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Evaluation of Time-Course Changes of Gingival Crevicular Fluid Glucose Levels in Diabetics

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Abstract. **A minute volume such as a few hundred nano-litres of gingival crevicular fluid (GCF) will evaporate within one minute at room temperature after collection. In order to investigate the feasibility of a method for noninvasive blood glucose measurement using this fluid, a fabricated GCF-collecting device, and the time-course changes of blood and GCF glucose levels in diabetics were evaluated. As a result of improvement in the GCF-collecting device such that the color of a filter paper changed from white to black upon collection of the sample, the completion of the collecting procedure could be visually confirmed. Using this device, we succeeded in collecting 200 nl of GCF within 30 s, despite the fact that GCF is usually secreted at a rate between 2 and 3 μl per tooth per hour. This method could be applicable for collecting small volume of other biological fluids. The time-course changes of blood and GCF glucose levels in ten type 2 diabetic subjects were examined, the correlation coefficient, *R*, between both glucose levels was 0.878. The result proved to be similar to that for normal healthy subjects in a previous study. A significant time difference between the two glucose levels was not observed. In diabetes period of 4.8 $\pm$ 1.7 years, the influence of the disease on the GCF glucose level was minimal. Furthermore, from the ROC curve analysis, both sensitivity and specificity showed favorable results. It was suggested that the measurement of GCF glucose levels was a promising screening method for diabetics.**

Key Words. **gingival crevicular fluid, noninvasive, blood glucose, capillary, diabetics**

1. Introduction

It is essential for a patient suffering from a disease such as diabetic mellitus to measure the concentrations of certain biochemical materials in blood, in order to maintain a high quality of life. Thus, the development of a noninvasive measurement assay for biochemical materials is an urgent necessity, and it will allow us to utilize the screening tests widely for many patients.

We have been focusing on the development of a method of noninvasive blood glucose measurement by analyzing painlessly collected gingival crevicular fluid (GCF) secreted from the gingival crevice. GCF is an extracellular fluid secreted from the epithelia of the gingival crevice, whose presence was first reported by Brill et al. in 1958. The components of GCF are mainly derived from plasma, and its flow rate is 2 to 3 μl per hour per tooth (Lenander-Lumikari and Loimaranta, 2000; Uitto, 2000; Griffiths, 2000; Goodson, 2000). It was reported that *in vitro* microassy analysis revealed that the GCF glucose level in healthy subjects was 70 $\pm$ 190 mg/l and in diabetics was 190 $\pm$ 290 mg/l (Cianter et al., 2002).

GCF has been collected using a filter paper (Brill and Krasse, 1958), a micropipette in commercial use (Kjellman, 1970) or a thin tube (Parker et al., 1993) directly inserted into the gingival crevice. Even though GCF was collected, it would evaporate within a minute at room temperature when using these methods. Therefore, a new reliable collecting method has been required. We have fabricated a GCF-collecting device, adopting a dry chemistry system using an enzymatic assay method. The device could be used for collection and analysis of GCF. Results have already reported that GCF could be collected within a few minutes and causing no pain to the subjects using the device; and it enables us to analyze the sample without evaporation (Yamaguchi et al., 2001, 2002a). Furthermore, the stimulating test using guinea pigs revealed that insertion of the GCF-collecting device into the gingival crevice would not cause any long-term problems (Yamaguchi et al., 2002b). Also, the results of the meal load test using healthy subjects demonstrated that the blood glucose level correlated well with the GCF glucose level, with a correlation coefficient of more than 0.9 (Yamaguchi et al., 2004). However, it was revealed that improvement in the device to allow visual confirmation of sample collection was required for the method to be practically applied.

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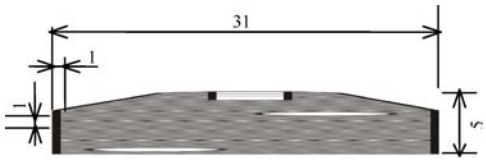
In this report, a new GCF-collecting device is proposed. It has a structure that enables us to visually confirm a quantitative collection of the sample and to decrease the volume of sample to 200 nl. Using ten type 2 diabetes subjects, the time-course changes in the blood and GCF glucose levels in fasting early morning are determined. Furthermore, based on the experimental results, a receiver operating characteristic (ROC) curve is performed. From all of the results, the clinical significance of the method of noninvasive blood glucose measurement is described.

