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Clinical Paper
TMJ Disorders

Effect of occlusal splint on interleukin 6, malondialdehyde and 8-hydroxydeoxyguanosine levels in the synovial fluid of patients with temporomandibular disorders

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Abstract. The actual role of splint therapy in preventing excessive loading of the temporomandibular joint (TMJ) is still debated. Lower intra-articular pressure levels have been measured in patients wearing occlusal splints, which may also reduce oxidative stress in the articular spaces. The aim of this study was to determine whether splint therapy reduces oxidative stress and inflammation in TMJ internal derangement patients by measuring interleukin 6 (IL-6), malondialdehyde (MDA), and 8-hydroxydeoxyguanosine (8-OHdG) levels in the synovial fluid (SF). Twenty-four patients with a temporomandibular disorder (TMD) were included in the study. TMJ SF samples were obtained prior to arthrocentesis. Twelve patients used a 2-mm hard acrylic, maxillary stabilization-type splint for 3 months after arthrocentesis. Twelve patients had no treatment after the SF aspiration. Second SF samples were obtained from all patients at 3 months post arthrocentesis. IL-6, MDA, and 8-OHdG levels in the samples were evaluated. All patients showed a significant symptomatic improvement after treatment ($P < 0.005$). No statistical correlation was found between the two groups concerning pre-treatment and 3-month SF levels of MDA, 8-OHdG, and IL-6. Although splint therapy was found to be successful in eliminating clinical symptoms of TMD, the results showed no beneficial effect on inflammation and oxidative stress markers in the synovial fluid.

Key words: arthrocentesis; inflammation; oxidative stress; synovial fluid; temporomandibular disorders.

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Internal derangement of the temporomandibular joint (TMJ) is defined as displacement of the articular disc from its normal relationship with the mandibular condyle and the articular eminence[1]. Although the biochemical and morphological processes that cause TMJ internal derangement have been studied well in the literature, the pathophysiological factors that lead to symptom-generating processes are still the subject of debate. In recent years, studies on the contents of the synovial fluid (SF) have shed some light on the pathophysiological nature of TMJ diseases[2–5].

It has been postulated that the possible aetiological factor of TMJ internal derangement is excessive mechanical loading, which leads to increased oxidative stress in the TMJ[6,7]. Oxidative stress is defined as any condition that leads to the accumulation of free radicals in a tissue[8,9]. Free radicals have an extremely reactive molecular configuration[7]. In healthy tissue, there is a balance between free radical production and the antioxidant mechanism. Antioxidants neutralize free radicals by binding to their free electrons[10,11]. Increased levels of free radicals in a tissue caused by either increased production or a defect in the antioxidant mechanism have been shown to be the first step in pathological diseases[11].

There is a growing interest in markers of inflammation in the SF of temporomandibular disorder (TMD) patients. A wide variety of molecules including cytokines, neuropeptides, arachidonic acid derivatives, matrix-degrading enzymes, and free radicals have been found to be associated with the pathogenesis of synovitis and resorption of cartilage and bone in the TMJ[10–20]. Interleukin 6 (IL-6), one of the most frequently measured cytokines in SF studies, has been found to be associated with internal derangement of the TMJ[14–17]. Malondialdehyde (MDA) is the last product of the lipid peroxidation reaction, which is a reaction generated by free radicals, and levels of MDA have been found to be higher in the SF of patients with TMD[19]. 8-Hydroxydeoxyguanosine (8-OHdG) has been reported to be a stable indicator of oxidatively damaged DNA in a tissue[13].

Occlusal splints are used frequently for the management of TMD, although their mechanism of action remains controversial. The beneficial effects of occlusal splints for masticatory muscle and TMJ pain, noise, and jaw mobility have been demonstrated in several studies[21–24]. Splint insertion causes a shift in the position of the intra-articular distance by increasing the vertical dimension both in the rest position and during jaw movements[25]. Studies have found lower intra-articular pressure levels in patients wearing occlusal splints[6,26]. The appliance decreases overloading inside the TMJ by providing a stable mandibular position and preventing parafunctional habits.

