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HbA1c adjusted by erythrocyte creatine is a useful glycemic control indicator in patients with hemolysis

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ABSTRACT

Objectives: HbA1c shows low in patients with hemolysis, whereas glycated albumin (GA) is not affected by hemolysis. Therefore, the GA/HbA1c ratio reflects hemolysis in diabetic patients with hemolysis. Erythrocyte creatine (EC) is an indicator of hemolysis that reflects the mean erythrocyte age. The aim of this study was to examine whether HbA1c adjusted by EC accurately reflected glycemic control in patients with hemolysis.
Materials and methods: A total of 238 individuals, consisting of 131 diabetic patients and 107 non-diabetic subjects, and consisting of 42 patients with hemolysis, and 196 subjects without hemolysis were selected for the study. HbA1c expressed in the IFCC units (iA1c) as well as in the NGSP units (A1C) were used. From the fact that EC and the GA/iA1c ratio showed a significant positive correlation, a formula for iA1c adjusted by EC (ECadj-iA1c) was created from a regression equation between EC and the GA/iA1c ratio.
Results: Significant correlations were observed between the GA/iA1c ratio and various hemolytic indicators but not between the GA/ECadj-iA1c ratio and those hemolytic indicators. The GA/iA1c ratio in individuals with hemolysis was significantly higher than in individuals without hemolysis, while no significant differences were observed in the GA/ECadj-iA1c ratio between the groups. Further, iA1c concentrations in non-diabetic patients with hemolysis were significantly lower than in the non-diabetic subjects without hemolysis, whereas ECadj-iA1c and GA concentrations showed no significant difference between the two groups.
Conclusions: These results suggested that ECadj-iA1c accurately reflected glycemic control in patients with hemolysis.

1. Introduction

Glycation of various proteins is increased in diabetic patients compared to non-diabetic subjects, suggesting that some glycated proteins may be responsible for the development and progression of chronic diabetic complications [1,2]. Among the glycated proteins, HbA1c is widely used as a glycemic control indicator for the diagnosis of diabetes mellitus in clinical settings [3–5]. The life span of erythrocyte is approximately 120 days, and HbA1c, therefore, reflects glycemic control for the past two month to three months [6]. However, as for diseases, such as hemolytic anemia, renal anemia, and liver cirrhosis in which the life span of erythrocyte is shortened, it has been known that HbA1c concentrations are low and do not accurately reflect glycemic control [7–9]. HbA1c concentrations are low not only in patients with hemolytic anemia but also in patients with compensated hemolysis without anemia [10,11]. If hemolysis with falsely low HbA1c is

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underlooked, the condition with poor glycemic control may result in the development or progression of diabetic complications.

Since glycated albumin (GA) is not affected in patients with hemolysis [9], the usefulness of GA has been reported as a glycemic control indicator in patients with hemolysis [11,12]. Moreover, a significant negative correlation (R = -710) between the GA/HbA1c ratio and hemoglobin was observed in patients with hemolysis, including diabetic patients. And the GA/HbA1c ratio could reflect the degree of hemolysis has been reported in diabetic patients with hemolysis [12]. Even with hemolysis, the conventional indicators of hemolysis, such as reticulocyte and haptoglobin, have been reported to be negative in patients with latent hemolysis with low HbA1c concentrations [13].

Since the creatine content in human erythrocytes is decreased with the aging of erythrocyte, erythrocyte creatine (EC) has drawn a lot of attention as an indicator of hemolysis reflecting the mean erythrocyte age [14–17]. In cases of compensated hemolysis, EC concentrations were significantly higher than the controls and have, therefore, been considered as an indicator of hemolysis with higher sensitivity compared to the conventional indicators [18,19].

In the present study, we hypothesized and investigated whether HbA1c adjusted by EC (ECadj-HbA1c) calculated with a regression equation for EC and the GA/iA1c ratio might not be affected by hemolysis and accurately reflect glycemic control in patients with hemolysis.

