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Evidence of Oxidative Stress in Temporomandibular Disorders: A Pilot Study

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INTRODUCTION

Temporomandibular muscle and joint disorders (TMJD) are characterized by persistent jaw pain associated with dysfunction and tenderness of the temporomandibular muscles and joints (1). Biomarkers have been studied in patients with TMJD to elucidate the mechanisms of pain and provide a basis for early detection of pain and degeneration and a potential target for therapeutic agents to prevent progression to more severe pain and dysfunction (2–10). Biomarkers of oxidative stress (OS) have been found significant higher in serum of rats in IL-1 α induced TMJ arthritis by Kawai *and colleagues* (2000) (11). Oxidative stress (OS) is caused by release of free radicals (FR) in concentrations that overwhelm the natural scavenging mechanism of the antioxidant defense system and initiates processes involved in the pathogenesis of many inflammatory diseases (3–7).

In the TMJ, trauma, mechanical stress, disk derangements, and degenerative changes can cause the release of FR resulting in OS and an imbalance in resultant biomarkers (12). Such deficiencies in the antioxidant defense system will lead to increased concentrations of OS biomarkers, such as 8-hydroxydeoxyguanosine (8-OHdG), malondialdehyde (MDA) and total antioxidant status (TAS) in both serum and saliva (3,9). The association between these biomarkers and TMJ pain has not been studied in saliva and, thus, warrants further investigation on whether this oral fluid may have diagnostic potential (3,13,14).

Saliva is a protective fluid of the oral cavity and has been proposed as a potential diagnostic medium for many conditions (6,7,8). Salivary assays, unlike blood, are less intrusive, and can be self administered without special equipment or personnel, and expense. Comprised of both enzymatic and non-enzymatic antioxidants, saliva plays a critical role as part of the antioxidant system (7,8). Its non-invasive collection makes saliva the biological fluid of choice to determine levels of biomarkers associated with OS and may prove to be an excellent diagnostic strategy to detect early TMJ degenerative changes.

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This study collected specimens from the saliva and serum of patients with TMJD disorders and subjects without TMJD disorders to determine the validity of saliva in measuring the level of OS biomarkers (8-OHdG, MDA, and TAS) and elucidate their relationship to pain from TMJDs. This paper presents the results comparing salivary and serum concentration levels of these OS biomarkers in TMJD pain patients for two stages of pain intensity compared to the non-TMJD controls.

MATERIALS AND METHODS

Study Design

This aim of this pilot study was to determine whether biomarkers of oxidative stress as measured in saliva and serum in patients with TMJD could be associated with increasing pain compared to healthy controls. The null hypothesis is that there is no relationship between biomarkers of oxidative stress and the presence and severity of pain of TMJD. Thus, a case-control study design was used compare OS biomarkers in both saliva and serum from patients with high and low TMJD pain intensity compared to non-TMJ controls.

Study Population

All subjects in this study were consented and recruited from the National Institute of Dental Research's TMJ Implant Registry and Repository (NIDR's TIRR) (15). Their data and specimens were collected using privacy and security methods that were approved by the IRB and consistent with HIPAA and federal guidelines. Since there is a high prevalence of TMJD in female populations, we selected 20 female TMJD pain subjects and 10 female control non-TMJD subjects. Following clinical examination by expert clinicians in the TMJD clinic, University of Minnesota, TMJD subjects were diagnosed using the Research Diagnostic Criteria for TMD (RDC-TMD) (16). All TMJD subjects had both RDC type III TMJ Arthralgia without any history of TMJ surgery and Type I Myalgia. The TMJD pain group included 10 subjects with mild pain and 10 with moderate pain. All participants were non-smokers to further avoid the smoking-induced elevation in oxidative biomarkers.

Pain intensity

The pain intensity score (PI) was procured from a self-reported reliable questionnaire, the Symptom Severity Index (SSI) (17,18). The PI is an average of two SSI questions: "Mean worst pain at moment" and "Mean worst pain over the past 6 months". These pain ratings were derived from the SSI and range between 0 and 1. Patients were assigned to groups I, II or III, according to their PI score; group I (no pain): PI=0, group II (mild pain): 0.1–0.5, while group III PI>0.5 (moderate to severe pain).

