

Correlation between Saliva Glycated and Blood Glycated Proteins

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Abstract

Objectives: Blood and saliva samples were obtained to examine if there is a correlation between saliva glycated protein and blood glycated protein.

Methods: Blood and saliva samples of 51 male workers were collected. The fructosamine and hydrazine methods were used to measure saliva glycated protein. HbA1c, fructosamine and blood glucose were measured as indices of blood glycated protein, and the correlation between blood glycated protein and saliva glycated protein was examined.

Results: Saliva fructosamine glycated protein showed a significant correlation with HbA1c and blood glucose ($r=0.449$; $p=0.001$ and $r=0.445$; $p=0.001$, respectively). No correlation was identified between saliva hydrazine glycated protein and the index of blood glycated protein.

Conclusions: Blood glycated protein and blood glucose could be estimated by measuring saliva glycated protein.

Key words: saliva, glycated protein, fructosamine, HbA1c, diabetes

Introduction

Glucose has an aldehyde group with strong reduction at its end, and this group can combine non-enzymatically with various kinds of protein (1). It was reported to combine with *in vivo* proteins and produce such glycated proteins as HbA1c (2), fructosamine (3) and glycoalbumin (4). HbA1c can be an index of average blood glucose levels up to 3 months, while fructosamine can show average levels up to 2 weeks. They are used as blood glucose control indices that are not influenced by diet. In addition, glycation of proteins in hair (5, 6), nail (7) and keratin (8) is also used as a control index of blood glucose.

Saliva contains abundant proteins secreted from salivary glands and from blood. If the glycation of saliva proteins is linked with glycated proteins in blood and blood glucose, it can be used to detect diabetes at an early stage. Since the collection of saliva samples is simple and painless, it is also useful for controlling blood glucose level for diabetics whose numbers are rapidly increasing.

Conventionally, proteins from saliva are regarded as having

a very quick turnover, and there is little research on saliva glycated proteins. However, Li (9) reported that a part of lactoferrin or lysozyme combines with advanced glycation end products (AGE) and antibacterial activity falls after combining with AGE. Dodds et al. (10) measured various proteins in saliva in healthy subjects and in people with Type II diabetics and found that in the diabetics the antibacterial activity of saliva is decreased despite an increase in lactoferrin. Belce et al. (11) also reported that the superoxide dismutase activity of saliva declines significantly in Type I diabetics compared with healthy persons. These results suggest that the saliva protein loses physiological activity following glycation, and that saliva glycated protein exists.

In this study, we applied two colorimetric methods to measure glycated proteins, the fructosamine method and the hydrazine method. The hydrazine method was reported by Kobayashi et al. (12), and the method's sensitivity differs from the former. Glycated protein in saliva as measured by the two methods was compared with HbA1c, plasma fructosamine and fasting plasma glucose in order to look for correlations. The fructosamine method utilizes glycated protein's reducing capacity in a strong alkaline solution and measures the color development reaction with nitroblue tetrazorium as a substrate. The hydrazine method utilizes glucosone released from glycated protein by adding hydrazine and heating for 30 min. at 100°C, then adding phenylhydrazine to the glucosone and measuring the derivatization reaction. The hydrazine method shows higher sensitivity than the fructosamine method.

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Materials and Methods

Subjects

The subjects of the investigation were about 500 male employees of a metalworking company based in Osaka Prefecture. They were divided into 3 groups based on blood glucose levels taken in a medical checkup the previous year: the normal group whose blood glucose was 109 mg/dl or lower, the impaired glucose tolerance (IGT) group whose glucose was between 110 and 125 mg/dl, and the diabetes group whose glucose was 126 mg/dl or higher. In order to examine the correlation between blood glycated and saliva glycated proteins in a wide range of blood glucose levels from normal to diabetic, three groups were created. In each group, 1 candidate (the first number being picked from a table of random numbers) was selected out of every 10 employees on a company list. The composition of sampled members was adjusted according to age. As a result, 51 candidates in total were selected: 31 in the normal group, 10 in the IGT group and 10 in the diabetes group. Sample collection was performed at the periodical medical checkup. The purpose of the investigation was explained to the candidates in advance and signatures were obtained on an informed consent document. An investigation of the Health Practice Index (HPI) (13–15); was also performed at the same time in the form of a questionnaire. The questionnaire was used to investigate the relation between the level of saliva glycated proteins and the lifestyle of each participant. Participants were requested to fast after the previous day's dinner. To secure the accuracy of the measurement, participants rinsed their mouths with water six times. After that, 3 ml of saliva was placed directly into a 50 ml centrifuge tube (Corning).