2. Materials and Methods

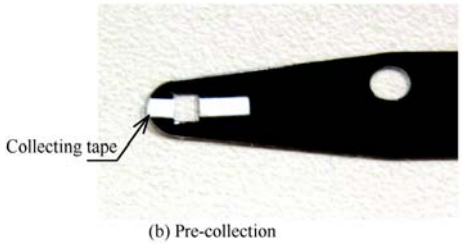
2.1. GCF-collecting device

The GCF-collecting device was designed enclosed in a collecting tape. Three sheets of film were laminated in order to make the capillary drain. The middle film had a drain and the plain films on each side were used as a cover (Figure 1(a)). The collecting tape was placed on the tip of the GCF-collecting device which was used to collect the GCF. In order to insert it in a gingival crevice with a width of about 0.2 mm, the thicknesses of the films were selected to be 38 μm - 120 μm - 38 μm (total thickness was 196 μm).

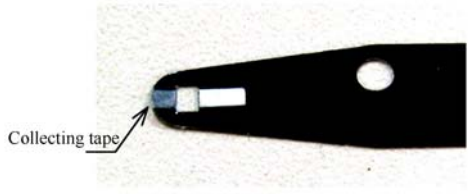
A black polyester film (Teijin Co. Ltd., Japan) was used for upper and lower plastic films. When GCF was collected, the color of collecting tape changed from white to black due to the refraction of light (Figure 1(b) and (c)). Thus the collection was visually confirmed. Since the volume of absorbed sample could be controlled by the volume of the collecting tape (Yamaguchi et al., 2001), the volume was set below 200 nl by making the tape size as $1 \times 1 \times 0.2$ mm^3.



(a) Structure of the GCF-collecting device (units in mm)



(b) Pre-collection



(c) Post-collection

Fig. 1. *A GCF-collecting device enclosed in a collecting tape and the developing in color from white to black.*

Previously, the authors used a testing tape saturated with enzyme, which was used for GCF glucose level analysis. In this study, however, since the purpose was to evaluate the time course changes in blood and GCF glucose levels in diabeics, a more accurate combined microplate fluorometer and luminometer (Fluoroskan Ascent FL, Thermo Electron Oy., Finland) was used for the analysis. This GCF-collecting device was used to collect samples alone.

2.2. In vivo evaluation

The meal load test was performed on diabetics, and the time-course changes of the blood and GCF glucose levels were measured using the GCF-collecting device. The subjects were 10 type 2 diabetics (7 male and 3 female, 55.1 ± 8.4 yr, hemoglobin A1c (HbA1c) 6.7 ± 0.7%, diagnosed diabetes period 4.8 ± 1.7 yr, BMI 25.6 ± 3.0 kg/m^2, mean $\pm$ SD). Prior to this evaluation, the salivary Hb of the subjects was measured, and their health with regard to oral diseases was assessed. The aim of the experiment was explained to the subjects and consent was obtained after confirmation that they fully understood the experiment. The local ethical committee at Toyama Medical and Pharmaceutical University approved the study.

During early morning fasting, the subjects brushed their teeth followed by gargling water two or three times prior to the GCF collection in order to remove residues in their mouths. The remaining fluid in the mouth was wiped out using two dental cottons (8×25 mm). The collecting tape was inserted into the gingival crevice several times to locate a position where the GCF was easily collected without bleeding. At the same times the residues inside the gingival crevice were cleaned and removed. The subjects were sat still for 10 minutes to wait for the secretion of the GCF. Then the GCF was collected using the GCF-collecting device being inserted into the gingival crevice by 1 mm (Figure 2). To avoid contamination of saliva to GCF, the sample was collected twice either from an incisor or a canine tooth in the upper jaw. Therefore, in a few subjects GCF was collected twice from almost the same location. When the color of the collecting tape changed from white to black, the collection was completed. The GCF-collecting device was sealed, kept in a test tube and refrigerated below –20°C until measurement. The collecting tape taken from the GCF-collecting device was soaked in 205 μl of distilled water and left for 30 minutes at 4°C, in order to extract the GCF. Glucose 1-dehydrogenase (EC 1.1.1.118) was added to the

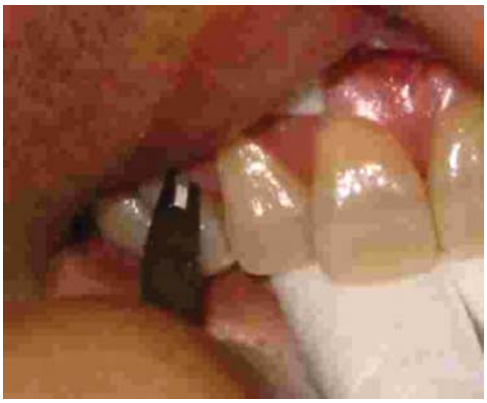


Fig. 2. *GCF-collecting device has a capillary drain and capillary action is used to pump GCF automatically.*

