

Interpretation of Oral Involvement in Patients with Sjögren's Syndrome by Using Salivary Markers

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Key words

Sjögren's syndrome – salivary flow rate – salivary protein.

Abstract

Sjögren's syndrome (SS) is a chronic inflammatory disorder of the salivary and lacrimal glands that leads to functional impairment. Hypofunction of salivary glands is the main cause of oral pathological changes and is associated with alterations in the constituents of the saliva.

This study aimed to test the hypothesis that proteomic approaches markers (notably peptides) are involved in the oral manifestations observed in Sjögren's syndrome (SS).

A total number of 71 participants were admitted in the study; 21 of them have Sjögren's syndrome (Group I), 43 non- Sjögren's syndrome cases referred or attended the dental clinic seeking for certain management (Group II) and 7 apparent healthy subjects without dental problems (Group III). Stimulated saliva was collected from each patients and participants for biochemical analysis including assessment of salivary protein and peptides.

Salivary flow rate was significantly ($p < 0.01$) reduced in SS patients by 33.4% and 24.1% of corresponding Group II and Group III respectively. The salivary protein concentrations (total albumin and peptides) per milliliter saliva fluid were increased in SS patients. Saliva peptides (2.09 ± 1.01 mg/ml) level was significantly ($p < 0.05$) higher than Group II (1.708 ± 0.649 mg/ml) and Group III (1.339 ± 0.517 mg/ml).

Hypofunction of salivary glands in SS is associated with significant changes in the saliva constituents particularly the peptides.

Introduction

Sjögren syndrome (SS) is an autoimmune exocrinopathy or also known as Micksiciz disease, first described in the late nineteenth century. It occurs alone as primary SS (pSS), or against a background of connective tissue disease as secondary SS (sSS). Primary SS is characterized by a chronic autoimmune attack involving both lacrimal and salivary glands.

Secondary SS is marked by an autoimmune attack against the lacrimal and/or salivary glands concomitantly with another autoimmune disease, most often a connective tissue disease like rheumatoid arthritis, systemic lupus erythematosus or scleroderma. SS frequently is described as a disease of middle-aged and elderly women. It is encountered more often in women than in men (at a rate of 9:1)⁽¹⁾. In both forms of the disease, the salivary glands are a target organ, as evidenced by the reduction in salivary output and the lymphocytic infiltration of the salivary

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glands⁽²⁾. The main oral complaint of SS patients is xerostomia. It has been suggested that the symptom of dry mouth appears when the salivary flow rate decreases to about 50% of the original "normal" level⁽³⁾. The range of normal salivary flow rates for healthy individuals is remarkably wide and differs according to the measurement technique used. The salivary flow range of rest and stimulated whole saliva are 0.08-1.83 and 0.2-5.7 ml/min respectively⁽⁴⁾. The hyposalivation disturbed the buffer system of saliva, the physicochemical integrity of tooth enamel, and the constituents of the saliva. In SS, decrease in small antimicrobial proteins (Defensins, Cathelicidins) may alter the microflora of the mouth and predispose to the oral complications of SS⁽⁵⁾. Recently several authors demonstrated a series of new proteins related to oxidative injury as well as proteins induced in response to pro-inflammatory cytokines. Two classes of antimicrobial proteins and peptides that have received the most attention in recent years are the defensins and cathelicidins⁽⁵⁾. This study aimed to test the hypothesis that proteomic approaches markers (notably peptides) are involved in the oral manifestations observed in Sjögren's syndrome (SS).

Material and Methods

This study was done in the Department of Pharmacology, College of Medicine, Al-Mustansiriya University in cooperation with the Department of Oral Medicine, College of Dentistry, Al-Mustansiriya University in Baghdad, Iraq, from October 2010 to May 2011. This study was approved by the Scientific Committee in the institution and a verbal consent form was obtained from each participant enrolled in the study.

Selection of participants

A total number of 21 female patients with SS fulfill the American-European Consensus Sjögren's Syndrome Classification Criteria (Group I)⁽⁶⁾. Two patients with pSS and 19 patients with sSS (three of them with systemic lupus erythematosus, and 16 of them with rheumatoid arthritis) were admitted in the

study, they were allocated from Rheumatology clinic in Baghdad teaching hospital in Baghdad city. A corresponding 43 non-Sjögren's syndrome subjects (41 male and 2 female) (Group II) attended the dentistry clinics at the outpatient clinic of the College of Dentistry, Al-Mustansiriya University in Baghdad seeking for management of dental problems including dental caries, periodontitis, gingivitis were randomly allocated to enroll in the study. Another 7 healthy male volunteers without any dental health problems served as control group (Group III) were admitted in the study.