Specimens' collection

Subjects were asked to restrain from using lipstick, chewing gum, eating or drinking any liquids except water for two hours before sampling saliva. They rinsed their mouth for 30 seconds with clean water and the water was discarded. Subjects were instructed not to try to generate saliva, talk, or think about foods. Unstimulated saliva was collected for 5 minutes into a graduated sterile tube kept on ice (4). Saliva aliquots, (1 ml), were stored at –80°C until analyzed. Venous blood (8.5 mg) was collected in BD Vacutainer® Plus Serum Tubes. Tubes were inverted 10 times and allowed to clot for 30 minutes. Serum was harvested after centrifugation at $1,200 \times g$ for 10 min. Serum aliquots, (1 ml), were stored at –80 °C until analyzed.

Salivary and serum analysis of 8-Hydroxydeoxyguanosine, Malondialdehyde and Total antioxidant status

Saliva and serum samples were pretreated by ultra-filtration thru 10,000 cut off molecular weight and centrifugation at $10,000 \times g$ and 8-OH-dG was determined using an ELISA assay kit as per manufacturer's protocol (Oxis International, Portland, OR). To increase the sensitivity of the 8-OHdG assays in saliva samples, the primary antibody reaction was incubated overnight at 4 °C and the secondary antibody reaction were incubated at room temperature for 1 hour. Absorbance values were measured ($\lambda=450$ nm) using a microplate reader, Multiskan-Spectrum-Instrument and Skanit Software 2.2 (Thermo Electron Corporation, Waltham, MA). A standard curve generated by plotting absorbance of standards vs. log of 8-OHdG concentrations was used to determine 8-OHdG concentrations.

Malondialdehyde was assayed using MDA-586 kit according to the manufacturer's protocol (Oxis International, Portland, OR). Absorbance was read ($\lambda=586$ nm) against a blank using a Beckman DU 640 spectrophotometer (Fullerton, CA).

Total antioxidant status was determined in saliva and serum samples using a TAS kit, as per manufacturer's protocol (Randox Laboratories, Oceanside, CA). Absorbances were read ($\lambda=600$ nm) using a (Beckman DU 640 spectrophotometer, Fullerton, CA). To aid in the interpretation of the results, the rOS were calculated dividing the concentrations of MDA or 8-OHdG by TAS (10).

Statistical Analysis

Descriptive statistics were performed to assess the mean, standard deviation (SD) and the percentage of patients in each study group. Since its distribution was skewed, 8-Hydroxydeoxyguanosine, in serum was natural log-transformed. Student's t-test, ANOVA (Proc-GLM) and chi-square were used to compare the continuous and categorical variables between study groups. Spearman rank correlation coefficient was used to assess the correlation between each biomarker and the correlation between same biomarker from saliva and serum.

By mean of multivariable linear modeling (SAS PROC GLM), regression model was used to assess the association of each biomarker with pain intensity. Crude and multivariable linear regression analyses (Proc-GLM) were performed to conduct a preliminary assessment of the degree of association of a specific biomarker (independent variables) and pain intensity (dependent variable). Crude and multi-variable ordinal logistic regression analyses (Proc-GENMOD) were performed to determine the independent association between each biomarker (independent variables) and study groups (dependent variable: TMJD pain groups: I, II, and III). ANOVA was performed to determine the difference between the ratios and TMJD pain groups I, II, and III. Analyses were performed with SAS (version 9.2, Cary, North Carolina, USA), and $p<0.02$ was considered significant.

RESULTS

Table 1 shows the demographic and pain characteristics between study groups. All patients had TMJD with both RDC-TMD type III arthralgia and type I myalgia. The mean age between 20 TMJD pain female subjects (40.50 ± 15.53 years) and 10 female controls (42.50 ± 11.95 years) was not significantly different ($P=0.72$). The mean pain intensity reported by all TMJD pain subjects averaged $0.55 (\pm 0.27)$ on a 0 to 1 scale. The mean pain intensity for the low pain group was 0.32 while for the high pain group, it was 0.78 and statistically different ($p \leq 0.0001$). The volume (ml) of saliva collected from TMJD patients (mean: 0.53 ± 0.19) and control subjects (mean: 0.59 ± 0.25) was not significantly different ($P=0.47$).

8-Hydroxydeoxyguanosine

Table 2 presents the 8-OHdG levels in saliva between the 2 pain groups and the healthy controls. The levels of salivary 8-OHdG in saliva from patients with TMJD pain (2.62 ± 0.39 ng/ml) were statistically higher than the controls (1.40 ± 0.43 ng/ml, $P < .0001$). This significant difference remained when comparing the mean serum levels of log 8-OHdG from serum (0.97 ± 0.70 ng/ml) with controls (0.04 ± 0.47 ng/ml $P = 0.0008$). Furthermore, the significant difference remained only when comparing levels from serum from TMJD subjects with severe pain and controls.