2. Materials and methods

2.1. Patients

A total of 238 individuals, consisting of 131 diabetic patients and 107 non-diabetic subjects, and consisting of 42 patients with hemolysis and 196 subjects without hemolysis were selected for the study (Table 1). For some studies, 107 non-diabetic subjects, consisting of 35 non-diabetic patients with hemolysis (Hb 10.1 ± 2.1 g/dL, EC 4.86 ± 2.55 μmol/g Hb) and 72 non-hemolytic subjects (Hb 14.4 ± 1.1 g/dL, EC 1.47 ± 0.49 μmol/g Hb) were used.

Some patients with hemolysis were recruited from patients who were older than 20 years old and had laboratory data of complete blood counts and reticulocytes for clinical reasons at Kochi Medical School Hospital and Kumamoto University Hospital [9]. We measured EC, HbA1c, GA, haptoglobin, and other biochemical screening items of the existing samples of the corresponding patients. Patients with type 2 diabetes mellitus at Kawanishi City Hospital and Jinnouchi Hospital and non-diabetic subjects selected from subjects who visited the Prevention Medical Center in Takagi Hospital for a health examination were also recruited for the study. Patients with liver disease, renal disease, or pregnancy, which may affect HbA1c and/or GA measurements, were excluded in all groups.

Table 1
Patient characteristics and results of various clinical laboratory tests in individuals with and without hemolysis.

	Hemolysis		
	–	+	P
n	196	42	
Age	63 [57, 69]	48 [37, 68]	< 0.001
Male (%)	115 (58.7)	23 (54.8)	0.641
Diabetes mellitus (%)	120 (61.2)	11 (26.2)	< 0.001
A1C (%)	6.8 [5.9, 7.8]	4.45 [3.5, 5.0]	< 0.001
iA1c (mmol/mol)	50.8 [41.0, 61.8]	25.2 [14.8, 31.2]	< 0.001
GA (%)	17.2 [13.8, 21.5]	13.7 [12.4, 16.9]	< 0.001
GA/iA1c ratio	3.47 [3.20, 3.77] (3.50 ± 0.48)	6.25 [4.75, 8.58] (7.27 ± 3.30)	< 0.001
Hb (g/dl)	13.9 ± 1.2	10.2 ± 2.4	< 0.001
Ret (%)	1.3 [0.9, 1.4]	6.9 [3.0, 9.5]	< 0.001
Hpt (mg/dl)	125 [100, 148]	0 [0, 11]	< 0.001
LDH (U/L)	176 [155, 201]	279 [219, 298]	< 0.001
I Bil (mg/dl)	0.3 [0.3, 0.4]	0.6 [0.2, 0.8]	0.146
EC (μmol/g Hb)	1.42 [1.26, 1.62]	4.61 [2.67, 6.36]	< 0.001

Abbreviations: A1C; HbA1c expressed in the NGSP units, iA1c; HbA1c expressed in the IFCC units, GA; glycated albumin, EC; erythrocyte creatine.

Hemolysis was defined as a condition with haptoglobin lower than the reference value (type 1–1: < 83 mg/dL, type 2–1; < 65 mg/dL, type 2–2: < 25 mg/dL). Anemia was defined as a condition with hemoglobin < 12.0 g/dL in female and < 13.0 g/dL in male. HbA1c, GA, EC, complete blood counts, indirect bilirubin, lactate dehydrogenase (LDH), reticulocytes, and haptoglobin were measured in the individuals. The GA/iA1c ratios and EC were compared between the groups.

The study was approved by the Institutional Research Board of each institute. All samples were prepared and analyzed in accordance with the protocols approved by the institutional responsible committee at Kumamoto University and other collaborating institutions. We provided the patients with type 2 diabetes mellitus and the healthy volunteers with detailed information about the study, and all participants without some patients with hemolysis provided written informed consent to participate.

