Temporomandibular disorders (TMD) are known to be more prevalent and severe in women than in men, especially in those who are in their reproductive age. In those patients reproductive hormones may play a vital role in the host adaptive capacity of the temporomandibular joint (TMJ). In order to clarify the relationship between TMD prevalence and estrogen cycle, a mandible deviated animal model was carried out, and the expression of inducible nitric oxide synthase (iNOS), an essential enzyme in the pathogenesis of inflammatory arthritis, was investigated in the rat’s synovial tissue. An appliance was attached to the rat’s incisors to produce a lateral deviation of the mandible during the metestrus phase, and the animals were sacrificed in the proestrus and estrus phase, when the estrogen was at the highest and lowest level, respectively. Immunostaining was then performed for 2 consecutive estrous cycles to demonstrate iNOS expression in the synovial membrane of the TMJ. The immunoreactivity for iNOS was more intense in the synovial membrane on the contralateral side in the proestrus phase (estrogen peak phase). These observations suggest that iNOS expression in the synovial membrane with mandibular deviation may be exacerbated in the presence of estrogen.

**Key words:** Temporomandibular disorders, mandibular deviation, estrogen, nitric oxide

**Introduction**

It is well known that various factors are involved in the development and progression of the temporomandibular joint (TMJ) disorders (TMD). Epidemiological studies have indicated an association between the female gender and the vulnerability to TMD, showing a higher prevalence in women than in men (1.5-2 times)\(^1\). And among those female TMD patients, who are in their reproductive years have the highest prevalence\(^2\). In addition, TMD pain increases systematically with pubertal development in girls and is less prevalent among older persons\(^3,4\). It is known that estrogen increases joint laxity, at least during pregnancy, and laxity of the temporomandibular joints have been related to TMJ dysfunction patients\(^5\). Moreover, other studies show that TMD symptoms vary in intensity over the menstrual cycle\(^6\). However, the biological basis for this possible gender-based disparity is still unclear.

Estrogen, as a representative reproductive hor-
mone, is known to regulate diverse physiological processes by binding to its respective receptor, and estrogen receptors (ER) have been revealed to be abundantly present in human symptomatic TMJ. Animal studies have also identified the distribution of ER in various cellular elements in the rat’s TMJ, including synovial lining cells, stromal cells in the articular disc, and chondrocytes. Those findings indicate that the TMJ can be a target for estrogen, which suggest that this female endogenous reproductive hormone may be involved in TMD.

Recent studies have identified that the activation of the inducible nitric oxide synthase (iNOS) pathway is involved in the pathogenesis of TMD. A study done in human samples showed iNOS expression in the synovium of TMJ with synovitis, which support a vital role for NO in the pathophysiology of synovitis. Yamaza et al. (2004) have demonstrated an enhanced iNOS expression in rat’s TMJ synovium after mechanical loading. Sato et al. (2006) demonstrated that the functional shift of the rat mandible during the growth period changes the morphology of the condylar cartilage. The different mechanical loading on the condylar cartilage of a deviated mandible lead to different morphological and histological changes between the ipsi-lateral side and the contra-lateral side.

Therefore, in this study, we focused on iNOS expression in the synovial membrane of the TMJ during the estrogen cycle, using an animal model with deviated mandible.

Materials and Methods

Experimental model

Thirty-two female Wistar rats (7 weeks old) were maintained under pathogen-free conditions. The rats were fed with powder diet (Rodent Diet CE-2; Japan Clea Inc, Shizuoka, Japan) and water ad libitum. All procedures were carried out under the guidelines of the Tokyo Medical and Dental University for Animal Research. During the proestrus and estrus phase, estrogen reaches the highest and the lowest level respectively. Vaginal smears were taken daily during the whole period of this study to identify the 4 different phases of the estrous cycle: proestrus, estrus, metestrus, and diestrus (Figure 1). Vaginal smears during the proestrus phase are characterized by nucleated epithelial cells (Figure 1A (a)) and by cornified cells in the estrus phase (Figure 1A (b)). In the metestrus phase, leukocytes, cornified cells and nucleated epithelial cells are abundantly in the vaginal smear (Figure 1A (c)). The diestrus phase consisted predominantly by leukocytes (Figure 1A (d)).

