Salivary Lysozyme and Glycosylated Lysozyme Levels of Type 2 Diabetic Patients in Comparison with Healthy Individuals

Jazaeri Jonghani, Mina
Assistant Professor of Department of Oral and Maxillofacial Medicine, School of Dentistry, Dental Research Center, Hamadan University of Medical Sciences , I.R. IRAN

Abdolsamadi, Hamidreza
Professor of Department of Oral and Maxillofacial Medicine, School of Dentistry, Dental Research Center, Hamadan University of Medical Science Hamadan , I.R. IRAN

Farmany, Abbas*[+]
Assistant Professor of Dental Research Center& Dental Implant Research Center, School of Dentistry, Hamadan University of Medical Sciences Hamadan, I.R. IRAN

Soltanian, Alireza
Professor of Department of Biostatistics, School of Health, Modeling of Noncommunicable Diseases Research Center, Health Sciences & Technology Research Institute, Hamadan University of Medical Sciences, Hamadan, I.R. IRAN

Borzouei, Shiva
Associate Professor of Department of Internal Medicine, School of Medicine, Shahid Beheshti Medical Educational Center, Hamadan University of Medical Sciences, Hamadan, I.R. IRAN

Samie, Lida
Assistant Professor of Department of Oral and Maxillofacial Medicine, School of Dentistry, Hamadan University of Medical Sciences, Hamadan, I.R. IRAN

Najar Karimi, Fereshteh*[+]
Assistant Professor of Department of Oral and Maxillofacial Medicine, School of Dentistry, Dental Research Center, Guilan University of Medical Sciences, Guilan, I.R. IRAN

ABSTRACT: *Diabetes is a complex metabolic disorder that can cause changes in the composition and function of saliva. Therefore, it seems that the study of saliva composition in patients with diabetes will help in its diagnosis, prognosis, and complications. The present study aimed to compare the saliva's lysozyme and glycosylated lysozyme levels of patients with type 2 diabetes (T2D) in comparison with healthy individuals.*

**To whom correspondence should be addressed.*
+ E-mail: a.farmany@ut.ac.ir , mastane_fk91@yahoo.com
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Lysozyme and glycosylated lysozyme levels of salivary samples were measured using ELISA method. The results of this study showed that salivary lysozyme levels were lower in T2D patients. Meanwhile, the salivary glycosylated lysozyme levels were higher in T2D patients compared to control ($p \leq 0.001$). Salivary lysozyme levels in patients with long-term diabetes (more than three years) were significantly lower than those of T2D patients having the disease for three years. Also, salivary glycosylated lysozyme levels were significantly higher in patients with long-term diabetes (more than three years) than in patients with short-term diabetes (less than three years). In conclusion, the results of this study demonstrated that the salivary lysozyme level in patients with T2D was lower than in healthy individuals. Also, the salivary glycosylated lysozyme level of the T2D patients was higher than healthy individuals. Increased duration of T2D affliction also appears to be associated with increased salivary glycosylated lysozyme levels. Moreover, the increase of salivary glycosylated lysozyme was greater in patients with T2D than in healthy individuals. These findings indicate the important role of salivary glycosylated lysozymes in diagnosing and predicting the complications of diabetic patients.

KEYWORDS: *Lysozyme; Glycosylated lysozyme; Saliva; Type 2 diabetes*.

INTRODUCTION

Diabetes is one of the most common endocrine and non-contagious diseases [1], affecting about 2-3% of the world's population [2]. Hyperglycemia, urinary glucose excretion, and decreased insulin secretion from pancreatic beta cells are the main characteristics of this disease [3]. Despite the fact that there are currently various oral and injectable medicines for the treatment of T2D, there is no definitive treatment for this disease and most of the medicines or treatment methods are only used to reduce the progression of Diabetes complications [4, 5].