extracted glucose to generate Glucono-δlactone. Then using diaphorase (EC 1.8.1.4), the quantitative assay chromogen, resazurin (CAS No. 550-82-3), was converted to the fluorescent, resorufin (Guilbault, 1975; Maeda et al., 2001). The chemical reactions catalyzed by glucose 1-dehydrogenase and diaphorase were a coupled reaction via NAD^+ and NADH. Finally, the fluorescence intensity of resorufin was measured using the combined microplate fluorometer and luminometer (538 nm excitation wavelength, 584 nm fluorescent wavelength), and then using the calibration curve, the value was converted to the GCF glucose level. The calibration curve of the glucose level and the fluorescence intensity was $R^2 = 0.99$ at the seven glucose levels such as 0, 2.25, 3, 10, 30, 100 and 300 mg/dl (2.25 mg/dl = 125 μmol/l, $n = 42$). Distilled water was used into which glucose ($C_6H_{12}O_6$, Wako Pure Chemical Industries, Ltd., Japan) as a substrate was dissolved. In addition, at 2.25 mg/dl of standard glucose solution, CV = 4.68% ($n = 6$).

Meanwhile the venous plasma glucose level was calculated by the whole blood measurement obtained by needling a finger using a portable glucose monitor (51.0 $\times$ 87.0 $\times$ 14.5 mm^3, Sanwa Kagaku Kenkyusho Co., Ltd., Japan). Immediately after a meal and at 30 minutes intervals for a two-hour period, GCF and blood were collected five times in total.

Finally, the values of the diagnostic standard of blood glucose during fasting, 126 mg/dl, and a standard for two hours after the meal, 200 mg/dl, were set as threshold levels (Expert committee, 1997), and a ROC curve for GCF glucose level was performed and the determination accuracy was evaluated. The ROC curve was defined as a curve obtained with sensitivity (positive rate) on the y-axis and the specificity (false positive, 1-specificity) on the x-axis, using a few threshold levels. It was used to compare effectiveness of the test methods. The left upper position of the curve indicates higher sensitivity and lower specificity (Mets, 1978). Five healthy subjects were added as the control subjects (2 male and 3 female, 38.6 $\pm$ 8.4 yr, fasting blood glucose (FBG) 88.2 $\pm$ 6.0 mg/dl, BMI 23.3 $\pm$ 2.9 kg/m^2, mean $\pm$ SD). GCF and blood were collected five times in total from the healthy subjects in the same manner as for the diabetics, immediately after a meal and with 30 minutes intervals over a two-hour period.

3. Results and Discussion

The meal-loading test was conducted on ten type 2 diabetic subjects, and the GCF was collected ten times per subject, giving one hundred samples in total. The improved GCF-collecting device indicated the completion of the collection procedure visually by the changed color of the collecting paper. Since the GCF was collected only from either the incisor or canine teeth gingival crevice, collection was easily performed without causing any pain to the subjects. In the previous study, GCF collecting took about one to two minutes; however, it could be shortened to 5 to 30 s (Yamaguchi et al., 2004). Consequently, variations in collecting time among the subjects could be reduced. This is due to decrease volume of sample from 500 to 200 nl.

The repeatability of the combined microplate fluorimeter and luminometer at 2.25 mg/dl was CV = 4.68%, which was a favorable result. Therefore, it was considered that the GCF glucose level was analyzed highly accurately. The blood glucose levels were in a range between 116 and 311 mg/dl (Table 1), while the GCF glucose levels were in a range between 12.8 and 278 mg/dl. Since the GCF is considered to be derived from serum, both glucose levels should be almost equal (Lenander-Lumikari and Loimaranta, 2000; Uitto, 2000; Griffiths, 2000; Goodson, 2000). The concentration of GCF glucose levels was in a range between 1/10 of and the same as blood glucose levels, which corresponded well with the reports made

Table 1. *Correlation coefficient between the blood and GCF glucose levels on 10 type 2 diabetic subjects*

Subject	No. 1	No. 2	Mean
A	0.959††	0.959††	0.959
B	0.969††	0.846††	0.908
C	0.941†	0.777††	0.859
D	0.944†	0.941††	0.942
E	0.931†	0.746†	0.838
F	0.905†	0.895†	0.900
G	0.976††	0.862††	0.919
H	0.972†	0.790††	0.881
I	0.943†	0.628††	0.786
J	0.966†	0.619††	0.792
Mean	0.950	0.806	0.878
±SD	0.022	0.118	0.070

†Incisor tooth, ††Canine tooth.

by Kjellman (1970), Parker (1993), and Cianter (2002). The concentration of GCF by evaporation was not observed. GCF glucose levels were 10-fold higher than the salivary glucose levels that the authors previously studied (Yamaguchi et al., 1998). A clear difference from the salivary glucose level was observed.