3. Laboratory methods

HbA1c was measured by high performance liquid chromatography and was expressed in the National Glycohemoglobin Standardization Program (NGSP) units (A1C: %) and the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) units (iA1c: mmol/mol) [20]. iA1c was calculated using the following equation. iA1c (mmol/mol) = 10.93 x A1C (%) - 23.5 [20]. GA was determined by enzymatic assay using albumin-specific protease, ketoamine oxidase, and albumin assay reagent (Lucica GA-L; Asahi Kasei Pharma Co., Tokyo, Japan) [21]. Erythrocyte creatine was assayed enzymatically in accordance with previous reports [18,22]. In brief, the plasma and buffy coat were aspirated from the blood sample after centrifugation. After lysis with 0.1% saponin and deproteinization of packed erythrocytes with 0.15 M $Ba(OH)_2$ and 0.15 M $ZnSO_4$, the supernatant was obtained by centrifugation and filtration. The creatine concentration in the supernatant was measured with the enzymatic assay involving creatine amidinohydrolase, sarcosine oxidase, and peroxidase. Measured data were expressed as micromole per gram of hemoglobin (μmol/g Hb).

Lactate dehydrogenase (LDH) and indirect bilirubin concentrations were determined with a commercially available kit [Ecudia XL Eiken LDH II-J (Eiken Chemical Co., Tokyo, Japan), Iatro LQ T-Bil II and Iatro LQ D-Bil II (LSI Medience Corp., Tokyo, Japan), respectively]. Hematologic examinations were carried out with a Sysmex SE 9000 (Sysmex Corp., Kobe, Japan), and reticulocyte counts were performed with a Sysmex R-3000 (Sysmex). Haptoglobin was measured by immuneturbidimetric assay using Cobas c 501 (Roche Diagnostics, Basel, Switzerland).

All laboratory tests were performed under the control of ISO15189 (RML00150), e.g., the within-run precision (CV; %) of the laboratory tests were; HbA1c 0.45–0.48, GA 0.25–0.37, EC 0.7–0.9, Hb < 5.0, reticulocyte < 5.0, haptoglobin < 5.0, LDH < 1.0 and indirect bilirubin < 5.0.

3.1. Statistical analysis

Kolmogorov-Smirnov normality tests showed normal distributions for Hb but not for other data. Continuous variables are shown as means ± SD when distribution was normal and as medians with interquartile range when distribution was skewed. For statistical analyses, the unpaired Student's *t*-Test, Mann-Whitney *U* test, or chi-square test was used to compare the two groups, as appropriate. To analyze

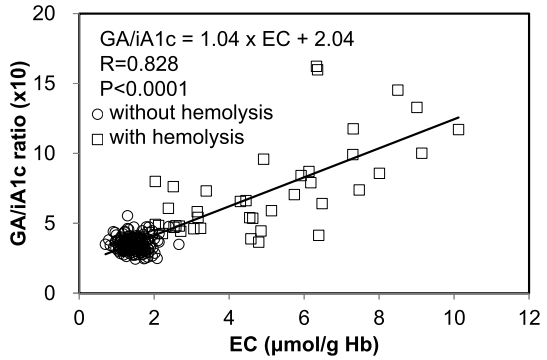

Fig. 1. Correlation of EC with the GA/iA1c ratio.
Correlation between EC and the GA/iA1c ratio is shown in 238 individuals with (open squares) or without (open circles) hemolysis is shown. A regression formula for EC and GA/iA1c ratio in 238 individuals is also shown. The 95% confidence interval of for the correlation co-efficient, slope and intercept was 0.783–0.864, 0.95–1.13, and 1.81–2.28, respectively.

correlations between two parameters, Pearson's correlation coefficient was performed with the StatFlex computer program (Version 6.0, Artec Co., Osaka, Japan). *P*-Values < 0.05 were considered to be statistically significant.