Oral assessment

Complete medical and oral health history was obtained from each participant and the epidemiological data related to the objectives of the study were obtained. Then the saliva was obtained from each participant for biochemical analysis (Appendix I).

Saliva collection

From each participant the whole stimulated saliva (using citric acid 2%) was collected for 10 minutes. Individuals were asked to stop eating or drinking two hours prior to saliva collection. Individuals rinsed their mouth with water. Samples were collected into 25-mL Falcon screw cap tubes and centrifuged (3000 rpm) for 10 min⁽⁷⁾. Supernatants were aliquoted into 1.5ml Eppendorff tube and frozen at (-20°C) until use.

Biochemical analysis

Salivary flow rate

The salivary flow rate (SFR) was estimated by dividing the total collected saliva volume (ml) by the collection time (min) that was measured during sample collection⁽⁸⁾. As in the following equation:

$$\text{SFR (ml/min)} = \frac{\text{Saliva sample volume (ml)}}{\text{collection time (min)}}$$

Determination of total saliva protein

The total saliva protein is based on the observation that the absorbance maximum for an acidic solution of Coomassie Brilliant Blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs

according to the Bradford method (1976)⁽⁹⁾.

Absorbance=0.0068 x conc. of albumin (μg) +0.505

Determination of peptide in saliva

A known value of saliva was centrifuged and the supernatant was obtained and then diluted with 0.9% sodium chloride (25 μl of saliva diluted with 3ml 0.9% NaCl). The absorbance of the diluted saliva was recorded at 205 nm using UV-visible spectrophotometer⁽¹⁰⁾. The concentration of the peptides (mg/ml) was calculated according to the following equation:

The concentration of peptide (mg/ml) = $[1/31] \times A_{205} \times [\text{Dilution factor (120)}]$

Statistical analysis

The results are expressed as number, percent, median, and whenever possible as mean \pm SD. The data were analyzed using unpaired two tailed student's "t" test and simple correlation test taking the $p \leq 0.05$ as the lowest limit of significance.

Results

The characteristics of participants admitted in the study were shown in table 1. All cases of SS were females and their ages ranged between 15 and 66 years with a mean of 44.52 years. Current smoking was reported in 60.5% of Group II and 4.8% in Group I. Family history of SS in first relatives of Group I was 42.9% and only two SS cases reported to use antihistaminic and β -adrenoceptor blocking agents. According to the American-European Consensus Sjögren's Syndrome Classification Criteria, 2 cases were previously diagnosed as (pSS) and 19 cases were diagnosed as (sSS) (Table 2). The duration of illness ranged between 1 month and 25 years with a median of 7 years. At the time of the study, sicca symptoms and signs (dry eye and dry mouth) were reported in all cases. All the patients were on medications that prescribed for the associated symptoms of autoimmune disorder. Methotrexate alone or in combination with prednisolone or chloroquine was the cornerstone treatment

(Table 2). Clinical and laboratory evidences of connective tissue diseases including rheumatoid arthritis and systemic lupus erythematosus were found in 18 and 3 patients of Group I respectively (Table 2). The results of stimulated whole saliva fluid were shown in Table 3. The salivary flow rate (0.285 ± 0.143) was significantly ($p < 0.01$) reduced in SS patients by 33.4% and 24.1% of corresponding Group II and Group III respectively (Table 3). The total saliva protein (0.777 ± 0.481), and total saliva peptide (2.09 ± 1.01) levels were significantly ($p < 0.05$) higher in SS patients than other participants in groups II and III as shown in table 3. In patients with SS, the salivary flow rate was significantly correlated with saliva peptides (Table 4). In non-Sjögren's syndrome (Group II) patients, the salivary flow rate was inversely correlated with saliva peptides whether the participants were smokers or not (Table 5 and Figures 1). The significant correlation ($p < 0.02$) was observed between salivary flow rate and saliva peptides in Group II-smokers.