When the rats were 9 weeks-old, they were randomly divided into 2 control groups (N = 3 each) and 2 experimental groups (N = 5 each) for one estrogen cycle, and in order to confirm the reliability, the experiment were carried out for another cycle. During the metestrus phase a guiding appliance was attached to the rat’s incisors of the experimental groups to induce a lateral functional shift of the mandible. Then the animals were sacrificed at the proestrus (5 experimental rats and 3 controls) and estrus phase.

Tissue preparation

The rats were sacrificed by transcardiac perfusion with saline solution followed by iced-cold 4% paraformaldehyde with 0.2% picric acid in 0.1 M phosphate buffer, pH 7.4. The specimens were immersed in the same fixative solution for an additional 12 hours at 4°C. After fixation, the tissues were decalcified in 10% ethylenediamine tetraacetic acid-2Na solution, pH 7.4, for 7 or 8 weeks, dehydrated in graded ethanols and embedded in paraffin. Serial sections of 6 μm thickness were made along the sagittal axis and stained with inducible nitric oxide synthase (iNOS).

Immunohistochemistry for iNOS

For each experimental and control group 5 of the mid-sagittal sections of the condyles were immunohistochimically processed with iNOS antibody. After the sections were dewaxed, rehydrated, rinsed with 0.01 M phosphate buffered saline (PBS) for 5 minutes, and treated with 0.3% H2O2 in absolute methanol for 30 minutes to inactivate endogenous peroxide activity. After washed in 0.3% Triton X-100 in PBS, the sections were incubated with 0.01 M phosphate buffered saline (PBS) for 5 minutes, and treated with a peroxidase-labeled anti-mouse antibody (Histofine Simplestain Max-PO (MULTI), Nichirei, Japan). Peroxidase activity was developed with 3,3’-diaminobenzidine hydrochloride (DAB, Sigma, USA). Microphotographs were taken under a microscope (100× and 400× magnification).

Immunohistochemical findings

iNOS immunostained sections were examined in the synovial membrane in the posterior-superior portion of the articular cavity, where the synovial membrane pro-
Fig. 1. A: Vaginal smears showing the characteristics of the 4 phases of the rat's estrous cycle. Magnification x 20. (a) Proestrus: moderate number of epithelial cells[E]. (b) Estrus: cornified cells[C]. (c) Metestrus: leukocytes[L], epithelial and cornified cells. (d) Diestrus: Abundant leukocytes. B: Estrogen cycle in rats.
trudes as a few folds, as indicated in the schematic dia-
gram (Figure 2). The immunohistochemical reactivity
was evaluated and graded according to the criteria of
Homma et al (2001): - (negative), no staining; + (pos-
itive), focally positive for a limited number of cells; and
++ (intensely positive), focally or diffusely positive for
numerous cells.

**Results**

**Determination of the estrous cycle phases of rats**

Estradiol level begins to increase at metestrus,
reaching peak levels during proestrus and returning to
baseline at estrus. In this study vaginal smear cytology
was used to determine the estrous cycle phases as
previously described. Figure 1 shows the cellular
types from the vaginal smear of rats.

**iNOS immunohistochemical localization in the
synovial membrane of the TMJ**

In the rat synovitis-induced model, the findings for the
posterior portion facing the upper joint compartment,
particularly in the synovial fold, were shown as typical
for all the regions of the synovitis-induced and non-
induced TMJs. As showed in the schematic diagram
of the rat TMJ (Figure 2), we examined the posterior
region of the ipsi-lateral side and contra-lateral side of
the TMJ. The immunoreactivity for iNOS was detected
in the membrane of the synovial fold of the TMJ of the
experimental groups (Figure 3, 4), and a negative
expression was observed for the control groups. No
obvious difference was identifiable for the 2 consecu-
tives estrous cycles (Figure 4).

The immunoreactivity for iNOS was more intense in
the synovial membrane of the contra-lateral side of the
proestrus phase (estrogen peak phase)(Figure 3B). On
the other hand, the expression of iNOS in the contra-
lateral side was less intense during the low estrogen
phase (Figure 3D). Vascular dilation was also found in
the subsynovial connective tissue. However, no
inflammatory change was evident in the synovial
membranes of all control TMJ (Figure 3A, 3C).

**Discussion**

A study done in human samples showed iNOS
expression in the synovium of TMJ with synovitis,
which supports a significant role for NO in the patho-
physiology of synovitis. Similar to other studies in this
experimental model, we also observed that iNOS was
most strongly expressed in the synovial lining layer.