A multivariable linear regression analysis was used to assess the degree of association of 8-OHdG levels in saliva or serum and the average of pain intensity. This analysis shows that a change of 0.28 units of the average of pain intensity (0–1) (95%CI: 0.13–0.43, $P = 0.0006$) was associated with a change in one unit of 8-OHdG levels in saliva, independent of MDA and TAS and patient's age. In the crude analyses, a significant association was noted, 8-OHdG serum and pain intensity ($\beta = 0.32$ units, 95% CI: 0.20–0.44, $P < .0001$), but not in the multivariable model ($\beta = 0.10$, 95%CI: -0.04 – 0.25 , $P = 0.16$), when the model was adjusted for the same covariates.

Higher levels of 8-OHdG from saliva (OR: 2.68, 95%CI: 2.11–23.41, $P < .0001$) or log 8-OHdG from serum (OR: 2.22, 95%CI: 1.71–2.88, $P < .0001$) were both related to severity of TMJD pain. When these crude analyses were adjusted for MDA, TAS levels and age, the significant association with 8-OHdG from saliva remained (OR: 1.92, 95%CI: 1.49–2.47, $P < .0001$), contrary to log 8-OHdG in the serum (OR: 1.24, 95%CI: 0.94 – 1.65, $P = 0.13$). The change in the magnitude of crude to adjusted odds ratios is explained by the correlation between 8-OHdG and MDA (serum $r = 0.68$, 0.0002, saliva $r = 0.55$, 0.001).

Tables 3 and 4, show a significant association between 8-OHdG and TMJD pain severity. 8-OHdG levels in the saliva was associated with increased odds of both mild pain (group II, $P < .0001$) or moderate-severe pain (group III, $P = 0.0004$) compared to non-TMJD group. Log 8-OHdG in the serum, however, was only related to an increased odds of moderate-severe pain (group III, $P = 0.002$). A strong positive correlation was noted between 8-OHdG from saliva and serum ($r = 0.68$, $P < .0001$) indicating that 8-OHdG may be easily accessed from saliva.

Malondialdehyde (MDA)

Table 2 present the MDA levels between the 2 pain groups and the healthy controls. The levels of salivary MDA among TMJD subjects (mean: 1.42 ± 0.40 pg/ml) were significantly higher than the controls (mean: 0.92 ± 0.34 pg/ml, $P = 0.002$). This significant difference remained when comparing the serum MDA levels between TMJD subjects (mean: 1.02 ± 0.22 pg/ml) and controls (mean: 0.76 ± 0.19 pg/ml, $P = 0.004$). Furthermore, the significant difference remained only when comparing MDA levels from saliva or serum in TMJD subjects with severe pain and controls but not between TMJD subjects with mild pain and controls (Table 2).

A multivariable linear regression analysis shows that a change of one unit of MDA in saliva ($\beta = 0.25$, 95%CI: 0.04 to 0.47, $P = 0.02$) or serum ($\beta = 0.60$, 95%CI: 0.22 – 1.00, $P = 0.004$) was associated with an increase in the average of pain intensity, independent of other biomarkers and patient's age.

In the crude model, MDA levels in saliva (OR: 3.66, 95%CI: 2.26–5.91, $P < .0001$) and in serum (OR: 13.29, 95%CI: 5.81–30.42, $P < .0001$) were related to severity of TMJD pain. The magnitudes of these effects were stronger than those noted between 8-OHdG and TMJD pain. When these crude analyses were adjusted for 8-OHdG, TAS and age, the significant

MDA associations in saliva (OR: 1.81, 95%CI: 1.25–2.62, $P = 0.002$) or in serum (OR: 3.72, 95%CI: 1.74–7.95, $P = 0.0007$) remained, but were weaker.

Tables 3 and 4, show a significant association between MDA and TMJD level of pain severity. MDA levels in saliva (group III, $P = 0.005$) and serum (group III, $P = 0.004$) were related to severe TMJD pain when compared to the non-TMJD control group. Our study failed to demonstrate a significant association between MDA levels in saliva (group II, $P = 0.29$) or serum (group II, $P = 0.26$) and mild pain intensity. A moderate positive correlation was noted between MDA from saliva and serum ($r = 0.45$, $P = 0.01$).

Total antioxidant status (TAS)

Table 2 present the TAS levels between the 2 pain groups and the healthy control. TAS means levels in saliva (1.34 ± 0.77 , nmol/L) or in serum (0.87 ± 0.49 nmol/L) were statistically significantly different between patients with TMJD pain and controls (salivary, 2.34 ± 0.26 nmol/L, $P = 0.005$; serum, 1.60 ± 0.46 nmol/L, $P = 0.001$). These significant differences were independent of the TMJD pain group.