Early screening for Type 2 Diabetes (T2D) is essential to improve the prognosis and delay diabetes-related clinical complications. In addition, it has been suggested as a critical strategy to reduce the incidence of this disease worldwide [6, 7]. Blood tests are currently the most common method for screening of T2D patients. Blood tests using needle finger pricks and blood draw are commonly applied as standard screening tests to diagnose and treat diabetes. However, these aggressive and unpleasant techniques disrupt daily life and lead to the anxiety of the patients. Furthermore, it still remains as a challenge to perform these tests in the long run due to scarring on the fingers, poor peripheral blood circulation, and increased risk of infection. On the other hand, many salivary compounds, like enzymes, can be reliably considered in patients' saliva for diagnosis and prognosis of the disease. Patient saliva sampling can overcome the problems related to the diabetes screening methods due to its non-invasive and painless nature (7). Recent studies have focused on developing saliva-based trials for screening or monitoring systemic diseases such as T2D [8, 9].

Lysozyme, as a non-immunological and antimicrobial agent, is one of the essential proteins in saliva that works as part of the immune system [10, 11]. Salivary lysozyme is secreted from neutrophils in the oral cavity in response to hyperglycemia and oral infections [12]. It exerts its antimicrobial activity by hydrolyzing and destroying the cell wall of bacteria. Lysozyme has an inhibitory effect not only on bacteria but also on *Candida Albicans* in the mouth, which indicates its antimicrobial effect on the tooth surface by binding to enamel hydroxyapatite [10, 13]. In diabetic patients, glycation of proteins and the formation of Advanced Glycated End Products (AGEPs) leads to changes in the function and structure of natural proteins due to hyperglycemia. Therefore, it plays a vital role in increasing diabetes complications such as retinopathy, nephropathy, neuropathy, and cardiomyopathy [14]. Lysozyme contains a chain with a high affinity for AGEPs and plays an essential role in their elimination in patients with diabetes [12]. It was reported that the increased affinity of lysozyme to bind to AGEPs can lead to the formation of glycosylated lysozyme in saliva and subsequently reducing the protective effects of lysozyme in diabetic hamsters [15].

Panchbhai et al. showed that the amount of this enzyme does not change significantly between the diabetic and control groups [16]. In contrast, some other studies have mentioned the decrease in salivary lysozyme levels and changes in its function that may be one of the causes

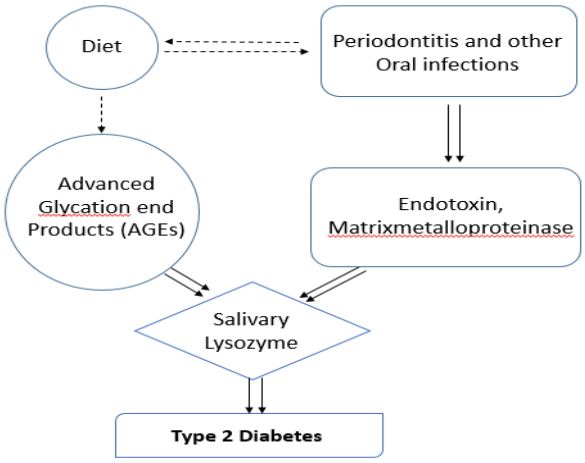

Fig. 1: A conceptual model for the association of salivary lysozyme with T2D

of the impaired salivary innate immune system and increased incidence of oral disease and infections in diabetic patients [17, 18]. Moreover, increased stress-oxidative reactions in T2D and the binding of nanoparticles such as ZnO to salivary proteins, lead to structural changes and dysfunction of serum and salivary proteins [19].

Considering the different results of past studies on salivary lysozyme levels in diabetic patients and the lack of previous studies on glycosylated lysozyme levels in human saliva, determining the amount of these proteins in saliva seems to be effective in screening T2D, considering the possibility of lysozyme depletion and glycosylated lysozyme formation in T2D [20]. Therefore, this study aimed to determine the amount of lysozyme and glycosylated lysozyme of saliva in patients with T2D.

EXPERIMENTAL SECTION

Ethical considerations of participants

Informed written consent was taken from all participants in the study after providing sufficient explanation regarding the cause and process of the study. This consent assured the volunteers that their information was confidential only for research purposes. Furthermore, volunteers in this study could end their participation in the study at any time. Therefore, the ethics committee has approved this research of Hamadan University of Medical Sciences with the ethics code 1398.38 IR.UMSHA.REC.