The characteristics of time course changes in the meal load tests of ten type 2 diabetic subjects were analyzed, with the time at which the meal commenced set as 0 minute (Figure 3). Both glucose levels indicated approximate curves by the 3-dimensional spline interpolation and the correlation coefficient (R) was calculated. R indicated a range between 0.619 and 0.976, with an average of 0.878 $\pm$ 0.070. In comparison with the blood glucose level, the GCF glucose level was assumed to fluctuate at almost the same time or a little later physiologically. However, no significant time difference was observed. No significant difference in the correlation coefficient between sample collection points was observed. The correlation coefficient was lower than the one Parker reported as 0.975 (1993). However, it was almost the same result as the correlation coefficient, between 0.705 and 0.877, that the authors indicated for healthy subjects (Yamaguchi et al., 2004). The diabetes period was not considered to influence the GCF glucose level with regards to the subject with the diabetes period for 4.8 $\pm$1.7 yr (mean $\pm$ SD).

The correlation coefficients (R) between the blood and GCF glucose levels were shown in Figure 4 for 10 type 2 diabetics. The R value was 0.52, the slope was 0.57, and the y-intercept was 10.63. These results proved the previous studies that the individual correlation between body fluid- and blood glucose levels is better than the correlations of the group (Ginsberg, 1992; Klonoff, 1997). It was expected that the estimation accuracy for the blood glucose level might be improved by using the individual correlation. On the other hand, with regard to the slope (0.57), it was reconfirmed that the GCF glucose level tends to be lower than the blood glucose level by 43%. However, the investigation of the mechanism will be a future study.

The usefulness of the GCF glucose level as a method for blood glucose measurement was evaluated using the ROC curve (Figure 5). The meal-loading test was conducted on five healthy subjects and the GCF was collected ten times per subject, to give fifty samples in total. The blood glucose levels of the healthy subjects were in

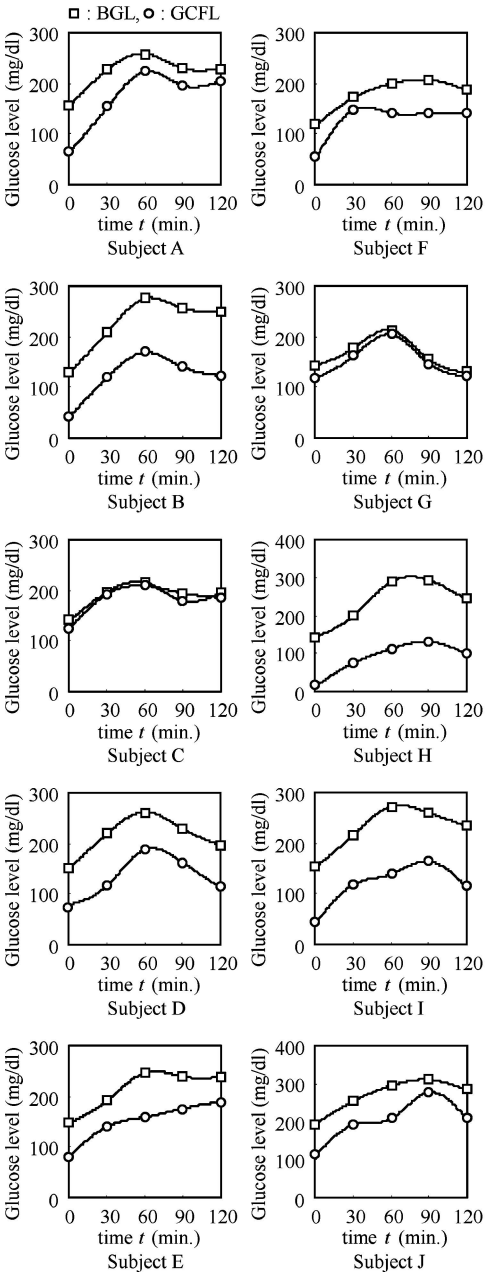


Fig. 3. *Time-course change characteristics between the blood and GCF glucose levels on type 2 diabetic subjects (data: No. 1).*