4. Results

The correlation between EC and the GA/iA1c ratio was examined in 238 individuals for the study, and a high correlation was observed (R = 0.828, $P < 0.0001$; Fig. 1). A regression equation for both indicators (GA/iA1c x 10 = 1.04 × EC + 2.04) was used to calculate the GA/EC-adjusted iA1c ratio with the following formula.

$$\text{GA/ECadj} - \text{iA1c} = \text{GA/iA1c} \times 3.5/(1.04 \times \text{EC} + 2.04) \quad (1)$$

The parameter 3.5 in Eq. (1) is the mean of GA/iA1c ratio in 107 non-hemolytic individuals in this study. In addition, a formula [ECadj-iA1c = GA/(GA / ECadj-iA1c)] was used to calculate ECadj-iA1c by Eq. (2).

$$\text{ECadj} - \text{iA1c(mmol/mol)} = \text{iA1c} \times [1.04 \times \text{EC}(\mu\text{mol/g Hb}) + 2.04]/3.5 \quad (2)$$

Indicators for the various clinical laboratory tests were compared among individuals with or without hemolysis. In this study, percent of diabetic patients (%) in group with hemolysis was significantly lower than those in group without hemolysis (Table 1). As a result, GA concentrations in group with hemolysis were also significantly lower than those in group without hemolysis. Both A1C and iA1c concentrations in patients with hemolysis were lower than in individuals without hemolysis. Indicators of hemolysis, such as reticulocytes, EC and LDH were significantly higher in patients with hemolysis than in patients without hemolysis, whereas haptoglobin was significantly lower. Indirect bilirubin showed no significant differences between the two groups.

The GA/iA1c ratio is not affected by glycemic control and is, therefore, useful as an indicator of hemolysis in the population that includes diabetic patients. For that reason, the correlations of the GA/iA1c ratio with various indicators of hemolysis were examined (Table 2). The GA/iA1c ratio had significant correlations with various indicators of hemolysis, including EC. On the other hand, the GA/ECadj-iA1c ratio had a weak correlation with only haptoglobin, while no significant correlations were observed with EC, hemoglobin, reticulocytes, LDH, and indirect bilirubin.

The GA/iA1c ratio in patients with hemolysis was significantly higher than in patients without hemolysis (Fig. 2A). In the meantime, the GA/ECadj-iA1c ratios in both groups were the reference range

Table 2
Correlations of the GA/iA1c ratio and the GA/ECadj-iA1c ratio with various indicators of hemolysis.

	GA/iA1c ratio		GA/ECadj-iA1c ratio	
	R	P	R	P
EC	0.828	< 0.0001	−0.047	0.466
Hb	−0.572	< 0.0001	−0.041	0.529
Reticulocytes	0.631	< 0.0001	−0.051	0.694
Haptoglobin	−0.502	< 0.0001	−0.282	0.037
Lactate dehydrogenase	0.345	< 0.0001	0.058	0.379
Indirect bilirubin	0.321	0.012	−0.027	0.835

Abbreviations: GA; glycated albumin, EC; erythrocyte creatine, iA1c; HbA1c expressed in the IFCC unit, ECadj-iA1c; iA1c adjusted by EC.

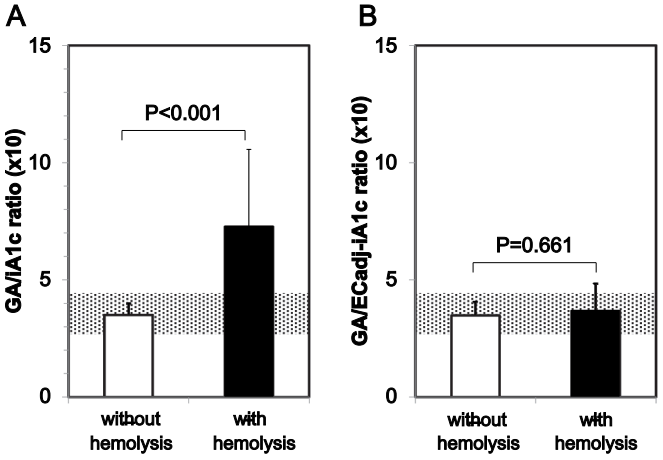

Fig. 2. Comparisons of the GA/iA1c ratio and the GA/ECadj-iA1c ratio between individuals with and without hemolysis.
Results of comparisons of the GA/iA1c ratio (A) and the GA/ECadj-iA1c ratio (B) between individuals with hemolysis (closed square) and without hemolysis (open square) are shown. The reference range of the GA/iA1c ratio (2.74–4.62) is indicated by shading.