Sample size collection

According to the previous study (Kumar, 2014)[21], the standard deviation of lysozyme in two healthy and diabetic groups is considered equal to 1.32 and 3.01, respectively. In this study, the non-response rate is considered as 20%. Therefore, at the confidence level of 95% and the power of the test is 90%, at least 77 samples of diabetic persons and 77 samples of healthy persons are needed (154 people in total). So, we collect the 82 samples in each group.

$$n=\frac{\left(Z_{1-\frac{\alpha}{2}}+Z_{1-\frac{\beta}{2}}\right)^2.(\sigma_1^2+\sigma_2^2)}{d^2}+(\text{non Response rate})=77$$

$Z_{1-\frac{\alpha}{2}}=1.96$ و $Z_{1-\frac{\beta}{2}}=1.28$ و $\sigma_2=3.01$ و $\sigma_1=$ [†]1.32 و $d=1.3$ و $non\ response\ rate=20\ \%$

Study population and related factors

This study included 82 T2D patients referred to the Hamadan Diabetes Center and 82 healthy people referred to the Oral Diseases Department of Hamadan Dental School for dental examinations.

Inclusion criteria

Participants in the case group were diagnosed as diabetic patients based on OGTT ≥200, FBS ≥126, plasma Random Glucose ≥ 200, at least two times, and the presence of clinical signs of polyuria, polydipsia, and polyphagia. The control group had FBS of 70 to 100 and no clinical signs of diabetes.

Exclusion criteria included

Immunodeficiency, AIDS, dry mouth, Sjögren syndrome, history of salivary gland surgery, head and neck radiotherapy and chemotherapy in the last month, and pregnancy. Also, the diabetic patients were matched with the healthy individuals in the control group in terms of age and sex.

Demographic information, duration of diabetes, history of smoking, blood pressure, blood lipids, FBS of diabetic patients on the day of saliva preparation, family history of diabetes, type of hypoglycemic drug, periodontal disease, dry mouth, and common complications of diabetes,

[†] Non-response rate: any samples in study that has failure to obtain information from a designated individual for any reason (death, absence or refusal to reply.

Table 1: Mean and standard deviation of age(y), weight (kg), height (cm) and BMI (kg/cm^2) of diabetic and non-diabetic individuals

Experimental groups / Value (Mean±SD)	Diabetic status		P value*
	Non diabetic	Diabetic	
Age	60.55±9.54	60.65±9.49	0.95
Weight	72.15±13.93	74.34±13.84	0.31
Height`	165.15±90	164.84±8.70	0.83
BMI	26.37±4.30	27.24±4.58	0.21

**Significance level is considered less than 0.05*

Table 2: Comparison between incidence of complications of T2D in diabetic patients and control group

Variable* % within Diabetic. Status (count/total)		Diabetic status		P value
		Diabetic	Non diabetic	
Periodontal Disease		29.3% (24/41)**	20.7% (17/41)	0.21
Kidney Disease		1.2% (1/2)	1.2% (1/2)	0.75
Hypertension		42.7% (35/69)	41.5% (34/69)	0.87
Smoking	past	13.4% (11/21)	12.2% (10/21)	0.82
	now	6.1% (5/7)	2.4% (2/7)	0.025
Heart Disease (except hypertension)		22.0% (18/22)	4.9% (4/22)	0.001
Eye disease		0	15.9% (13/13)	P<0.001
Hyperlipidemia		24.4% (20/21)	1.2% (1/21)	P<0.001
Xerostomia		69.5% (57/82)	30.5% (25/82)	P<0.001
Positive family history		54.9% (45/64)	23.2% (19/64)	P<0.001

**In the above table, only the positive values of each of the complications mentioned above are given as the total positive values (total number of people with the mentioned complication in all 164 participants).*

*** % within Diabetic. Status (count/total)*

including heart, kidney and eye infections and Body Mass Index (BMI) of participants [22] were recorded in questionnaires. Furthermore, this information was attached to the patients' informed consent form, and then was thoroughly reviewed and recorded along with the medical records of the diabetic patients.