There appears to be no report in the literature on the association between splint therapy and its effects on biochemical markers in the SF. There is a need to understand the effectiveness of splint therapy with regard to the elimination of oxidative stress and inflammation. The aim of this study was to investigate whether the insertion of an occlusal splint decreases oxidative stress and inflammation through an analysis of the levels of IL-6, MDA, and 8-OHdG in the SF.

Materials and methods

This prospective randomized trial was conducted in accordance with the Declaration of Helsinki on medical protocol. The procedure was approved by the Institutional Review Board of Ondokuz Mayıs University in Samsun (Clinical Research Ethics Committee of Ondokuz Mayıs University Experimental Medicine Research and Application Centre). After a detailed clinical and radiological examination, 24 patients with a TMD, who had disc displacement (DD) with reduction with intermittent locking or DD without reduction with limited opening according to the Diagnostic Criteria for Temporomandibular Disorders (DC/TMD) classification, were included in the study[27]. The main symptoms of the patients were pain in the TMJ region and limited mouth opening.

The following exclusion criteria were applied: other local diseases and/or systemic disorders; the use of anti-inflammatory drugs, analgesics, and/or muscle relaxants, vitamin C and/or vitamin E; smoking, which increases the levels of free radicals; and individuals who had already been under treatment for a TMD.

Written informed consent was obtained from the patients after a detailed explanation of the study. All patients were clinically examined by the same clinician and the clinical diagnosis was confirmed by magnetic resonance imaging (MRI). The patients completed a visual analogue scale (VAS) to assess their pain, with marked endpoints of score 0 (no pain) and score 10 (worst pain ever experienced). Maximum mouth opening (MMO) was measured with a ruler. These measurements were recorded at baseline and at each clinical visit for 12 weeks.

The patients were allocated to two groups by computer-generated randomization draw, performed immediately before the procedure: group 1 comprised 12 patients who were provided with a stabilization splint for use during the 3 months after the SF sample was obtained; group 2 comprised 12 patients who were not provided with a stabilization splint after the SF sample was obtained.

The TMJ SF samples were obtained by arthrocentesis, according to the technique of Nitzan et al.[28], under sedation. Two millilitres of saline solution was injected into the upper joint space after subcutaneous local anaesthesia. To allow the saline solution to mix with SF, the solution was aspirated and then re-injected 10 times, and the mixture of SF and saline solution was re-aspirated into a syringe for biochemical analysis. The SF samples were immediately centrifuged at 3000 rpm for 10 min and stored at −80 °C before analysis.

Patients in group 1 received a 2-mm hard acrylic, maxillary stabilization-type splint with flat surfaces and occlusal contacts in centric occlusion for all opposing teeth. All splints were made and adjusted by the same clinician. After the appliance was seated comfortably, the occlusion was adjusted until the desired centric contacts were obtained. Patients were instructed to wear the splint 24 h per day for 12 weeks, except during oral hygiene activities and at mealtimes.

The primary outcome variables of this study were SF levels of IL-6, MDA, and 8-OHdG. Secondary outcome parameters were the VAS score to assess pain and the MMO measured with a ruler. All variables were evaluated at baseline and at the 3-month follow-up. SF samples were evaluated by measuring IL-6, 8-OHdG, and MDA levels. During the period of splint therapy, the patients were regularly followed-up to check that the splint was successfully accepted. At the 3-month follow-up, a second SF sample was obtained from all patients and a second arthrocentesis was performed if indicated.

High-performance liquid chromatography with fluorescence detection (HPLC-FLD) for the analysis of MDA

Instrumentation and chromatographic conditions

The HPLC system consisted of multichannel pumps (LC 20AT), an autosampler

(SIL 20ACHT), a degasser (DGU 20A5), and a fluorescence detector (RF-10AXL) (Shimadzu, Kyoto, Japan). A reversed-phase C18 column (Inertsil ODS-3V, 5 μm, 4.6 × 250 mm; GL Science, Tokyo, Japan) was used for separation. The mobile phases consisted of a 40:60 ratio (v/v) of methanol to 50 mM potassium monobasic phosphate at pH 6.8 using potassium hydroxide. All chemicals used were purchased from Merck, Darmstadt, Germany. The flow rate was 1 ml/min and the column oven temperature was set to 30 °C. The fluorescence detector was set at an excitation wavelength of 515 nm and emission wavelength of 553 nm. The injection volume was 20 μl and run time was 10 min per analysis[29].