Discussion

The results of this study clearly pointed out the following effects of hyposalivation; physiologically including low saliva flow rate and biochemically including changes in the constituents of the saliva. Therefore, the explanations of these findings are discussed from the following two lines:

1. Physiological determination
2. Biochemical alteration

First, the low saliva flow rate in SS is thoroughly investigated. It resulted from irreversible damage of salivary acini. Low un-stimulated salivary flow rate is well documented in both pSS and sSS. In this study significant low stimulated-saliva flow rate was reported. Salivary flow rate test is used as a diagnostic aid for SS diagnosis, and thus as a basis for inclusion within the subsidy net for dental care⁽¹¹⁾. Recently, hydroxychloroquine therapy improved the un-stimulated saliva flow rate in pSS patients but stimulated saliva flow rate values, objective and/or

subjective complaints did not change considerably⁽¹²⁾. As mentioned earlier that the acini are irreversibly destroyed, therefore it is expected to find a number of patients not responded to cholinomimetic agents like pilocarpine and this reflected to the clinical oral presentation of cases⁽¹³⁾. Second, total protein (in form of albumin) and peptides are determined in saliva of SS patients and their higher concentrations did not reach to the level of significance as compared with non-Sjögren's participants except the peptides concentration. It is important to mention here that these concentrations are expressed per milliliter rather than per total volume of saliva. If the total amount of protein is calculated per total volume of saliva, the results, therefore, decrease rather than increase in the protein concentration. Most authors referred to decrease rather than increase saliva protein in SS. The level of total protein in saliva is influenced by the duration of disease and the treatment. In one study, the level of IgG tended to be significantly high in early onset of disease compared with late onset or long standing duration of illness and the use of steroids or disease modifying antirheumatic drugs like methotrexate and hydroxychloroquine reduced the level of IgG⁽¹⁴⁾. The significant high level of peptides reported in this study may be attributed to the increased proteolytic enzyme activity which involved in degradation of saliva protein⁽¹⁵⁾. Proteomic studies of human saliva characterized four major salivary families of specific secretory proteins: proline-rich proteins (PRPs), statherins, cystatins and histatins that differ significantly from other host defence salivary proteins, as the former group has specific functions in the oral environment⁽¹⁶⁾. Therefore, one of the limitations of this study is determination of the specific peptide that responsible for significant high level. Recent technological advances have greatly increased the amount of information and the number of proteins that can be investigated in any given system and put into a scientific context simultaneously. These technologies, termed transcriptomics, proteomics,

metabolomics and other '-omics', were followed by the increase in systems-based thinking across different scientific disciplines⁽¹⁷⁾. In the last few years, a considerable interest has grown in the application of proteomic analysis in Sjögren's syndrome characterized by involvement of salivary gland function. This pathological condition influences the composition of the human saliva *in toto* and to investigate the effect of pilocarpine on the salivary peptide and protein profile in patients with primary Sjögren's syndrome. Saliva was analyzed using HPLC-ESI-MS (High performance liquid chromatography electrospray ionization tandem mass spectrometry). Before pilocarpine, approximately 60% of salivary proteins, in samples from primary Sjögren's syndrome patients, could not be identified or showed lower levels than those in healthy controls. However, 30-60 minutes after pilocarpine treatment, approximately one-third of the less represented proteins were found to be present in a similar percentage to that of primary patients and controls⁽¹⁸⁾. Almost all the proteins detectable, at the lower levels, in primary patients compared with controls, reached levels similar to those in controls at 30-60 minutes after pilocarpine. It is important to exclude the effect of nicotine in studying the correlation between the different determinants used in this study. Non-Sjögren's patients who are smokers show significant inverse correlation between saliva flow rate and saliva peptide. Therefore, the nicotine acts in this study as a co-founding factor in non-Sjögren's syndrome patients and not in Sjögren's patients. In conclusion, the significant high concentration of saliva peptides level indicates that proteins undergo catalytic process due to increase proteolytic enzymes activity in Sjögren's syndrome. Nicotine as confounding factor should be taken in consideration in any study. Further research is recommended to elucidate that Proteomics studies utilizing mass spectroscopy are useful for diagnosis and differential diagnosis of primary and secondary Sjögren's syndrome.

Appendix-I: Case Sheet for Sjögren's syndrome patients.

