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OPEN Estimation of the hemoglobin glycation rate constant

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In a previous study, a method of obtaining mean erythrocyte age (M_{RBC}) from HbA1c and average plasma glucose (AG) was proposed. However, the true value of the hemoglobin glycation constant (k_g dL/mg/day), required for this model has yet to be well characterized. Another study also proposed a method of deriving M_{RBC} from erythrocyte creatine (EC). Utilizing these formulae, this study aimed to determine a more accurate estimate of k_g. One hundred and seven subjects including 31 patients with hemolytic anemia and 76 subjects without anemia were included in this study. EC and HbA1c data were analyzed, and M_{RBC} using HbA1c, AG and the newly-derived constant, k_g were compared to M_{RBC} using traditional ^{51}Cr in three patients whose data were taken from previous case studies. A value of 7.0 x 10^{-6} dL/mg/day was determined for k_g. M_{RBC} using HbA1c, AG and k_g were found to no be significantly different (paired t-test, $p = 0.45$) to M_{RBC} using traditional ^{51}Cr. k_g enables the estimation of M_{RBC} from HbA1c and AG.

Abbreviations

AG	Average plasma glucose
CGM	Continuous glucose monitoring
DM	Diabetes mellitus
EC	Erythrocyte creatine
GA	Glycated albumin
Hb	Hemoglobin
HPLC	High performance liquid chromatography
Hpt	Haptoglobin
IFCC	International Federation of Clinical Chemistry
k_g	Hemoglobin glycation constant
MCDP	10-N-methylcarbamoyl-3,7-bis (dimethylamino) phenothiazine
M_{RBC}	Mean erythrocyte age
NGSP	National Glycohemoglobin Standardization Program
Ret	Reticulocyte
SMBG	Self-monitoring of blood glucose.

HbA1c is widely used as both an indicator of glycemic control, as well as a diagnostic index, for diabetes in clinical settings[1,2]. Hemoglobin glycation is assumed to obey a three compartment model (Fig. 1). The rate constant of the total glycation reaction (k_g) is as follows.

$$k_g = \frac{k_1 k_3}{k_2 + k_3} \tag{1}$$

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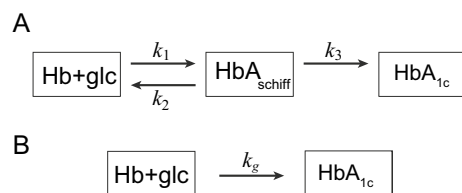
A

B

Figure 1. Hemoglobin glycation. (**A**) HbA1c is produced from Schiff base by amadori rearrangement. (**B**) Simplified two compartment model. HbA$_{schiff}$ is aldimine complex (intermediate product). k_1, k_2, k_3, k_g are kinetic constants.

	Non-hemolysis	Hemolysis	p
n (M/F)	76 (30/46)	31 (17/14)	0.1463
Age (years)	62.3 ± 7.9	45.6 ± 15.0	1.37×10^{-6}
HbA1c (%)	5.78 ± 0.25	4.05 ± 0.78	2.27×10^{-13}
iA1c (mmol/mol)	39.7 ± 2.7	20.8 ± 8.5	2.27×10^{-13}
GA (%)	13.57 ± 1.07	13.06 ± 1.75	0.151
GA/iA1c	0.343 ± 0.032	0.762 ± 0.363	5.85×10^{-7}
Hb (g/dL)	14.26 ± 1.16	9.75 ± 1.97	2.44×10^{-14}
EC (μmol/g Hb)	1.40 ± 0.21	5.47 ± 2.13	1.42×10^{-11}
EC-M_{RBC} (days)	58.5 ± 3.41	29.0 ± 10.0	4.46×10^{-17}

Table 1. Participants characteristics. Results are expressed as mean ± standard deviation (SD). Sex ratio was examined by χ^2 test. Other items were examined by t-test (bilateral). GA, glycated albumin; EC, erythrocyte creatine.

Although HbA1c is generally indicative of recent glycemic control over the past 1–2 months, it is known to show reduced correlation to glycemic control status in the presence of diseases which result in a shortened erythrocyte lifespan such as hemolytic anemia[3].

Erythrocyte creatine (EC) is a good marker that reflects the mean erythrocyte age[4]. We proposed a method that compensates glycated albumin (GA)/IFCC-HbA1c ratio for hemolysis by EC[5].

We have recently proposed a simple method to obtain mean erythrocyte age (M_{RBC}) from HbA1c and average glucose (AG)[6], which has theoretically derived based on Γ-like function model of erythrocyte lifespan[7]:

$$M_{RBC} \simeq \frac{HbA1c}{(1 - \frac{2}{3}HbA1c)k_g AG} \tag{2}$$

This formula provides meaningful information for the diagnosis of anemia. We estimated k_g to be 6–10×10^{-6} dL/mg/day based on past literature[6]. However, a more accurately estimated value of k_g would provide more useful information.

