Free-radical Oxidation and Superoxide Dismutase Activity in Synovial Fluid of Patients with Temporomandibular Disorders

Heng-Xing Cai, DDS*
Graduate Student

Jing-Mei Luo*
Associate Professor

Xing Long, DDS, PhD
Professor and Chairman

Xiao-Dan Li, DDS, PhD
Associate Professor

Department of Oral and Maxillofacial Surgery

Yong Cheng, DDS, PhD
Associate Professor
Department of Oral and Maxillofacial Radiology

College and Hospital of Stomatology
Key Laboratory for Oral Biomedical Engineering
Ministry of Education
Wuhan University
Wuhan, China

Correspondence to:
Dr Xing Long
Department of Oral and Maxillofacial Surgery
College and Hospital of Stomatology
Key Laboratory for Oral Biomedical Engineering
Ministry of Education
Wuhan University
237 Luoyu Rd
Wuhan 430079
People's Republic of China
E-mail: longxing_china@hotmail.com

*Primary investigators

Aims: *To measure the activity of oxygen free radicals and the level of antioxidant enzyme superoxide dismutase (SOD) in the synovial fluid (SF) of the temporomandibular joints (TMJs) of patients with temporomandibular disorders (TMD).* ***Methods:*** *Thirty-two patients were divided into 3 subgroups: anterior disc displacement with reduction, anterior disc displacement without reduction, and osteoarthrosis. Six healthy volunteers served as controls. A pumping procedure was used to take SF from the superior TMJ space. The concentration of lipid peroxidation products was assessed by means of the thiobarbituric acid reaction, and the level of SOD was assessed with spectrophotometry.* ***Results:*** *SF levels of lipid peroxides (LPO) in the control and patient groups were 0.010 (± 0.004) × 10^{-3} nmol/mg protein and 0.61 (± 0.121) × 10^{-3} nmol/mg protein, respectively. SF SOD levels were 4.61 (± 1.30) NU/mg protein and 9.83 (± 2.66) NU/mg protein, respectively. Both the concentration of LPO and the level of SOD activity were significantly higher in the TMD patients than in the normal control subjects (*$P < .001$ *and* $P < .01$*, respectively). There were no significant differences in the level of LPO or SOD between the 3 subgroups.* ***Conclusion:*** *These results demonstrate oxygen free radicals and antioxidant enzymes may be connected in the pathogenesis of TMD.* J OROFAC PAIN 2006;20:53–58

Key words: lipid peroxides, oxygen free radicals, superoxide dismutase, synovial fluid, temporomandibular disorders

It has been suggested that oxygen free radicals may play a role in the pathogenesis of degenerative joint diseases and that oxidative stress may be an important aspect in the mechanism of temporomandibular disorders (TMD).[1,2] A free radical is a chemical species (atom, ion, or molecule) that contains an unpaired or odd number of electrons. By far the most common source of free radicals in biological systems is oxygen. The term *oxidative stress* is used to designate any condition that results in an accumulation of free radicals in a tissue.[3] Free radicals are extremely reactive by virtue of their particular molecular configuration. Although oxygen free radicals participate in many physiological processes, they can be very harmful to tissues when their action is left uncontrolled.[3–5] Both cellular and extracellular molecules may be destroyed.[5–8]

One site that is particularly susceptible to peroxidative reaction is the cellular membrane.[3] Lipid peroxides (LPO), which may serve as indicators of oxidative stress, are oxidation products of the unsaturated fatty acids on these membranes. Increased lipid peroxidation as well as increased levels of thiobarbituric acid-reactive

material in the synovial fluid (SF) have been reported in cases of rheumatoid arthritis.[9–11] Superoxide dismutase (SOD) is 1 of the enzymes engaged in converting highly reactive free radicals to less reactive molecular species.[12] The study of lipid peroxidation is often combined with that of SOD.[3] This study was designed to measure the activity of oxygen free radicals and the level of SOD in the SF of the temporomandibular joints (TMJs) of patients with TMD.