The samples were immediately put on ice and were then centrifuged in a cooling centrifuge in the laboratory for 15 minutes at 1,200 g and 4°C. The separated supernatant fluid was kept at -80°C until analysis.

Chemicals

All reagents except for the fructosamine kit were analytical reagent grade products purchased from Wako Pure Chemical Industries.

Apparatus

Absorbance of samples except for fructosamine was measured with a spectrophotometer (Shimazu UV-160A).

Measurement methods

Saliva protein (SP):

Saliva protein (g/l) was measured at the wavelength of 595 nm by the Bradford (16) method using bovine serum albumin as a standard.

Saliva glucose (SG):

Saliva glucose (mg/dl) was measured with a "Glucose B Wako kit (Glucose oxidase method)" at the wavelength of 505 nm.

Plasma fructosamine:

Samples were first thawed at room temperature and then measured with "Fructosamine test Roche II liquid" and a Hitachi 7150 automatic biochemical analyzer.

Saliva fructosamine glycated protein (Saliva fructosamine GP):
Saliva samples were first thawed at room temperature and then measured using the same method as plasma fructosamine. Value of saliva fructosamine concentration (SF, μmol/l) was divided by the saliva protein concentration, which was measured separately.

Equation: Saliva fructosamine GP (μmol/g protein)=SF/SP

Saliva hydrazine glycated protein (Saliva hydrazine GP):

Measurement was performed according to the method reported by Kobayashi et al. (12). The acquired value (SH, μmol/l) was divided by the saliva protein concentration, which was measured separately.

Equation: Saliva hydrazine GP (μmol/g protein)=SH/SP

Other measurements

The Association for Preventive Medicine of Japan analyzed fasting plasma glucose and HbA1c measured by HPLC and other factors (blood pressure, weight, height, GOT, GPT, cholesterol, triglyceride, HDL, uric acid, etc.) taken from samples obtained in periodical medical checkups. Fasting plasma glucose was collected in test tubes containing NaF.

Statistical analysis

Statistical analysis was performed with SPSS ver. 10.0 (17). The statistically significant level was set to p<0.05. Pearson's correlation coefficients were used to examine the relation between the variables.

Results and Discussion

Some research studies on diabetes using saliva samples have estimated blood glucose levels with saliva glucose (18–20). However, saliva glucose is rapidly decomposed by bacteria

Table 1 Characteristics of subjects (n=51)

	means±SD
Age (yr.)	46.2±8.1
BMI (kg/m ²)	23.5±2.6
Systolic blood pressure (mmHg)	124.5±18.3
Diastolic blood pressure (mmHg)	81.7±12.2
Health practice index	4.3±1.7
Fasting plasma glucose (mg/dl)	115.7±35.7
Plasma fructosamine (μmol/l)	279.9±58.3
HbA1c (%)	5.7±1.5
Saliva fructosamine GP (μmol/g protein)	25.2±11.6
Saliva hydrazine GP (μmol/g protein)	80.0±22.8
Saliva glucose (mg/dl)	1.5±2.0

Table 2 Correlation between saliva glucose and glucose related index items in blood and saliva (n=51)