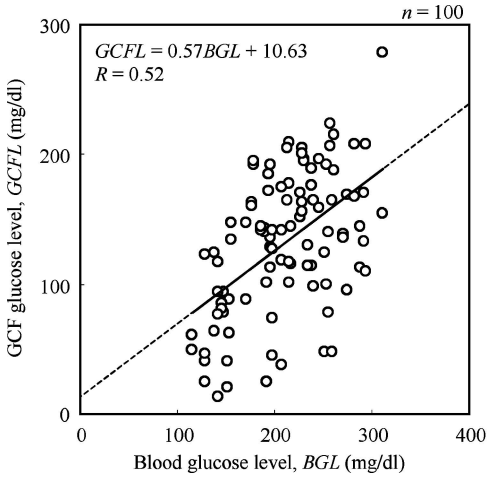


Fig. 4. *Calibration curve of the GCF and the blood glucose levels in type 2 diabetic subjects.*

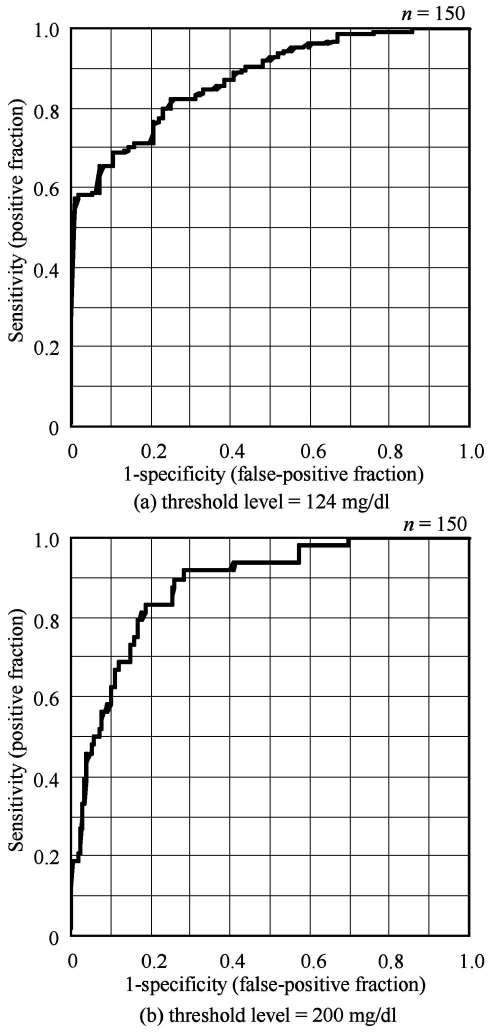


Fig. 5. *Receiver operating characteristic (ROC) curves of the GCF-glucose levels.*

a range between 51 and 207 mg/dl. When the threshold blood glucose level was set at 126 mg/dl, the optimum threshold level of the GCF glucose level was revealed at 93.1 mg/dl. The sensitivity was 0.69 and the specificity was 0.90. When the threshold blood glucose level was set at 200 mg/dl, the optimum threshold level of the GCF was revealed at 110.3 mg/dl. The sensitivity was 0.83 and the specificity was 0.81. It was considered that application of GCF to a screening test could obtain high accuracy in judgments.

4. Conclusion

In order to study the feasibility of a non-invasive blood glucose measurement method using a few hundred nano-litres volume of GCF samples, the time-course change characteristics of the blood and GCF glucose levels of diabetics were evaluated using the fabricated GCF-collecting device. The GCF device was improved to change the color of the collecting-tape from white to black by absorbing the sample. As a result, the completion of sample collection procedure was visually confirmed. With the use of this device, concentration of the sample by evaporation was not observed. Furthermore, collecting 200 nl of GCF was completed within 30 s, although the usual secretion rate is between 2 and 3 μl (per hour and per tooth). Our method could be applied to the collection of other small volume biological fluids.

Using ten type 2 diabetics, fasting early morning blood and GCF glucose levels with time-course changes were analyzed. The correlation coefficient of both glucose levels (R) was 0.878, which was almost equal to the ones of healthy subject. No significant time difference between the two glucose levels was observed. The effect of diabetes period on the GCF glucose level was small for subjects with a diabetes period of 4.8 $\pm$ 1.7 yr. Consequently it was considered that this method could be applicable to diabetics. The usefulness as a blood glucose measurement method was evaluated by ROC curve. Both sensitivity and specificity showed favorable results and it was revealed that this might be a promising screening method.

The use of GCF-collecting device with a testing tape saturated with enzyme as a collecting tape, simultaneous analysis of GCF could be possible (Yamaguchi et al., 2001, 2002a, 2002b, 2004). In addition, not only the use of the non-invasive blood glucose level measurement via GCF, but also the application to qualitative and quantitative analyses for various diseases will be the subject of further study.

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