Sample preparation

The method described by Yoshioka et al. was used to determine the MDA level in SF[30]. Protein precipitation was performed via the addition of 2.5 ml of 20% trichloroacetic acid solution to 0.5 ml of SF. Subsequently, 1 ml of 0.67% thiobarbituric acid solution was added. After being kept in a boiling water (95 °C) bath for a period of 30 min, the mixture was rapidly cooled in an ice-cold water bath. Following the addition of 4 ml of *n*-butanol, the mixture was vortexed and centrifuged at 3000 rpm for 10 min. Finally, the supernatant was injected into the HPLC-FLD system.

Analysis of IL-6 and 8-OHdG

IL-6 and 8-OHdG levels in the SF were measured using commercial human enzyme-linked immunosorbent assay (ELISA) kits (Shanghai YL Biotech Co., Ltd, Shanghai, China) following the instructions provided by the manufacturer. Briefly, samples, standards, and ELISA solutions were added to the wells, which were pre-coated with human IL-6 or 8-OHdG monoclonal antibody, and then incubated at 37 °C for 60 min. After washing the plate five times, chromogen solutions A and B were added. The plate was then incubated at 37 °C for 10 min for colour development. Finally, stop solution was added to each well to stop the reaction. The absorbance of each well was measured within 10 min at a wavelength of 450 nm.

Statistical analysis

IBM SPSS Statistics version 21.0 (IBM Corp., Armonk, NY, USA) was used for the data analysis. The Shapiro–Wilk test was applied to evaluate the normality of the data distribution. The statistical analysis of differences between groups was done using the Mann–Whitney *U*-test (between two experimental groups that were not normally distributed) or independent-samples *t*-test (for normally distributed variables). A *P*-value of <0.05 was considered as significant.

Results

A total of 24 patients diagnosed with TMJ internal derangement were included in this study (age range 15–53 years; mean age 30.04 ±10.93 years). In group 1, seven patients were diagnosed with DD without reduction and five patients with DD with reduction and intermittent locking. In group 2, eight patients were diagnosed with DD without reduction and four patients with DD with reduction and intermittent locking. The demographic variables of the patients are listed in Table 1. The mean age and sex distribution of the participants did not differ significantly between the groups ($P > 0.05$).

All patients showed a significant symptomatic improvement after treatment. The average pain score decreased from 6.79 to 4.12 ($P = 0.000$) and MMO increased from 29.91 mm to 33.33 mm ($P = 0.004$). On general comparison, the difference in VAS and MMO scores between the baseline measurements and those obtained at 3 months after the arthrocentesis were statistically significant in both groups ($P < 0.05$), whereas the differences between the two groups were non-significant ($P > 0.05$) (Table 2).

Table 3 illustrates the SF levels of MDA, IL-6, and 8-OHdG in each group at baseline and at 3 months. The differences between the two-time points were non-significant for both groups ($P > 0.05$).

Table 1. Descriptive statistics for the study population.

Demographic variables	Group 1 (Stabilization splint) ($n = 12$)	Group 2 (No stabilization splint) ($n = 12$)	Total ($n = 24$)
Age (years)			
Mean ± SD	31.41 ± 11.46	28.66 ± 10.68	30.04 ± 10.93
Range	18–53	15–47	15–53
Sex			
Male	2	3	5
Female	10	9	19

SD, standard deviation.

Discussion

The purpose of this study was to determine the effect of splint therapy on the synovial fluid levels of MDA, IL-6, and 8-OHdG in patients with TMD. It was hypothesized that if splint insertion has an effect on decreasing occlusal stress, it may also decrease the markers of oxidative stress and inflammation in the SF. According to the study results, the two groups improved similarly after arthrocentesis, regardless of whether a splint was used or not. This was evident by evaluating MMO and VAS, and not by evaluating SF.