Materials and Methods

Subjects

The subjects included in this study were recruited from the Clinic of the Stomatological Hospital of Wuhan University; 6 healthy volunteers served as controls. SF samples were taken from 40 patient TMJs and 10 control TMJs; samples were taken bilaterally from 8 patients and 4 control subjects. Clinical examination was performed, and signs and symptoms were recorded at the patient's initial visit. Subjective pain during mouth opening and eating was rated using a visual analog scale (VAS) of 0 (no pain) to 100 (maximum pain imaginable). Maximal mouth opening (MMO) was measured with a millimeter ruler and defined as the maximal distance between the incisal edges of the maxillary and mandibular central incisors. Radiographic examination included transcranial views (open and closed mouth) and tomography of the TMJ for determination of bony changes such as osteophytes and erosion. Superior joint space arthrography was used to determine any anterior disc displacement. Clinical diagnoses of TMD were made according to the Clinical Diagnostic Criteria for Temporomandibular Disorders.[13] The clinician applied these guidelines with his own clinical judgment to arrive at a diagnosis. Joint sounds were evaluated using a stethoscope and palpation and were recorded as "click," "friction," or "crepitus." The patients were divided into 3 subgroups according to the imaging findings: anterior disc displacement with reduction (ADDwR), anterior disc displacement without reduction (ADDw/oR), and osteoarthrosis (OA) of the TMJ. All the data were collected at the patient's first visit, and none of the patients had had any treatment previously. The Ethics Committee of the College and Hospital of Stomatology, Wuhan University, approved the study protocol, and informed consent was given by each participant before participation in the study.

Collection of Samples

Samples were obtained from the patients during TMJ arthrography. After subcutaneous infiltration with 2% lidocaine in the preauricular region, 2 mL of saline solution was injected into the superior joint space. The patient was then asked to open and close his or her mouth to mix the saline solution with SF. After the mixture of SF and saline was aspirated and reinjected 5 times, the sample was aspirated and collected, but the tip of the needle remained in the superior joint space; later the syringe was replaced by another one which contained the proper contrast medium so that arthrography could be performed at the same time. Samples from the control subjects were collected in a similar procedure, except that they did not receive TMJ arthrography. After being centrifuged at 4°C (1,000*g*, 5 minutes) to remove the cells and tissue debris, all the samples were stored at –70°C until the assays were performed.

Protein Quantification

Protein-containing solutions were quantified using a bicinchoninic acid (BCA) protein assay kit (Pierce), according to instructions provided by the manufacturer, except that the assay was downscaled to a total volume of 200 μL.

Thiobarbituric Acid Test

The concentration of LPO products was assessed in samples by means of the thiobarbituric acid (TBA) reaction. The method of Rowley et al[10] was modified for this study; briefly, 125 μL of SF was added to 250 μL of TBA solution (1% wt/vol in 50 mmol/L sodium hydroxide), 250 μL of hydrochloric acid (HCl; 25% vol/vol) and 200 μL of water; 125 μL of water was used in place of the sample as a negative control. The tubes were tightly capped and heated at 100°C for 1 hour, after which they were allowed to cool to room temperature before they were extracted into 1.5 mL of 1-butanol and vigorously mixed for 2 minutes. The samples were then centrifuged at 1500*g* at 4°C for 15 minutes, and the absorbance of the upper organic layer was determined at 532 nm by spectrophotometry.

Assay of SOD Activity

The superoxide anion was formed through the xanthine oxidase reaction system and then the superoxide anion was oxidated to form nitrite, which manifested as light purple under the action of a

Table 1 Characteristics of the TMD Patients and Control Subjects

Group	No. of subjects	Sex		Age		Pain VAS score		No. of TMJs
		M	F	Mean	Range	Mean	Range	
Control	6	4	2	28.5	25–35	0	0	10
ADDwR	10	4	6	30.2	17–52	48	0–90	12
ADDw/oR	10	4	6	26.4	19–49	56	25–95	13
OA	12	5	7	36.25	20–62	45	20–85	15

Table 2 Results of the Clinical Examinations

Group	MMO (mm)	Deviation		Joint noises				Pain	
		Yes	No	No	Click	Friction	Crepitus	Yes	No
Control	38.22 ± 7.56	2	4	6	0	0	0	0	6
ADDwR	42.39 ± 5.22	8	2	2	8	0	0	6	4
ADDw/oR	31.24 ± 7.76	7	3	8	0	1	1	7	3
OA	40.26 ± 4.68	7	5	2	0	7	3	8	4

color-developing agent. The maximum absorbance value was reached at a wavelength of 550 nm. All the agents were purchased from the Nanjing Jiancheng reagent company, and the tests were carried out strictly in accordance with its instructions. The activity of the enzyme was defined such that when the SOD inhibiting rate amounted to 50% per milliliter of reaction solution, the corresponding amount of SOD was 1 nitrite unit (NU).