	Saliva glucose (mg/dl)	
	r	p value
HbA1c (%)	0.054	0.712
Plasma fructosamine (μmol/l)	0.019	0.895
Fasting plasma glucose (mg/dl)	0.032	0.826
Saliva fructosamine GP (μmol/g protein)	0.276	0.050
Saliva hydrazine GP (μmol/g protein)	0.043	0.764

Table 3 Correlation between saliva glycated protein and blood glycated protein (n=51)

	Saliva fructosamine GP ($\mu\text{mol/g protein}$)		Saliva hydrazine GP ($\mu\text{mol/g protein}$)	
	r	p value	r	p value
HbA1c (%)	0.449	0.001	0.204	0.155
Plasma fructosamine ($\mu\text{mol/l}$)	0.260	0.065	0.090	0.529
Fasting plasma glucose (mg/dl)	0.445	0.001	0.147	0.305

and enzymes in the mouth (21), so it is very difficult to measure it without using ultrafiltration (22). Therefore, we assumed that saliva glycated proteins, i.e., proteins combined with glucose, are relatively stable against bacteria, and there may be a correlation with glycated proteins and glucose in the blood.

Table 1 describes the basic characteristics of the 51 subjects that were investigated. Table 2 shows the correlation between saliva glucose and glucose related index items in blood and saliva. No correlation was identified between saliva glucose and blood glucose. This result is identical with those of previous studies (18, 20) and implies that bacteria decomposed saliva glucose or it was decomposed during the transfer from blood to saliva. Saliva glucose showed a weak correlation with saliva fructosamine GP ($r=0.276$, $p=0.050$), but showed no correlation with saliva hydrazine GP. Saliva hydrazine GP was not compensated by glucose, hence the effect of glucose should be stronger in saliva hydrazine GP than in saliva fructosamine GP. In the fructosamine method, a preparatory reaction is performed to eliminate to remove the effect of glucose and ascorbic acid, so the effect of saliva glucose should be small. Therefore, the weak correlation between saliva glucose and saliva fructosamine GP is probably due to saliva glycated protein produced from saliva glucose rather than the saliva glucose itself.

The correlation between saliva glycated protein and blood glycated protein is shown in Table 3. A significant correlation only appeared between “saliva fructosamine GP and HbA1c” ($r=0.449$; $p=0.001$) and “saliva fructosamine GP and fasting plasma glucose” ($r=0.445$; $p=0.001$). HbA1c is usually described as the ratio (%) of stable HbA1c in total hemoglobin. On the other hand, in plasma fructosamine measurements, no protein compensation is performed and the concentration of total

plasma glycated proteins is measured ($\mu\text{mol/l}$). As saliva fructosamine GP was compensated with saliva protein, it had a correlation with HbA1c but no correlation with plasma fructosamine, which had no protein compensation. Though HbA1c showed a correlation with saliva fructosamine GP, HbA1c in erythrocytes cannot be measured with saliva fructosamine GP. Some plasma proteins with a long half-life may be glycated and exuded into saliva. Considering that there is no correlation between saliva glucose and blood glucose, the correlation between saliva fructosamine GP and blood glucose reflects glycated protein that was produced in the blood or in saliva over a short period of time. Therefore, a relation between various glycated proteins from blood and saliva can be determined with measurements of saliva fructosamine GP. Research on the kind of protein related to saliva fructosamine GP is ongoing.

Fig. 1 shows the correlation between saliva fructosamine GP and HbA1c, and Fig. 2 shows the correlation between saliva fructosamine GP and fasting plasma glucose. Distribution of saliva fructosamine GP cannot be seen in the area of 45–60 $\mu\text{mol/g}$ protein, though a significant correlation is identifiable. The analysis of the correlation between saliva fructosamine GP and HbA1c/fasting plasma glucose showed a significant correlation in the diabetes group ($r=0.645$; $p=0.044$, $r=0.675$, $p=0.032$, respectively), but there was no correlation in the normal and IGT groups (Table 4). This implies that saliva glycated protein is secreted when the blood glucose level stays at a high level. Further research using more samples is needed.