Masticatory muscle hyperactivity due to malocclusion, physical stresses, anxiety, and oral habits leads to increased stresses in the TMJ[3]. During clenching, as well as jaw movements, forces that are transported to the TMJ structures are compressive and tangential in nature and increase to high levels of magnitude[21,22,31]. In a study by Ettlin et al., intra-articular distances were evaluated before and after the insertion of occlusal splints in 20 human TMJs[25]. They reported that the occlusal splints increased the intra-articular distance in habitual closure, as well as during sliding movements. Occlusal splints are non-invasive and reversible treatment modalities if applied correctly. Although several studies have reported the therapeutic success of occlusal splints on clinical symptoms, it appears that no study has assessed their effects inside the joint cavity.

The aim of this study was to determine whether the use of occlusal splints reduces the markers of oxidative stress and inflammation in the joint cavity. The levels of oxidative stress indicators MDA and 8-OHdG and of the proinflammatory cytokine IL-6 were measured to evaluate free radicals and inflammation grade in the joint cavity. When the baseline and 3-month results were compared, no significant difference in MDA, IL-6, or 8-OHdG levels were found in patients who used splints after the arthrocentesis. Furthermore, regarding the clinical symptoms, no statistically significant differences in pain or MMO were found between the two groups. However, a significant decrease in

Table 2. Inter- and intra-group comparisons of the mean pain scores (VAS) and maximum mouth opening (MMO) at baseline and the 3-month follow-up; mean ± standard deviation (range) values.

Groups	Group 2 (No stabilization splint)	Group 1 (Stabilization splint)	*P*-value (Inter-group comparison)
VAS			
Baseline	6.75 ± 2.05 (2–9)	6.83 ± 1.52 (5–10)	0.226
3 months	4.16 ± 2.16 (0–8)	4.08 ± 2.84 (0–10)	0.936
P-value (compared to baseline)	0.005	0.003	
MMO			
Baseline	28.33 ± 7.17 (16–40)	31.5 ± 5.10 (24–40)	0.713
3 months	31.75 ± 7.5 (25–50)	34.91 ± 6.15 (25–43)	0.16
P-value (compared to baseline)	0.041	0.05	

Table 3. MDA, IL-6, and 8-OHdG levels in the synovial fluid at baseline and the 3-month follow-up in the two groups. No significant difference between the baseline and 3-month synovial fluid MDA, IL-6, and 8-OHdG levels were found in either group.

	Mean ± standard deviation		*P*-value
	Baseline	3 months	
MDA (μM)			
Group 1	0.68±0.54	0.55±0.42	0.433
Group 2	0.36±0.19	0.44±0.37	0.424
IL-6 (ng/ml)			
Group 1	55.65±14.60	62.78±14.07	0.336
Group 2	59.71±11.91	62.17±13.35	0.508
8-OHdG (ng/ml)			
Group 1	33.61±4.83	31.48±4.10	0.359
Group 2	30.53±6.72	32.20±10.04	0.754

MDA, malondialdehyde; IL-6, interleukin 6; 8-OHdG, 8-hydroxydeoxyguanosine.

pain and increase in MMO were observed in both groups when baseline and 3-month follow-up scores were compared. It is believed that this was the positive effect of arthrocentesis treatment, regardless of the use of occlusal splints.

There is increasing evidence that free radical-induced oxidative damage leads to DNA damage[32]. 8-OHdG is one of the reliable biomarkers of oxidative stress, and is formed through the oxidation of guanine from damaged DNA[33,34]. Increased levels of 8-OHdG in blood, urine, and saliva samples from patients with different metabolic and inflammatory disorders have been reported[35,36]. Hajizadeh et al. investigated 8-OHdG in the SF of patients with rheumatoid arthritis and found a significant correlation between mitochondrial DNA (mtDNA), 8-OHdG and rheumatoid factor[20]. Rodríguez de Sotillo et al. investigated the salivary and serum levels of 8-OHdG in TMD patients and found a significant association between TMJ pain and 8-OHdG levels in saliva[37]. The present study is novel in investigating the levels of 8-OHdG in the SF of TMD patients.