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Fig. 2. A schematic diagram of the rat TMJ on a sagittal section. The synovial fold corresponds to the observed portion in the histopathological analysis. (Boxed area).
Since epidemiological studies have indicated a higher prevalence of TMD in women than in men\(^1,19\), and the highest prevalence was observed in those female patients in their reproductive years\(^2\). In this study we aimed to clarify the relationship between TMD and estrogen cycle. The onset of pain for TMD is almost always after puberty and it is lower for postmenopausal women\(^19\). In addition, other studies have showed that TMD symptoms vary in intensity during the menstrual cycle\(^5\). Those findings suggest that the female endogenous reproductive hormones may play an important role in TMD. Our results may advocate this hypothesis, and may also link the mechanical-stress dependent causes of TMD into those factors\(^20\).
In this study, we used an appliance designed to produce a deviation of the mandible towards the left side as described in a previous study done by Sato et al., where the functional shift of the mandible changed the morphology of the condylar cartilage\(^{11}\). Buranastidporn et al. (2006) have reported that symptoms confined to the ipsi-lateral side were primarily found in the subjects with mild asymmetry, whereas symptoms on both sides and those on the contra-lateral side were greater in those with moderate and severe asymmetry, respectively\(^{21}\). However, we found that iNOS expression was detected in the synovial membrane of the contra-lateral side of the deviated mandible during the high estrogen phase. Because of the structural difference of the condyle and glenoid fossa between human and rat\(^{15}\), the condyle of the contra-lateral side might have deviated not only forward and laterally but also downward, leading to the result of this experiment. These findings suggest that mechanical stress, due to occlusal disharmonies, may contribute to the development or progression of TMJ dysfunction bearing differences on iNOS expression by the changes in estrogen level.

As many other studies have mentioned the implication of the female hormones\(^ {1,2,5,6,22,23}\), our data also indicated that estrogen exerts an influence on iNOS. We identified that iNOS expression was enhanced during the proestrus phase, when the level of estrogen reaches the peak; and found a decreased or negative staining during the estrus cycle, when the level of estrogen was low. We also observed a negative expression for the control group, which correlates to those findings suggested by other reports that the synovia of symptomatic TMD patients expressed iNOS, while an absent or weak staining of iNOS was found in asymptomatic patients\(^ {8}\). Some studies have reported a relationship between INOS and estrogen, where estrogen exerts an up-regulation of iNOS expression\(^ {24}\) to release NO in a dose dependant manner in peritoneal macrophages through the ER activation\(^ {25-26}\). Another report revealed that estrogen treated mice markedly up-regulate the levels of iNOS mRNA, iNOS protein, and nitric oxide in activated splenocytes\(^ {28}\). This may also indicate that high level of estrogen may exacerbate the production of NO via iNOS on an overloaded TMJ.

The presence of functional ER in the synovial tissue of animals and human TMJ\(^ {6,7,23,27}\) may provide evidence that TMJ is a target for estrogen. Moreover, animal studies have also identified the distribution of estrogen receptors (ER) in various cellular elements in the rat’s TMJ, including synoviocytes, stromal cells in the articular disc, and chondrocytes\(^ {7}\). Estrogen increases iNOS expression in macrophages, and this effect appears to be the consequence of ER activation\(^ {25}\). However, the role of this female hormone in the synovial tissue is still not well understood. There are controversies regarding the anti-inflammatory and pro-inflammatory effects of estrogen on different tissues. For example, some studies showed that the estrogen exerts an anti-inflammatory activity on primary cultures of rat’s microglia\(^ {26}\). They also suggested that estrogen modulates local inflammation in various joint diseases via synoviocytes as well as sublining macrophage-like cells\(^ {8}\). Further investigation may be needed to elucidate other function of estrogen on TMJ.

In conclusion, this study may have provided new and important information about the influence of estrogen cycle on the symptom of TMD via iNOS expression in the synovial membrane of the TMJ. Our data also suggested that malocclusions, which may cause mandibular deviation, could trigger the development or progression of TMJ dysfunction in susceptible patients\(^ {20,29}\), especially female in their reproductive years. And further investigations for the functional significance of estrogen are needed to clarify the relationship between estrogen and the etiology of TMD.

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