In Table 3, saliva hydrazine GP showed no correlation with blood glycated protein and blood glucose, but there was a significant correlation with blood pressure and HPI ($r=0.395$;

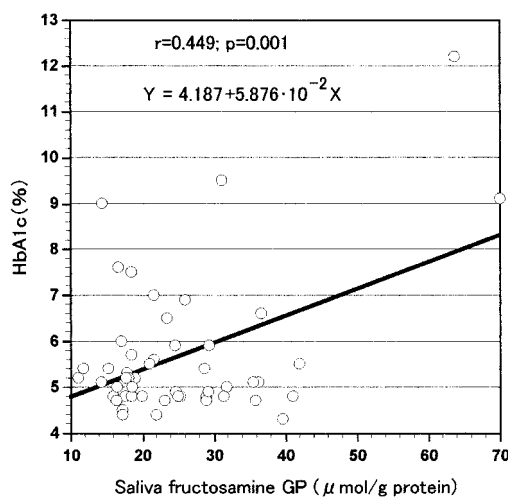


Fig. 1 Correlation between saliva fructosamine GP and HbA1c.

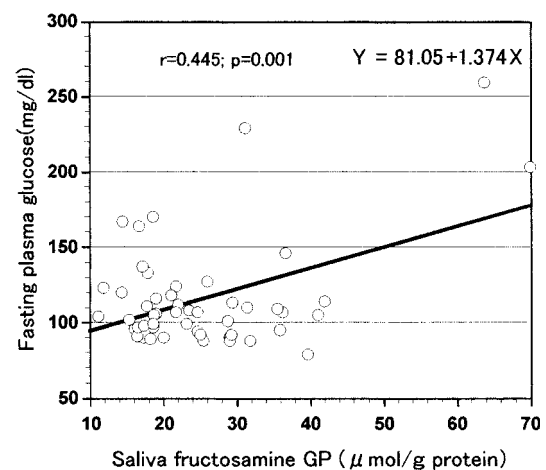


Fig. 2 Correlation between saliva fructosamine GP and fasting plasma glucose.

Table 4 Correlation between saliva fructosamine GP and HbA1c/fasting plasma glucose in each group of blood glucose levels

		Saliva fructosamine GP ($\mu\text{mol/g protein}$)	
		r	p value
Normal group (n=31)	HbA1c (%)	-0.123	0.518
	Fasting plasma glucose (mg/dl)	-0.082	0.661
IGT group (n=10)	HbA1c (%)	0.147	0.684
	Fasting plasma glucose (mg/dl)	-0.511	0.131
Diabetes group (n=10)	HbA1c (%)	0.645	0.044
	Fasting plasma glucose (mg/dl)	0.675	0.032

$p=0.004$ and $r=-0.311$; $p=0.026$, respectively). This result is likely because the hydrazine method is very sensitive against various reducing substances such as anti-hypertension drugs. Kobayashi et al. (12) also reported that the hydrazine method tends to be affected by reducing substances, so the hydrazine method is not appropriate for measuring saliva glycated proteins. Age showed no correlation with saliva and blood glycated protein. There were some problems in this study such as “no female participants in the research”, “the number of samples was small”, and “variation within one day cannot be traced”. These problems should be eliminated in future studies.

The presence of saliva glycated proteins had been suggested indirectly through the decline in the anti-bacterial capacity of saliva proteins. We directly measured saliva glycated proteins

and found a correlation between saliva fructosamine GP and HbA1c, and blood glucose. Since diabetes is increasing not only in Japan but also in developing countries, it is important to find new forms of diagnosis. Though there are still some problems to solve, if a method of estimating blood glycated proteins by using saliva fructosamine GP is developed, it will be very useful for the prevention of diabetes because of such merits as easy sample collection, simple measurements, low cost and high accuracy.