Saliva collection method

Saliva samples of the present study were collected at 8-10 a.m. People in both groups were instructed to abstain from eating, drinking, and smoking for 1 h before collecting their saliva. Complete non-stimulating saliva was compiled by the spitting method [22]. Each patient emptied his saliva into a sterile 5 mL sterile microtubule every 2 sec for 2-5 min after initial swallowing [23]. They were in a sitting posture on a chair in a quiet environment with their head bent forward. Next, all micro-tubes were placed in ice and frozen at -20 °C to determine the amount of and Glycosylated lysozyme in the saliva samples.

Lysozyme and glycosylated lysozyme analysis

Human lysozyme ELISA Kit 96-Strip-Wells and Human lysozyme Hangzhou Eastbiopharm China (Glycosyled) ELISA Kit 96-Strip-Wells were used for measuring salivary glycosylated lysozyme and lysozyme via the ELISA method.

Statistical analysis

All data were entered into the computer under the SPSS software (version 18). Data with normal distribution were analyzed using a t-test, and data with non-normal distribution were performed using the chi-square test ($P \leq 0.05$).

Table 3: Comparison between glycosylated and non-glycosylated lysozyme levels in patients with diabetes and healthy subjects in control group

Diabetic. status	Non-Diabetic (Mean ± SD)	Diabetic (Mean ± SD)	P- value
Lysozyme	16.68± 3.60	8.32± 3.63	<0.001
Glycosylated lysozyme	0.054± 0.15	2.45± 1.67	<0.001

Table 4: Glycosylated and non-glycosylated lysozyme levels in diabetic and non-diabetic subjects by presence or absence of hypertension, heart disease, periodontal disease, and dry mouth

0.26		0.52		0.71		0.44		P value	Glycosylated lysozyme	diabetic
2.57± 1.81	2.07± 1.06	2.37± 1.58	2.63± 1.89	2.58± 2.41	2.41± 1.42	2.57± 2.08	2.29± 0.9	Mean±SD		
0.47		0.05		0.79		0.56		P value	lysozyme	
8.49 ± 3.80	7.81± 3.10	7.80 ± 3.17	9.6 ± 4.39	8.38± 3.81	8.11± 2.10	8.53± 4.10	8.04± 2.93	Mean±SD		
0.753		0.982		0.515		0.551		P value	Glycosylated lysozyme*	Non diabetic
0.06 ± 0.16	0.05± 0.10	0. 05± 0.16	0.05± 0.09	0.05± 0.15	0.10± 0.14	0.04± 0.17	0.17± 0.11	Mean±SD		
0.29		0.47		0.001		0.02		P value	Lysozyme*	
16.97± 3.75	16.05± 3.27	16.11± 3.23	16.83± 3.71	17.01± 3.40	11.18± 3.21	17.43± 3.46	15.61± 3.59	Mean±SD		
No	Yes	No	Yes	No	Yes	No	Yes	Complication status		
Xerostomia		Periodontal disease		Heart disease		Hypertension				

** Lysozyme and glycosylated lysozyme levels are given in ng/ml*

** Values of P <0.05 are considered significant*

RESULTS AND DISCUSSION

Results

In this study, 164 people participated in two groups of T2D and healthy individuals (82 T2D patients and 82 healthy people). The diabetic patients included 39 men and 43 women (82 subjects), and the control group had 40 men and 42 women (82 subjects). The chi-square test results showed that the sex distribution of the two experimental groups was not statistically significant (P = 0.876). The mean and standard deviation of age (y), weight (kg), height (cm), and BMI (kg/cm^2) of the two experimental groups are given in Table 1. The chi-square test results showed no statistically significant difference between the two groups of diabetes and control in terms of mentioned variables (P> 0.05).

Table 2 shows the incidence of complications of T2D, including periodontal, renal, hypertension, cardiovascular diseases (excluding hypertension), eye diseases, dry mouth, smoking history, and positive family history of T2D in the two experimental groups. According to the results of the present study, there was no significant difference between the diabetic and control groups

(P=0.0.001), except for the rate of cardiovascular diseases (except hypertension), the incidence of dry mouth (P≤0.001), and a positive family history of T2D (P≤0.001).