A decrease in the average of pain intensity was associated with a change in TAS in serum ($\beta = 0.25$, 95%CI: -0.44 to -0.05 , $P = 0.01$). No significant association was noted between a change in the average of pain intensity and TAS from saliva ($\beta = -0.03$, 95%CI: -0.44 to -0.05 , $P = 0.69$), when taking into account differences of other biomarkers and age between subjects. The loss of statistical association between pain intensity and TAS from serum was due to the MDA being moderately correlated with TAS ($r = 0.50$, $P = 0.005$).

TMJD pain was negatively associated with TAS in saliva (OR: 0.54, 95%CI: 0.51–0.85, $P < .0001$) and serum (OR: 0.34, 95%CI: 0.23–0.49, $P < .0001$). These negative associations related to TAS in saliva (OR: 0.81, 95%CI: 0.66–0.98, $P = 0.03$) and serum (OR: 0.47, 95%CI: 0.33–0.69, $P < .0001$) persisted when the model was adjusted for 8-OHdG, MDA and age. Moreover, we noted that moderate-severe pain group was associated with TAS in the saliva (group III, $P = 0.009$) and serum (group III, $P < .0001$). A significant relationship was also observed between weak TMJD pain and TAS from serum (group II, $P = 0.001$). Our study failed to demonstrate a significant association between TAS levels in saliva (group II, $P = 0.07$, Table 3) and non-severe pain intensity. A moderate correlation was also found between TAS from saliva and serum ($r = 0.50$, $P = 0.005$).

Oxidative stress index ratios (rOS)

The rOS in saliva or in serum were statistically significantly different between patients with TMJD pain and controls. These significant differences were independent of the TMJD pain group (Table 4).

DISCUSSION

This pilot study found that TMJD patients had significantly different salivary concentrations of biomarkers indicating oxidative stress when compared to control patients. Furthermore, these salivary biomarkers were a valid diagnostic predictor of pain severity and correlated with serum levels. Higher levels of both 8-OHdG and MDA were related to higher scores of pain intensity. Decreased concentrations of TAS, in saliva or serum samples, were associated with pain intensity. The decreased TAS found among subjects with TMJD pain may be indicative of total antioxidants imbalance due to mechanisms of pain. The significant differences of MDA/TAS and 8-OHdG/TAS ratios are also indicative that oxidative stress plays a role in pathophysiology of TMJD disorders.

Although nature of peripheral pathological and/or dysfunctional processes of temporomandibular joint or muscle pain is not fully understood, this study suggests that OS may play a role in the mechanism of both. Previous studies that report decreased concentrations of TAS, and increased concentrations in MDA and/or 8-OHdG in patients suffering from inflammation and/or acute pain (5,19–21). It is possible that the mechanical stress of both TMJ and masticatory muscles can generate free radicals through a number of mechanisms, triggering a cascade of reactions that can exacerbate tissue damage, inflammation, and pain (6). An accumulation of free radicals in the joint can occur from mechanical strain, internal derangements, and injury of the joint tissues that can overwhelm the local antioxidant defenses resulting in protective peripheral nociception and reactive degeneration (2,6,12).

Furthermore, hypoxia-reperfusion injury from repetitive strain in muscles can also generate free radicals, oxidative stress, and pain (3). Studies of chronic muscle pain suggest that localized progressive increase of oxidative metabolism, particularly in muscle fiber type I with depleted energy supply, increases metabolic oxidative by-products and results in peripheral nociception (22–26). In both joint and muscle conditions, agents that enhance endogenous scavenging of free radicals may be sufficient to prevent their accumulation and development of a pathological condition such as TMJD. This suggests that antioxidant scavenger agents may be a potential therapeutic intervention in preventing early TMJD from progressing if strain is reduced.

Despite the promising nature of the results of this study, the limits of this study suggest caution in interpreting the statistical analysis, results, and implications. This study is a pilot study with small sample size and limits the statistical analysis and suggests the need for future investigations with larger sample sizes to determine the diagnostic utility of OS biomarkers in saliva. Furthermore, the lack of determining whether OS biomarkers are correlated with joint or muscle pain is unclear. Further studies to determine diagnostic specificity for subgroups are also needed. In addition, the influence of periodontal disease on biomarkers levels need to be controlled to ensure it does not confound results (27). Although the diagnostic data collection was performed by trained TMJD examiners with standardize data and specimen collection procedures, the chances of misclassification of subjects or inaccurate measurement of OS biomarkers is possible. Furthermore, the change in concentration of OS biomarkers over time was not studied and the relationship to the temporal aspect of TMJ disorders progression needs to be studied.