MDA is a biomarker of lipid peroxidation and has been found to be associated with oxidative stress in several studies[38]. Fleifel and Alkhiary reported increased levels of MDA and superoxide dismutase in TMD patients compared to healthy controls[39]. Demir et al. investigated the serum and saliva samples of TMD patients and found increased MDA levels when compared to the control group[40]. In the present study, MDA levels were evaluated as an indicator of oxidative stress due to the results of previous studies on TMD patients. However, no difference in MDA levels was found with the use of occlusal splints.

Studies have shown a direct relationship between inflammation and the increased production of free radicals in a tissue. Several cytokines including IL-1, IL-6, and tumour necrosis factor (TNF) have been shown to be present in the SF of patients with TMD[14–19]. Kaneyama et al. and Sato et al. found higher levels of IL-1β, TNF-α, IL-6, and IL-8 in symptomatic patients with DD with reduction, DD without reduction, and osteoarthritis than in asymptomatic patients[16,41]. IL-6 has also been shown to correlate with the degree of synovitis and symptom chronicity[42]. Gulen et al. showed that arthrocentesis was efficient for eliminating IL-1, IL-6, IL-8, IL-11, and TNF-α from the SF[43]. In their study, they obtained SF samples from patients with TMD before and 2 weeks after arthrocentesis and found a significant decrease in proinflammatory cytokines over the 2-week period[43]. In contrast to that study, the second arthrocentesis procedure in the present study was performed 3 months after the first procedure; no difference was found in either group, regardless of splint use.

A known major limitation of studies on free radicals is that individual factors (e.g., metabolic events, lifestyle factors such as smoking and alcohol consumption) and environmental factors (e.g., UV radiation) can affect the levels of biomarkers[44]. To eliminate this risk, the SF contents of the same patients were compared, at baseline and 3 months. Due to ethical limitations, this study did not include a control group consisting of patients without TMDs. It is not appropriate to obtain SF samples from healthy volunteers who have no TMJ symptoms, from an ethical point of view. Another limitation of the study is that the results are highly related with patient cooperation. The patients were strongly advised about the importance of wearing the splint 24 h per day except at mealtimes for the full 12 weeks. The patients were checked at 15-day intervals in the first month to control the adaptation of the splint. They were then controlled regularly at monthly intervals. Patients who mentioned that they could not use the splint properly were excluded from the study.

Arthrocentesis has been shown to be an effective treatment for alleviating pain and dysfunction and re-establishing MMO in TMD patients. The necessity of splint use after arthrocentesis is not clearly demonstrated in the literature[45]. In a recent meta-analysis, Nagori et al. reported that splint therapy may not improve outcomes after arthrocentesis[46]. In accordance with the literature, the present study found that the improvement in clinical symptoms after arthrocentesis was not associated with the use of a splint. Through the arthrocentesis technique, proteins and biochemical mediators causing the joint pathology are washed away and healthy

SF production is promoted[47]. In this study, in order to observe the effect of splint therapy on the SF content, arthrocentesis was performed after taking the first SF samples from the patients. In this way, the new production of inflammation and free radical mediators in the relatively healthy synovial fluid of patients at 3 months after the arthrocentesis was evaluated. However, 3 months after arthrocentesis, it was found that the levels of mediators of oxidative stress and inflammation were the same as at baseline. Although clinical symptoms were improved after arthrocentesis, this was not correlated with cytokine levels in the SF. It is believed that washing away the cytokines from the SF helps relieve the clinical symptoms in the early period. In the later period, new production of cytokines is observed in the SF. Thus, the improvement in clinical symptoms could be mainly attributed to the lysis effect of arthrocentesis rather than the washing away of the inflammatory mediators inside the joint cavity. From the results of the present study, it can be concluded that SF is not a good tool to evaluate the success of treatment modalities in TMD.

In conclusion, this study demonstrated that arthrocentesis has positive effects on patient clinical symptoms, regardless of postoperative splint use. Clinical success after arthrocentesis does not correlate with the markers of inflammation and oxidative stress in the SF. However, further studies with larger samples and longer follow-up periods are needed.

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Competing interests

None declared.

Ethical approval

The procedure of this study was approved by the Institutional Review Board of Ondokuz Mayıs University Research Ethics Committee.

Patient consent

Not required.

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