The mean and standard deviation of lysozyme and glycosylated lysozyme values of experimental groups are shown in Table 3. Based on the results of the independent t-test, salivary lysozyme levels in people with T2D were significantly lower compared to those of healthy people (P≤0.001). Also, the amount of salivary lysozyme in people with T2D (7.76±3.62) for more than three years was significantly lower than those who had this disease for less than three years (10.31±2.97) (P=0.01). The amount of salivary lysozyme in patients with T2D receiving injectable insulin was 7.10 ± 3.03.

The results of data analysis showed that salivary lysozyme levels in patients treated with the above treatments were not significantly different from each other (P=0.08).

The mean level of salivary glycosylated lysozyme in patients with T2D was significantly higher than that in the control group (P<0.001) (Table 3). The amount of salivary glycosylated lysozyme in people who had the disease for a more extended period (more than three years) was (2.64±1.72). This statistically significant (P=0.04) value was 1.72±1.27 in those who had diabetes for less than three years.

Salivary glycosylated lysozyme (mean ± standard deviation) in diabetic patients treated with insulin, oral anti diabetic drugs, and combination (insulin with oral hypoglycemic medicines) was 3.19±2.65, 2.22±1.42, and

2.54±1.45, respectively. However, there was no significant difference between them based on data analysis (P=0.32).

The amounts of lysozyme and salivary glycosylated lysozyme in diabetics and the control group by the complications of diabetes are listed in Table 4. The amount of salivary lysozyme in the control group with hypertension (15.61±3.59) was significantly lower than that of the control group (17.43±3.46) with normal blood pressure (P=0.024). Also, the amount of salivary lysozyme in non-diabetic patients with cardiovascular diseases (11.18±3.21) (except hypertension) was markedly lower than the amount of salivary lysozyme in non-diabetic patients without cardiovascular disease which was 17.01±3.40 (P=0.001).

Discussion

To date, various studies have focused on saliva composition in patients with diabetes, depending on the type of saliva (excitatory and non-excitatory), different types of diabetes, and other factors, which different results have been reported [24-28]. In this study, whole non-excitatory saliva was used as the test sample, which is more challenging to collect in the laboratory for analysis than the excitatory saliva [29]. Therefore, the saliva samples should be collected strictly under constant conditions and at regular intervals to be valid for diagnostic tests.

In the present study, the control and study groups (i.e., T2D) were matched according to age and sex [13, 30-32]. According to some previous studies, age and sex were mentioned as two critical variables affecting saliva composition. In general, in the present study, interfering factors such as age, sex, and smoking have the same distribution between control and diabetic groups [13]. Besides, obesity is a significant risk factor for T2D [22]. There was no statistically significant difference between the mean BMI of the control group (26.3 kg/m^2) and the comparison group (27.2 kg/m^2) in the study population.

Inflammation and periodontal disease also affect the amount of salivary lysozyme [23]. Nevertheless, in the present study, the statistical difference of this factor was not significant in the two groups. In comparison, the rate of periodontal problems was slightly higher in the diabetic group.

According to the results, salivary lysozyme levels in patients with T2D were lower than in the control group. However, salivary glycosylated lysozyme levels in patients with T2D were higher than in healthy individuals. This difference is due to the increased tendency of lysozyme to bind to AGEPs and the formation of advanced glycation products (due to hyperglycemia in diabetes). According to the present study, as the value of salivary lysozyme decreases and the amount of glycosylated lysozyme in saliva increase the protective effect of lysozyme in saliva declines. On the other hand, the binding of lysozyme to AGEPs leads to changes in the structure and function of lysozyme, which can be a reason for the disruption of the innate and acquired immune system of saliva and functional changes in the salivary glands of people with diabetes. It is also a factor in increasing the susceptibility of these patients to various infections and periodontal diseases. These findings are consistent with the results of previous studies.

For instance, *Mirmiranpour et al.* (2016) reported a reduction in salivary lysozyme levels and a change in its structure in patients with T2D [11]. Moreover, *Muratsu et al.* also observed a 56% decrease in salivary lysozyme activity of hamsters with T2D compared to the control group [15].