Statistical Analysis

One-way analysis of variance (1-ANOVA) was used for comparison of LPO or SOD levels between different groups, and the Student *t* test was used for comparison of LPO or SOD levels between the control and patient groups. Distribution of the sample values, whether normal or not, was tested by Kolmogorov-Smirnov 3 method. To eliminate the possibility of different amount of SF on the results of these assays, all the data were compared with their protein concentration. These analyses were done with statistical software SSPS for Windows 11.0. A probability level of .05 was considered statistically significant. Values are expressed as mean ± SD.

Results

Clinical Findings

Table 1 shows characteristics of the TMD patients and control subjects, and Table 2 shows the results of the clinical examinations.

Table 3 Protein Concentration in SF of TMJ

Group	No. of TMJs	Protein concentration (mg/mL)
Control	10	0.705 ± 0.258
ADDwR	12	0.777 ± 0.251
ADDw/oR	13	0.838 ± 0.175
OA	15	0.870 ± 0.241

Protein Quantification

Protein concentrations for each group are shown in Table 3. There was no significant difference in protein concentration between the groups ($F = 1.163$; $P > .05$).

LPO and SOD Assays

Results of LPO and SOD assays are listed in Table 4. As shown in Table 4, LPO and SOD were detected in TMJ SF samples of all control subjects and all patients. No correlation was found between SF LPO and SOD. Both the concentration of LPO and the level of SOD in SF samples of the TMD patients were significantly higher than those of the control subjects ($P < .001$, $P < .01$, respectively). There was a significant difference in LPO concentration between each patient group and the control group ($P < .01$; Fig 1a). There was a significant difference in the level of SOD between each group and the control group ($P < .05$; Fig 1b). In addition, no significant differences were found either in LPO or in SOD level between the 3

Table 4 Levels of LPO and SOD in SF of TMJ

Group	No. of TMJs	LPO (10^{-3} nmol/mg protein)	SOD (NU/mg protein)
Control	10	0.010 ± 0.004	4.61 ± 1.30
ADDwR	12	0.619 ± 0.145	9.29 ± 2.37
ADDw/oR	13	0.616 ± 0.110	10.96 ± 2.96
OA	15	0.538 ± 0.108	9.29 ± 2.85

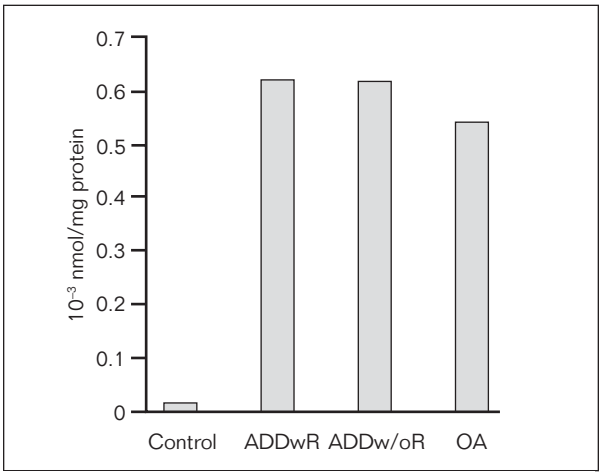

Fig 1a Graph showing the level of LPO in each group. The level of LPO was significantly higher in each patient subgroup than in the control group ($P < .01$).

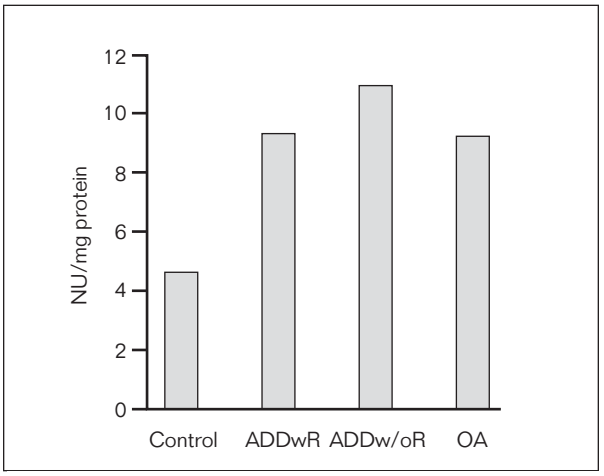

Fig 1b Graph showing the level of SOD in each group. The level of SOD was significantly higher in each of the patient subgroup than in the control group ($P < .05$).

patient groups. The level of LPO or SOD did not correlate with any of the clinical parameters such as MMO or VAS (data not shown).