The relationship between M_{RBC} and EC was previously established based on a model and the data[8] from 21 patients, which included EC and ^{51}Cr, as following[9]:

$$M_{RBC} = -22.84 \log_e EC + 65.83 \tag{3}$$

This study aimed to determine the accurate value of k_g from EC-derived M_{RBC} and HbA1c.

Results

Participant characteristics. Participant demographics are shown in Table 1. All participants had no more than 16% GA. There was no significant difference in the GA of anemic and non-anemic subjects. However HbA1c, Hb, EC and their derivatives showed significant variation between the two groups.

The demographic information on the 3 patients from the previous cases are shown in Table 2.

Estimation of k_g. EC derived M_{RBC} and $\frac{iA1c}{1000 - \frac{2}{3}iA1c}$ are shown in Fig. 2. A linear relationship was successfully observed.

k_g calculated by the two methods outlined previously, for non-hemolytic participants and the entire study population are seen in Table 3. All 4 numbers can be approximated to 7×10^{-6}. Figure 2 shows that data from severe hemolytic patients is less stable. Thus, the value derived from the direct method for calculating k_g is likely to be the least accurate. Excluding this value as an outlier, the 3 remaining figures were 6.94–6.99×10^{-6} (average 6.970×10^{-6}). Therefore, considering significant figures, k_g can be said to be 7.0×10^{-6}.

Case	Herranz[10]	Ishii[11]	Hiratani[12]
Age/sex	30F	72M	58F
Disease	AIHA	AIHA	HSt
DM	Type 1	Type 2	Type 2
HbA1c (%)	5.4	6.5	5.8
GA (%)	–	26.1	23.3
Hb (g/dL)	Normal	13.5	11.5
Ret (%)	Normal	1.3	1.3
Hpt (mg/dL)	Normal	82	58

Table 2. Characteristics of three reported patients with latent hemolysis and DM in literature. These patients showed normal Hb, reticulocyte, and haptogloblin. AIHA, autoimmune hemolytic anemia; HSt, hereditary stomatocytosis, DM, diabetes mellitus; GA, glycated albumin; Hb, hemoglobin; Ret, reticulocyte; Hpt, haptoglobin.
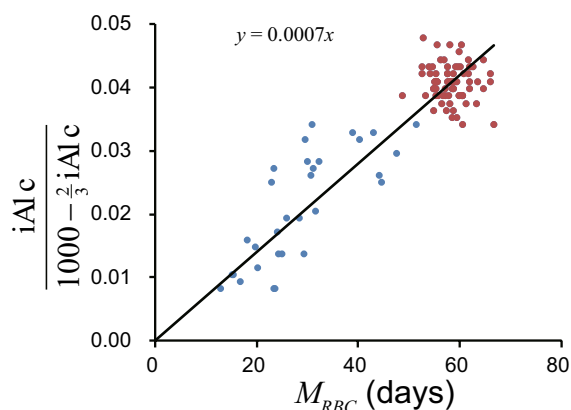
Figure 2. Relationship between EC derived M_{RBC} and iA1c/(1000−(2/3)iA1c). Red circles denote non-hemolytic participants and blue circles denote hemolytic patients. Black line denotes regression line through origin.

Population	Slope	Straight forward
The whole	6.973×10^{-6}	$(7.073 \pm 1.229) \times 10^{-6}$
Non-hemolytic	6.942×10^{-6}	$(6.994 \pm 0.662) \times 10^{-6}$

Table 3. k_g estimation. Results obtained by the direct method are expressed as mean ± standard deviation (SD).

Confirmation of derived k_g. The M_{RBC} using the derived k_g, 7.0×10^{-6} and M_{RBC} using ^{51}Cr half-life are shown in Table 4.

M_{RBC} derived from iA1c were 36.95 ± 5.93, M_{RBC} derived from ^{51}Cr half-life were 41.29 ± 2.22. Paired t-test: t, −0.9278; df, 2; p (bilateral), 0.4514. Thus, M_{RBC} derived from iA1c and M_{RBC} using ^{51}Cr half-life were not significantly different.

Discussion

Based on EC-derived M_{RBC} and HbA1c data, a more accurate value for the constant k_g was obtained. Though k_g was previously determined to be 6–10×10^{-6} dL/mg/day[6], the more accurate value of 7.0×10^{-6} improves the usefulness of the proposed model allowing closer approximation of M_{RBC} based on AG and iA1c.