In contrast to the results of this research, two studies (i.e., Reuterving and Rathnayake) reported non-significant differences in salivary lysozyme levels in T2D patients and the control group. However, it is noteworthy that in the study of *Rathnayake et al.* saliva samples were collected from stimulated saliva by chewing 0.5 mg of paraffin for 5 min (unlike the present research, in which non-stimulated saliva samples were used). Moreover, in the mentioned study no attention has been paid to details of collecting saliva samples such as the posture of the participants (standing or sitting) or sampling at certain times of the day [33]. In the Reuterving study, parotid stimulatory saliva flow was used for the analysis [34].

The results of *Dodds et al.* (2000) study were inconsistent with the findings of the present study. The amount of salivary lysozyme in people with T2D was 26% higher than in the control group in their research [3]. In this study, the amount of lysozyme was measured using the double sandwich ELISA 2 method. Furthermore, the samples of salivary stimulation of parotid and submandibular glands were used; thus, it was different from the sampling method in the present study.

Moreover, in the present study, a significant difference was observed between salivary lysozyme levels in diabetic patients with hypertension and diabetic patients without

hypertension. (P=0.024). Hence, lysozyme levels were lower in diabetic patients with hypertension, but the difference between glycosylated lysozyme levels in the two groups was not statistically significant. This result was not consistent with of the findings of *Qovarnstorm et al.* study, since the amount of salivary lysozyme in people with hypertension was higher than in healthy people [12]. Lysozyme levels were lower in diabetic patients with hypertension, but it was no significant difference between glycosylated lysozyme levels in the two groups. This finding was not consistent with the results of *Qovarnstorm et al.* study. According to the results of the mentioned study, the amount of salivary lysozyme in people with hypertension was higher than that in healthy people [12].

Based on our findings, salivary lysozyme level was associated with the duration of T2D. The salivary lysozyme level of people with T2D with a more extended period (more than three years) was significantly less than that in people with a shorter duration (3 years or less). On the other hand, the amount of salivary glycosylated lysozyme in people with T2D with a longer period (more than three years) was significantly higher than in patients with a shorter duration (3 years or less).

Increasing the duration of T2D raises HbA1c levels, increases insulin resistance [35], and deteriorates diabetic complications such as increased cardiovascular disease and kidney disease in diabetic patients. Therefore, the role of glycosylated lysozyme in the pathogenesis of diabetes complications and the relationship between increasing the amount of glycosylated lysozyme and decreasing salivary lysozyme with the duration of diabetes can be a kind of predictor of diabetic complications. However, further studies are required in this area.

The present study showed a lack of any significant difference between the levels of lysozyme and salivary glycosylated lysozyme in T2D patients treated with various therapies, including insulin therapy, receiving hypoglycemic drugs, or a combination of the both methods. However, the amount of lysozyme in diabetic patients with insulin use was lower than in diabetic patients receiving concomitant insulin and oral antidiabetic drugs. Also, it was lower than in diabetic patients receiving oral antidiabetic drugs alone. On the other hand, the amount of glycosylated lysozyme in patients taking insulin was higher compared to that of patients taking concomitant insulin and oral medications. In addition, it was higher compared to that of patients taking oral drugs alone. In *Ben-Aryeh et al*., study, the amount of salivary lysozyme in people with non-insulin T2D was higher than that of people with T2D receiving insulin [36].

According to our findings, the prevalence of cardiovascular disease, eye diseases, dry mouth, and family history of diabetes in people with T2D was significantly higher than that of the controls. About 57% of the studied diabetic subjects suffered from dry mouth. These results are consistent with the findings of previous studies, including those of *Carramolino et al*. [23, 37, 38]. Moreover, previous studies have suggested the presence of a link between family history and the incidence of T2D that may alter the sensitivity of insulin receptors, IGFBP-1 reduction, insulin dysfunction, and β cells [39-41].

CONCLUSIONS

According to the findings of the present study, the increase in salivary glycosylated lysozyme was higher in people with T2D than in healthy individuals compared to the decrease in salivary lysozyme. Also, the duration of diabetes was associated with a decrease in salivary lysozyme levels and an increase in salivary glycosylated lysozyme levels. This finding show that there is a relationship between lysozyme and glycosylated lysozyme with T2D and the measurement of these salivary factors can be useful for the prediction and diagnosis of complications in diabetic patients, however, more studies are needed to use these results.

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