the condylar collagen fibers, worn strips of different size in the collagen fiber on the articular disk, and cracks on the surface of the condyle (Fig. 2). As to the mechanism by which psychological stress causes changes in the TMJ, it is widely believed that psychological stress can cause excessive masticatory muscle movements that lead to TMJ damage. It was reported that oral habits (e.g., teeth grinding) probably provide a behavioral link between stress and the development of TMD symptomatology. In a recent study, it was observed that psychological stress resulted in evidence of swollen mitochondria with cristae loss, and it reduced matrix density in the masticatory muscles after 3 weeks of stimulation. After 5 weeks of psychological stress stimulation, severe vacular changes to the mitochondria were observed. Increased vascular permeability of the masticatory muscle capillaries was observed following 5 weeks of psychological stimulation. In addition, we observed decreased activity of Na+/K+ATPase and Ca2+-ATPase and a simultaneous increase in the activity of lactate dehydrogenase and lactic acid in the masticatory muscles of PS rats. Together, these results indicate that psychological stress induces alterations in the ultrastructure and energy metabolism of the masticatory muscles in rats. Previous studies revealed that TMD was related to deregulation of the hypothalamic-pituitary-adrenocortical axis and increase in basal cortisol secretion. In general, stressors affect the hypothalamus through neural pathways, which results in the release of corticotropin-releasing hormone, which in turn promotes the secretion of ACTH from the pituitary and finally ACTH acts in the adrenal cortex to enhance the synthesis and release of glucocortico-ids. There are additional factors aside from psychological stress, such as gender, age, depression, and general health, that influence TMD, so understanding the specific relationship between psychological stress and TMD still requires further investigation. Whether these alterations are reversible or reducible needs to be further examined with countermeasures, such as drugs, physiotherapy, and psychological stress-free environments.

A number of studies have reported that the expression levels of inflammatory cytokines are correlated with degenerative changes in the TMJ. The cytokines in the synovial fluid may participate in the pathogenesis of TMD. Previous studies have shown that the concentrations of IL-1β, tumor necrosis factor-α, IL-6, and IL-8 were significantly higher in the synovial fluid of patients with TMJ than in healthy subjects. Moreover, the concentration of IL-6 was significantly higher in patients with degenerative changes than in other patients. Therefore, we chose to examine 2 typical inflammatory cytokines, IL-1 and IL-6, to understand the correlation between psychological stress and changes in the TMJ. Soon after introducing psychological stress, the expression of IL-1 and IL-6 increased significantly, indicating that inflammation of the TMJ was induced by psychological stress. After longer periods of stimulation, the TMJ slowly acclimatized to the stress and began to reconstruct itself, and the expression of IL-1 and IL-6 began to return to normal levels after 5 weeks of psychological stress. Soon after removal of the psychological stress stimulus, the expression of IL-1 and IL-6 increased slightly, but as the recovery time continued, the expression of IL-1 and IL-6 returned to a normal level.

The antianxiety drug diazepam was used in this study to observe the effect of diazepam on the changes in the TMJ that were produced by psychological stress. The results were satisfying because the PS+DI group showed significant differences compared with the PS group and was similar to the control group. Stress-provoking stimuli are reported to activate the dopaminergic system. Diazepam has been suggested to induce inhibitory effects on the activation of mesoprefrontal dopamine neurons, which play an important role in the control of negative states, such as fear and/or anxiety, and the hypothalamic-pituitary-adrenocortical axis. The drug also has been used as an anxiolytic drug in several stress-related experiments using the communication box. Therefore, diazepam appears to antagonize psychological stress to protect the TMJ. Along with drug treatment, emotional therapy can be an important treatment for TMD.
In conclusion, psychological stress can induce alterations in the serum concentration of cortisol and ACTH, the TMJ ultrastructure, and increase the expression levels of the inflammatory cytokines IL-1 and IL-6. Psychological stress plays an important role in inducing TMD in rats. Using the antianxiety drug diazepam, which can reduce psychological stress, would alleviate excessive masticatory muscle movement and calm the dopaminergic system and hypothalamic-pituitary-adrenocortical axis which are activated by psychological stress, and so protect the TMJ indirectly.

REFERENCES


Reprint requests:
Yongjin Chen
School of Stomatology
Fourth Military Medical University
Xi’an 710032
China
cyj1229@fmmu.edu.cn

Correspondence to:
Yanliang Wang
School and Hospital of Stomatology
The Wenzhou Medical College
No. 113 West Xueyuan Road
Wenzhou 325027, China
wangyanliang@hotmail.com