Moreover, the validity of k_g has been confirmed through comparison of M_{RBC} derived from iA1c and k_g with M_{RBC} derived from ^{51}Cr half-life. Of the three patients with hemolytic anemia and comorbid DM analyzed, data from two patients showed a remarkable correlation with the model derived figures. Data from one patient showed a 1.47 times difference in values however, this may be attributable to the use of SMBG instead of CGM, and the difficulty of standardizing ^{51}Cr data containing elution.

Variant hemoglobin should be distinguished from hemolysis when M_{RBC} determined by Eq. (2) is low. Glycated variant hemoglobin will exhibit different peaks in HPLC from normal HbA1c, resulting in erroneously low

Case	Herranz[10]		Ishii[11]		Hiratani[12]
HbA1c (%)	5.8	4.9	6.0	6.7	6.5
iA1c (mmol/mol)	39.9	30.0	42.3	50.2	47.5
AG (mg/dL)	203	148	138	177	184
iA1c derived (days) M_{RBC}	28.7	29.6	45.3	41.9	38.1
	29.2		43.6		38.1
^{51}Cr half-life (days)	20		20		17.8
^{51}Cr derived M_{RBC} (days)	42.9		42.9		38.1

Table 4. M_{RBC} of 3 cases in literature. HbA1c value is different from Table 2. The AG in Herranz[10] and Ishii[11] were calculated from blood glucose values using self-monitoring of blood glucose (SMBG).

values for HbA1c (some variants show an artefactually high value). It has previously been reported that variant hemoglobin can be detected by the dissociation between HbA1c measured by HPLC and by immunoassay[13]. Moreover, some variant hemoglobins such as Hb Himeji[14] have different k_g values from normal Hb. In patients with these variant hemoglobins, Eq. (2) is likely to provide a falsely low M_{RBC}.

There are a number of limitations to this study. The data used to calculate a more specific estimate of k_g contained EC and HbA1c, but lacked CGM data, necessitating the use of 100 mg/dL as an approximation of AG. However, participants were confirmed to be free of DM through GA, an indicator of glycemic control that is independent of mean erythrocyte age, with a cut off of GA no more than 16%. Further study with more complete data including CGM, HbA1c and EC would provide an even more definitive value for k_g. Another limitation is that the value for k_g derived in this study is totally dependent on Eq. (3) that derives M_{RBC} from EC. This equation was based on old published data[8], which used less sensitive and poorly specific chemical methods of measuring creatine which were prone to cross-reactivity with other guanidino compounds. This may reduce the reliability of the system. In contrast, in this study creatine was measured using an enzymatic method which was sensitive and specific to creatine in erythrocytes which uses 10-N-methylcarbamoyl-3,7-bis(dimethylamino) phenothiazine (MCDP), an N-methylcarbamoyl derivative of methylene blue, with a high molar absorption coefficient (9.6×10^7 L mol^{-1} cm^{-1})[4], as a chromogen.

Methods

Participants. One hundred and seven subjects including 31 patients with hemolytic anemia and 76 subjects without anemia were included in this study. All samples were prepared and analyzed in accordance with the protocols approved by the institutional committees at Kumamoto University and other collaborating institutions.

Patients with hemolytic anemia were recruited from 115 patients who were older than 20 years old and required laboratory tests including complete blood counts and reticulocyte counts (Ret) for clinical reasons. Those who were suspected of having diabetes mellitus (DM) based on history, a low 1,5-Anhydroglucitol (1,5-AG) value (male, < 14.9 μg/mL; female, < 12.4 μg/mL), or had comorbid liver or renal diseases, were excluded, as liver and renal diseases affect HbA1c and GA. EC, HbA1c, GA, haptoglobin, and other biochemical screening items were measured using the existing plasma samples from these patients. Use of existing plasma samples from anemic patients without written consent was approved by the institutional review board.

Participants without anemia were recruited from medical examination checkup recipients at Takagi Hospital. Those who had anemia, DM, liver disease, renal disease or who were pregnant were excluded to avoid confounding effects on HbA1c or GA value. We provided the healthy volunteers with detailed information about the study, and all participants without anemia provided written informed consent to participate.

Data interpretation. EC was measured enzymatically in accordance with a previous report[4], HbA1c was measured by high performance liquid chromatography (HPLC) method[15], and GA was measured by enzymatic method using albumin-specific protenase, ketoamine oxidase, and albumin assay reagent (Lucica GA-L; Asahi Kasei Pharma Co., Tokyo, Japan)[16].