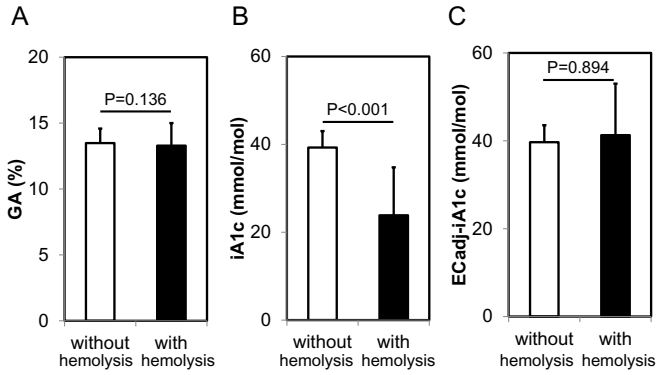

Fig. 3. Comparisons of GA, iA1c and ECadj-iA1c between non-diabetic patients with hemolysis and non-diabetic subjects without hemolysis.
Comparisons of GA (A), iA1c (B) and ECadj-iA1c (C) between 35 non-diabetic individuals with hemolysis (closed square) and 72 non-diabetic individuals without hemolysis (open square) are shown.

(2.74–4.62) and these were not significantly different between the both groups (Fig. 2B). Further, various indicators in the 35 non-diabetic individuals with hemolysis were compared with the 72 non-diabetic individuals without hemolysis. The iA1c concentrations in non-diabetic patients with hemolysis were significantly lower than in the non-diabetic subjects without hemolysis, whereas ECadj-iA1c as well as GA concentrations showed no significant difference between the two

groups (Fig. 3).

5. Discussion

Significant correlations were observed in the GA/iA1c ratio with various indicators of hemolysis but not in the GA/ECadj-iA1c ratio with the most indicators of hemolysis. Further, in the non-diabetic individuals, ECadj-iA1c concentrations in the individuals with hemolysis were not significantly different from that of the individuals without hemolysis. From these findings above, it was suggested that ECadj-iA1c accurately reflected glycemic control in patients with hemolysis.

EC has been reported to be highly correlated with HbA1c in non-diabetic patients [23]. This is because both reflect the mean erythrocyte age. Therefore, EC decreases and HbA1c increases with the associated aging of erythrocyte, and both age-associated changes show a mirror image [9,23]. HbA1c can be an indicator of hemolysis in non-diabetic subjects. However, determination of hemolysis is not feasible based on HbA1c because HbA1c concentrations are also affected by plasma glucose in diabetic patients. In the meantime, GA is a glycemic control indicator that is not affected by hemolysis. Therefore, the GA/HbA1c ratio can be an indicator of hemolysis because the GA/HbA1c ratio was not affected by plasma glucose [12].