Discussion

TMD are some of the most common diseases of the TMJ and have a broad spectrum of subgroups. Analysis of TMJ SF from TMD patients has become popular since TMJ SF is closely related to joint metabolism and pathology. In the selection of subjects, many investigators have singled out patients with internal derangement (ID) and/or OA.[14,15] The authors of the present investigation presumed that there may be some differences between patients with ADDwR and those with ADDw/oR, since these are 2 subgroups of ID which can be easily diagnosed from the patient's history and a comprehensive clinical and radiographic examination of the patient. However, no significant differences in the level of LPO or SOD were found between these 2 subgroups in the present study.

The exact molecular mechanism of TMD remains obscure.[16] Three of the models that have been established to explain the molecular development of this condition are *(1)* direct mechanical damage, *(2)* hypoxia and recuperation injury, and *(3)* neurogenic inflammation.[2] These 3 models are based on the concept that the initial step to TMD is excessive mechanical stress. Many authors believe that cytokines and matrix-degrading enzymes play the major role in the pathology of TMD,[14,15,17–20] but they often overlook the transduction mechanism from excessive mechanical stress to the production and activation of the cytokines and matrix-degrading enzymes. In recent years, several authors found that oxygen free radicals and oxidative stress may participate in this process.[1,2] Studies have shown that oxygen free radicals are able to take part in many important pathologic changes in TMD.[21–26]

In this study, both LPO and the antioxidant enzyme SOD were identified in the SF of patients with TMD. The investigators' expectation was that there might be an increase of LPO and a decrease of SOD activity in SF of patients with TMD, as oxygen free radicals are often responsible for similar changes in the pathologic processes of other diseases, such as coronary heart disease.[27]

However, in the present study, both LPO and SOD levels were significantly higher in TMD patients than in healthy control subjects. The authors suppose that there should be a low-level balance between oxygen free radicals and antioxidant enzymes in SF. When joint diseases such as infection or injury occur, both sides are stimulated, and their interaction may direct the course of a pathological process. It may be a reasonable explanation that the overactivity of oxygen free radicals leads to the overgeneration of antioxidant enzymes. Although there are little published data of SOD activity in TMJ SF, and there may be many methods to evaluate SOD, the SOD value of the control subjects in this study probably was only relatively close to the value in physiologic conditions, since SF was collected using a dilution method and the enzymes may behave differently in physiologic conditions. A study of SOD in a normal knee joint SF by the direct extraction method and using the same reagent from the same company revealed an SOD value of 120.65 ± 15.42 NU/mL, which was almost 40 times the level of TMJ SF.[28]

A limitation of the present study was that the control subjects were not comparable in age to the OA group. This limitation arose because of the extreme difficulty in obtaining SF samples from volunteers who had no symptoms. The effect of age on the metabolism of LPO and SOD has been widely studied in other fields. However, there is little information about the effect of age on free radical activities in TMJ SF. Strictly controlled studies are needed before the possibility that age effects on free radical production within the TMJ in healthy subjects can be excluded.

Another limitation of this study was the small number of control subjects (n = 6; 10 joints). Most previous studies of TMJ SF included 10 to 15 joints from control subjects.[18–20] The collection of normal SF samples may represent a common challenge in such studies.

When SF samples are collected from the TMJs, a concern is that, in addition to other factors, different amounts of saline aspirates will influence the results. Thus, a new method has been designed to compensate for this influence.[29] In this study, the activity of enzymes was first calculated against their protein concentration and then compared between groups. This method is feasible because enzymes are proteins which have been diluted proportionally. Many kinds of proteins, such as proinflammatory cytokines and growth factors, are upregulated in the SF of patients with TMD[14,15,18–20]; however, there is no significant difference in the total protein concentration in the SF.[30,31] It is possible that the changes are so small that significant changes cannot be detected.

SOD has been used in TMD patients who are unresponsive to traditional treatments, and the results have been fairly encouraging.[32] However, caution is advised before this approach is used clinically, because a certain degree of oxygen free radical activity is indispensable to the physiological metabolism of normal tissues, and the long-term loss or repression of its activity in a tissue may lead to other diseases.[33]

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