HbA1c expressed in International Federation of Clinical Chemistry (IFCC) units (iA1c) was used for calculations in this study. While the National Glycohemoglobin Standardization Program (NGSP) is used to express HbA1c in many clinical research and medical care settings, NGSP is measured by an old standardized method and at the time of conception, HPLC was not able to distinguish true HbA1c from other products. HPLC technology later advanced, however the derived HbA1c value is adjusted to NGSP in the interest of consistency. IFCC provides a strict definition of iA1c as hemoglobin with a glycated valine in the N-terminal β-chain. Thus, iA1c value is preferred value for estimation of hemoglobin glycation.

To acquire iA1c from HbA1c expressed in NSGP unit, we used the following equation[17]:

$$\text{HbA1c}_{\text{NGSP}} \ (\%) = 0.0915 \times \text{iA1c (mmol/mol)} + 2.153 (\%) \tag{4}$$

$$\Longleftrightarrow \text{iA1c(mmol/mol)} = 10.93 \times \text{HbA1c}_{\text{NGSP}} \ (\%) - 23.53 \tag{5}$$

M_{RBC} was acquired from EC by the aforementioned Eq. (3).

An AG value of 100 mg/dL was substituted for plasma glucose values derived using CGM. This number was based on the average AG of non-diabetic participants and the previously reported findings from a study which showed the median AG in healthy subjects to be reported to be 101.0 (96.3–106.0) mg/dL[18] and another ADAG (A1c-derived average glucose) study which found that the AG of the non-diabetic group of their study was similarly 100 mg/dL[19,20].

M_{RBC} was also determined using ^{51}Cr half-life. As the reference range for ^{51}Cr half-life was described as 28–30 days[10], 30 ± 5 days[11], and 26–40 days[12], M_{RBC} was calculated by multiplying ^{51}Cr half-life and 2.14 (= 60/28), 60 days being the normal value for M_{RBC}.

Data analysis. *EC* and M_{RBC} data were analyzed using a spreadsheet software, Excel 365 (Microsoft Corporation, Redmond, WA, USA).

Estimation of k_g. The following two methods were used to estimate k_g. The slope method—the following Eq. (6) derived from Eq. (2) shows that the slope of the line connecting a point and the origin is k_g AG.

$$\frac{\text{iA1c}}{1000 - \frac{2}{3}\text{iA1c}} = k_g \text{AG} \times \text{M}_{\text{RBC}} \tag{6}$$

Estimating the slope of the regression line through the origin by the least square model:

$$\frac{\sum_i^n x_i y_i}{\sum_i^n x_i^2} \tag{7}$$

where x_i, y_i are M_{RBC} and $\frac{\text{iA1c}}{1000 - \frac{2}{3}\text{iA1c}}$ of each participant, respectively.

The direct method—the k_g of each participant was calculated by the following equation:

$$k_g = \frac{\text{iA1c}}{(1000 - \frac{2}{3}\text{iA1c})M_{RBC}\text{AG}} \tag{8}$$

Then, average and standard deviation of each k_g was calculated.

Confirmation of derived k_g. The method of obtaining M_{RBC} from AG and iA1c was applied to data from three patients with latent hemolysis who were presented in a previous case studies[10–12].

Data of Herranz[10] and Ishii[11] showed changes in HbA1c during the course of the study. Therefore, M_{RBC} was calculated separately for each period. For the Ishii case[11], AG was calculated by averaging self-monitoring of blood glucose (SMBG) data for each period. The Hiratani study[12] examined ^{51}Cr erythrocyte lifespan measurement during hospitalization in Oct 1999 and CGM in Feb 2016. While HbA1c and plasma glucose concentrations fluctuate routinely, RBC lifespan remain comparatively constant, especially when influenced by a certain diseases (stomatocytosis). Furthermore, supply of ^{51}Cr was ceased in Japan in 2015 and thus it can no longer be used to study erythrocyte lifespan.

Ethical approval and consent to participate. The work was conducted in accordance with Ethical Guidelines for Medical and Health Research Involving Human Subjects in Japan and conformed to the Helsinki Declaration. All samples were prepared and analyzed in accordance with the protocols approved by the institutional committees at Kumamoto University and other collaborating institutions.

Data availability
The data supporting the findings can be obtained on reasonable request to the corresponding author.

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Acknowledgements

Author contributions

M.Ka. contributed to study design, discussing the results, statistical analysis, project administration, the figures and initial draft manuscript preparation. T.O. contributed to organization of data acquisition, measurement of erythrocyte creatine, and advised the project. S.T., Y.M., H.M., Y.O., T.I. contributed to acquisition of data. K.H. advised the project. M.Ko. contributed to the conceptualization, study design, data curation, discussing the results, statistical analysis, and project administration. All the authors discussed the project and have read and approved the final manuscripts.

Competing interests

Additional information

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