No.
 Name
 Age
 Sex
 Smoking previous passive active (current)
 Family history of Sjögren syndrome 1st 2nd

History of drugs intake:
 Antihistamines
 Antidepressants (tricyclic and atypical)
 Antipsychotics
 Anticholinergics
 Antiarrhythmics

Known case of Sjögren disease
 Duration
 Current drug therapy:

Presence of systemic lesions:
 Dry eye
 Dry mouth
 Purpura
 Evidence of other conditions: e.g. SLE, RA...

Salivary gland biopsy:

Serum autoantibodies:

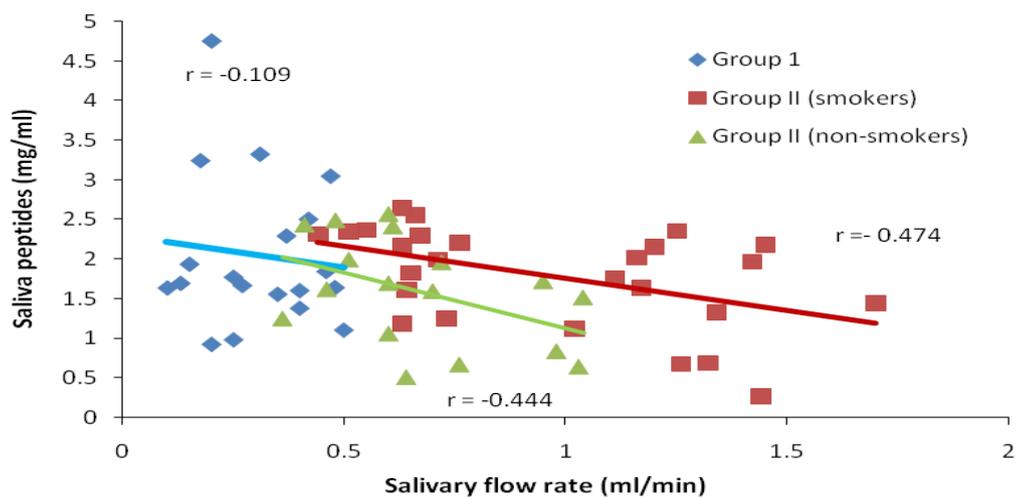


Fig.(1):- Correlation between salivary flow rate (ml/min) and saliva peptides (mg/ml).

Table (1):- The characteristics of the study.

	Group I (n=21)	Group II (n=43)	Group III (n=7)
Gender			
Male	0	40	7
Female	21	3	0
Age(year)	44.52±11.50	33.16±13.04	26.85±5.61
Smoking			
Previous	0	5	0
Current passive	0	0	3
Current active	1	26	4
Family history of Sjögren's disease			
first degree relatives	9	0	0
second degree relatives	3	0	0
both first and second relatives	1	0	0
History of drug intake			
Antihistamines	1	0	0
Beta-adrenoceptor blockers	1	0	0

The results are expressed as number and mean ± SD

Table (2):- The Characteristics of patients with Sjögren's disease.

Characteristics	Frequency
Type of Sjögren's syndrome	
Primary	2
Secondary	19
Duration of disease (year)	
Range	1 month-25 year
Median	7
Current drug therapy	
Methotrexate	7
Prednisolone	3
Methotrexate +Prednisolone	6
Chloroquine	1
Methotrexate +Chloroquine	2
Chloroquine +Salazopyrine	1
Pericilianine	1
Clinical presentation	
Dry eye	20
Dry mouth	21
Purpura	0
Evidence of other conditions:	
Rheumatoid arthritis	18
Systemic lupus erythematous	03

Table(3):- Assessment of different variables in stimulated whole saliva among there studying groups.

Variables	Group I (n=21)	Group II (n=43)	Group III (n=7)
Salivary flow rate(SFR) (ml/min)	0.285±0.143*†	0.848±0.343	1.182±0.483
Total protein(mg/ml)	0.777±0.481	0.468±0.389	0.295±0.226
Total peptide(mg/ml)	2.09±1.01††	1.708±0.649	1.339±0.517

The results are expressed as mean ± SD

*p < 0.001 compared with Group II,

†p < 0.01, †† p < 0.05 compared with Group III

Table(4):- Correlations between salivary flow rate and other variables related to the oral health in patients with Sjögren's syndrome.