HbA1c expressed in NGSP units (A1C) is a value standardized on the basis of HbA1c concentrations measured by high-performance liquid chromatography (HPLC) for the Diabetes Control and Complications (DCCT) Trial in the 1980s. The resolution of HPLC was poor at that time and included components other than HbA1c in the HbA1c fraction (approximately 2.2%). Later, although the ability of HPLC was refined, the A1C value was not changed for the purpose of consistency. Therefore, the low GA/A1C ratio has been pointed out under conditions with a favorable glycemic control status (i.e., state with low HbA1c and GA concentrations) due to mathematical issues. On the other hand, HbA1c expressed in IFCC units (iA1c) is the value of the HbA1c component alone, and the GA/iA1c ratio is, therefore, not affected by the glycemic control status. In the present study, we used the GA/iA1c ratio for analyses because the population, including diabetic patients, was selected. Since the individuals in the present study including diabetic patients, we used GA/iA1c ratio for analysis to avoid the interference of glycemic control status. A positive correlation was observed between EC and the GA/iA1c ratio with a high correlation coefficient of 0.828 (Fig. 1). Although EC was significantly correlated with the GA/A1C ratio, the correlation coefficient 0.523 was lower compared to that with the GA/iA1c ratio.

In diabetic patients with hemolysis, HbA1c concentrations are falsely low and do not accurately reflect the glycemic control status; the glycemic control cannot be determined by HbA1c. Therefore, determination of their glycemic control based on GA has been recommended [11,12]. However, GA concentrations become abnormal in patients with an abnormality in albumin metabolism. For example, in patients with autoimmune hemolytic anemia treated with glucocorticoid, liver cirrhosis, and chronic kidney disease accompanied with proteinuria, GA as well as HbA1c show abnormal concentrations and these cannot be used as glycemic control indicators [11]. In these patients, ECadj-iA1c is expected as a useful glycemic control indicator.

In the present study, we hypothesized and investigated whether iA1c adjusted by EC (ECadj-iA1c) might accurately reflect glycemic control. Significant correlations were observed in the GA/iA1c ratio with various indicators of hemolysis but not in the GA/ECadj-iA1c ratio with all indicators of hemolysis other than haptoglobin. Consequently, the GA/ECadj-iA1c ratio was considered not to be affected by hemolysis because of the adjustment by EC.

Then, comparisons of the GA/ECadj-iA1c ratio between individuals without and with hemolysis were performed to examine whether the values accurately reflected glycemic control. Since HbA1c in individuals with hemolysis is significantly lower than it in individuals without hemolysis, the GA/iA1c ratio in individuals with hemolysis was significantly higher than it in individuals without hemolysis. On the other hand, no significant differences in the GA/ECadj-iA1c ratio were observed between the two groups and those in both groups were in the reference range (Fig. 2). From the findings above, HbA1c might be useful as a glycemic control indicator by adjustment by EC in patients with hemolysis. However, because HbA1c concentrations in diabetic patients with hemolysis are affected by glycemic control, it is difficult to determine whether it accurately reflects glycemic control. Therefore, we studied using non-diabetic individuals. As a result, iA1c concentrations in non-diabetic patients with hemolysis were significantly lower than in the non-diabetic subjects without hemolysis, whereas ECadj-iA1c and GA concentrations showed no significant difference between the two groups (Fig. 3). These findings suggested that ECadj-iA1c is not affected by hemolysis, and reflects accurately glycemic control in patients with hemolysis.

This article had some limitations. First, ECadj-iA1c reflecting glycemic control was indicated by comparisons with GA. Although comparisons with mean plasma glucose obtained by continuous glucose monitoring (CGM) could not be performed, such an investigation is warranted in the future. Second, HbA1c was examined using iA1c expressed in the IFCC units, not using A1C expressed in the NGSP units. A1C has been used in the examination of diabetes mellitus in many countries, and the number of institutions using iA1c is limited. However, a conversion formula for A1C to iA1c has already been reported. Therefore, ECadj-A1C can be obtained with the formula $[(10.93 \times A1C - 23.5) \times (1.04 \times EC + 2.04)/3.5]$ using a formula for ECadj-iA1c shown in this study and for a formula by Hoelzel et al. (iA1c = 10.93 x A1C -23.5) [20].

ECadj-iA1c had no significant correlation with the various indicators of hemolysis, and no significant differences in ECadj-iA1c were observed between patients with and without hemolysis. It is suggested that ECadj-iA1c is a useful glycemic control indicator in patients with hemolysis.

Declaration of Competing Interest

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