In conclusion, this research demonstrates a significant association between TMJD pain and OS biomarkers in saliva and the null hypothesis was rejected. This supports the potential that OS biomarkers in saliva can be both a diagnostic strategy and a target for therapeutic agents to detect and prevent progression of early TMJ disorders into more severe pain and dysfunction. Despite the limited sample size of this pilot study, the results suggest that further larger studies should be completed.

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Table 1

Means and SD of age and pain intensity between TMJD pain subjects and healthy controls. All subjects were female.

	Controls	TMJD subjects		
	Group I n = 10	Low Pain Group II n = 10	High Pain Group III n = 10	<i>P-value</i>
Age, average (SD)	42.50 (11.95)	38.60 (16.30)	42.40 (15.36)	0.80
Pain intensity, average (SD)	-	0.32 (0.16)	0.78 (0.10)	.0001

Table 2
Means and SD of each biomarker levels between TMJD pain groups and controls

Biomarkers	Controls		TMJD subjects		
	Group I n = 10	Group II n = 10	Group III n = 10	P-value I vs II	P- value I vs III
Saliva Concentrations					
8-OHdG, Mean (SD) ¹	1.40 (0.43)	2.45 (0.24)	2.78 (0.40)	.0001	.0001
MDA, Mean (SD) ³	0.92 (0.34)	1.17 (0.39)	1.66 (0.23)	0.14	.0001
TAS, Mean (SD)	2.24 (0.26)	1.48 (0.91)	1.18 (0.62)	0.01	.0001
Serum Concentrations					
Log 8-OHdG, Mean (SD) ²	0.04 (0.47)	0.53 (0.74)	1.41 (0.26)	0.09	.0001
MDA ¹ , Mean (SD) ⁴	0.76 (0.19)	0.85 (0.08)	1.19 (0.16)	0.23	.0001
TAS, Mean (SD) ⁵	1.60 (0.46)	1.09 (0.39)	0.64 (0.31)	0.01	.0001

Note: differences between groups II and III:

¹ $P = 0.04$,

² 0.002,

³ $P = 0.003$,

⁴ $P < .0001$,

⁵ $P = 0.02$

Table 3

Models assessing the association between each saliva biomarker and TMJD pain among 30 subjects.

Saliva biomarkers	Group II/ Group I OR (95%CI)	Group III/ Group I OR (95%CI)
8-OHdG	1.76 (1.45– 2.14, $P < .0001$)	1.76 (1.29–2.39, $P = 0.004$)
MDA	1.18 (1.45–2.14, $P = 0.29$)	2.00 (1.24–3.23, $P = 0.005$)
TAS	0.87 (0.75–1.01, $P = 0.07$)	0.67 (0.50–0.91, $P = 0.009$)
Serum biomarkers	Group II/ Group I OR (95%CI)	Group III/ Group I OR (95%CI)
Log 8-OHdG	1.06 (0.76– 1.47, $P = 0.74$)	1.54 (1.17–2.01, $P = 0.002$)
MDA	1.94 (0.61–6.18, $P = 0.26$)	2.48 (1.33–4.61, $P = 0.004$)
TAS	0.52 (0.35–0.77, $P = 0.001$)	0.40 (0.28–0.57, $P < .0001$)

Table 4

Means and SD of each log biomarker ratio between TMJD pain groups and controls

Log biomarker ratio	Controls		TMJD subjects			P-value I vs III
	Group I n = 10	Group II n = 10	Group III n = 10	P-value I vs II		
Saliva concentration						
8-OHdG/TAS, Mean (SD)	-0.55 (0.36)	0.73 (0.85)	1.10 (1.00)	0.0003	.0001	
MDA/TAS, Mean (SD)	-1.01 (0.52)	-0.05 (0.85)	0.59 (0.97)	0.007	.0002	
Serum concentration						
8-OHdG/TAS, Mean (SD)	-0.40 (0.52)	0.53 (0.92)	1.98 (0.73)	0.01	.0001	
MDA/TAS, Mean (SD) ¹	-0.74 (0.32)	-0.17 (0.49)	0.73 (0.60)	0.007	.0001	

¹ Note: the difference between groups II and III are also significant at $P = 0.002$.

² Negative values indicate that 8-OHdG and MDA log ratios have negative ratios.