Variables	Salivary flow rate (ml/min)
Saliva peptides (mg/ml)	-0.109

The results are expressed as correlation factor. $p < 0.05$

Table (5):- Correlations between salivary flow rate (ml/min) and other variables related to the oral health in Group II in respect to the current smoking habit.

Variables	Salivary flow rate (ml/min)	
	Smokers (n=26)	Non-smokers (n=17)
Saliva peptide (mg/ml)	-0.474*	-0.444

The results are expressed as correlation factor. * $p < 0.02$

References

- 1-Talal NM: Sjögren's syndrome: Historical overview and clinical spectrum of disease. *Rheum Dis Clin North Am*, 1992; 18:507-515.
- 2-Fox RI, Robinson CA, Curd JG, Kozin F, Howell FV. Sjögren's syndrome proposed criteria for classification. *Arthritis Rheum*, 1986; 29: 577-585.
- 3-Dawes C. Physiological factors affecting salivary flow rate, oral sugar clearance, and the sensation of dry mouth in man. *J Dent Res*, 1987; 66: 648-653.
- 4-Oxholm P, Asmussen K. Primary Sjögren's syndrome: the challenge for classification of disease manifestations. *J Intern Med*, 1996; 239: 467-474.
- 5-Ouellette AJ. Paneth cell alpha-defensin synthesis and function. *Curr Top Microbiol Immunol*, 2006; 306: 1-25.
- 6-Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE et al. European study group on classification criteria for Sjögren's syndrome. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. *Ann Rheum Dis*, 2002; 61(6):554-558.
- 7-Iijima K, Fyfe V, McColl KE. Studies of nitric oxide generation from salivary nitrite in human gastric juice. *Scand J Gastroenterol*, 2003; 38(3): 246-252.
- 8-Jensen SB, Mouridsen HT, Reibel J, Brüner N, Nauntofte B. Adjuvant chemotherapy in breast cancer patients induces temporary salivary gland hypofunction. *Oral Oncol*; 2008; 44(2): 162-173.
- 9-Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*, 1976; 72: 248-254.
- 10-Stoscheck CM. Quantitation of protein. In: John N. Abelson, Melvin I. Simon, Murray P. Deutscher (Editors). *Methods in enzymology*. Volume 182: Guide to protein purification. 1st ed. San Diego: Academic Press. 1990; pp 50-68.
- 11-Jorkjend L, Johansson A, Johansson AK, Bergenholtz A. Resting and stimulated whole salivary flow rates in Sjögren's syndrome patients over time: a diagnostic aid for subsidized dental care? *Acta Odontol Scand*, 2004; 62(5): 264-268.
- 12-Cankaya H, Alpöz E, Karabulut G, Güneri P, Boyacioglu H, Kabasakal Y. Effects of hydroxychloroquine on salivary flow rates and oral complaints of Sjögren patients: a prospective sample study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod*, 2010; 110(1): 62-67.
- 13-Jorkjend L, Bergenholtz A, Johansson AK, Johansson A. Effect of Pilocarpine on impaired salivary secretion in patients with Sjögren's syndrome. *Swed Dent J*, 2008; 32(2): 49-56.
- 14-Pijpe J, Kalk W, Bootsma H, Spijkervet F, Kallenberg C, Vissink A. Progression of salivary gland dysfunction in patients with Sjögren's syndrome. *Ann Rheum Dis*, 2007; 66(1): 107-112.
- 15-Patamia M, Messana I, Petruzzelli R, Vitali A, Inzitari R, Cabras T et al. Two proline-rich peptides from pig (*Sus scrofa*) salivary glands generated by pre-secretory pathway underlying the action of a proteinase cleaving Pro-Ala bonds. *Peptides*, 2005; 26:1550-1559.
- 16-Lamkin MS, Oppenheim FG. Structural features of salivary function. *Crit Rev Oral Biol Med*, 1993; 4:251-259.
- 17-van der Greef J, Martin S, Juhasz P, Adourian A, Plasterer T, Verheij ER et al. The art and practice of systems biology in medicine: mapping patterns of relationships. *J Proteome Res*, 2007; 6:1540-1559.
- 18-Scarano E, Fiorita A, Picciotti PM, Passali GC, Calò L, Cabras T et al. Proteomics of saliva: personal experience. *Acta Otorhinolaryngol Ital*, 2010; 30(3): 125-130.