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References

- (1) Maillard LC. Action des acides amines sur les sucres; formation des melanoidines par voie methodique. C. R. Acad. Sci. Gen. 1912; 154: 66.
- (2) Little RR, Wiedmeyer HM, England JD, Rohlfing CL, Madsen RW, Goldstein DE, et al. International standardization of glycohemoglobin measurements: practical application. Clin. Chem. 1993; 39: 2356.
- (3) Goldstein DE, Little RR, Lorenz RA, Malone JI, Nathan D, Peterson CM. Tests of glycemia in diabetes. Diabetes Care 1995; 18: 896–909.
- (4) Shima K, Ito N, Abe F, Hirota M, Yano M, Yamamoto Y, et al. High-performance liquid chromatographic assay of serum glycated albumin. Diabetologia 1988; 31: 627–631.
- (5) Oimomi M, Maeda Y, Hata F, Kitamura Y, Matsumoto S, Hatanaka H, et al. Increased fructose-lysine of hair protein and blood glucose control in diabetic patients. Horm. Metab. Res. 1988; 20: 654–655.
- (6) Kobayashi K, Igimi H. Glycation index of hair for non-invasive estimation of diabetic control. Biol. Pharm. Bull. 1996; 19: 487–490.
- (7) Bakan E, Bakan N. Glycosylation of nail in diabetics: possible marker of long-term hyperglycemia. Clin. Chim. Acta 1985; 147: 1–5.
- (8) Delbridge L, Ellis CS, Robertson K, Lequesne LP. Non-enzymatic glycosylation of keratin from the stratum corneum of the diabetic foot. Br. J. Dermatol. 1985; 112: 547–554.
- (9) Li YM. Glycation ligand binding motif in lactoferrin. Implications in diabetic infection. Adv. Exp. Med. Biol. 1998; 443: 57–63.
- (10) Dodds MW, Yeh CK, Johnson DA. Salivary alterations in type 2 (non-insulin-dependent) diabetes mellitus and hypertension. Community Dent. Oral Epidemiol. 2000; 28: 373–381.
- (11) Belce A, Uslu E, Kucur M, Umut M, Ipbuker A, Seymen HO. Evaluation of salivary sialic acid level and Cu-Zn superoxide dismutase activity in type 1 diabetes mellitus. Tohoku J. Exp. Med. 2000; 192: 219–225.
- (12) Kobayashi K, Yoshimoto K, Hirauchi K, Uchida K. Determination of glycated proteins in biological samples based on colorimetry of 2-keto-glucose released with hydrazine. Biol. Pharm. Bull. 1994; 17: 365–369.
- (13) Morimoto K. Lifestyle and health. Japanese Journal of Hygiene 2000; 54: 572–591.
- (14) Hagihara A, Morimoto K. Personal health practices and attitudes toward nonsmokers’ legal rights in Japan. Soc. Sci. Med. 1991; 33: 717–721.
- (15) Morimoto K. Stress-kiki no yobo-igaku (in Japanese). Tokyo: NHK books; 1997.
- (16) 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.
- (17) SPSS Inc. SPSS Base 10.0 Brief Guide. Chicago: SPSS Inc.; 2000.
- (18) Kjellman O. The presence of glucose in gingival exudate and resting saliva of subjects with insulin-treated diabetes melli-

- tus. Sven. Tandlak. Tidskr. 1970; 63: 11–19.
- (19) Shannon TL. Blood and saliva glucose levels in relation to gingival health. *J. Indian Dent. Assoc.* 1973; 45: 299–302.
- (20) Forbat LN, Collins RE, Maskell GK, Sonksen PH. Glucose concentrations in parotid fluid and venous blood of patients attending a diabetic clinic. *J. R. Soc. Med.* 1981; 74: 725–728.
- (21) Sandham HJ, Kleinberg I. Utilization of glucose and lactic acid by salivary sediment. *Arch. Oral Biol.* 1969; 14: 597–602.
- (22) Yamaguchi M, Kimura H, Kano Y, Egusa G, Kita J. Individual correlation of blood glucose and saliva glucose. *J. Japan Diab. Soc.* 1997; 40: 335–340.