Research Paper

A novel method for calculating mean erythrocyte age using erythrocyte creatine

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ABSTRACT

Estimating the lifespan of erythrocytes is useful for the differential diagnosis of anemia. However, measuring the lifespan of erythrocytes was very difficult; therefore, it was seldom measured. Erythrocyte creatine (EC) decreases reflecting erythrocyte age. We developed a method to obtain mean erythrocyte age (M_{RBC}) from *EC*. We reanalyzed the previously published data from 21 patients with hemolytic anemia, which included *EC* and the half-life of ^{51}Cr.
M_{RBC} and $\log_e EC$ showed excellent significant linearity ($r = -0.9475$, $p < 0.001$), proving that it could be treated as a mono-exponential relationship within the studied range (*EC*: 1.45 – 11.76 µmol/g Hb). We established an equation to obtain M_{RBC} (days) from *EC* (µmol/g Hb): $M_{RBC} = -22.84 \log_e EC + 65.83$.
This equation allowed calculation of M_{RBC} based on EC which has practical applications such as the diagnosis of anemia.

INTRODUCTION

Estimating the lifespan of erythrocytes is useful for the differential diagnosis of anemia, as it is known that the erythrocyte lifespan in hemolytic patients is shortened [1]. Previously, obtaining the lifespan or mean age of erythrocytes was very difficult; therefore, it was seldom measured. Furthermore, supply of ^{51}Cr, which is needed for measuring erythrocyte lifespan, was ceased in Japan in 2015 due to low demand. This left Japanese doctors unable to measure the erythrocyte lifespan of patients by means of ^{51}Cr. Biotin-labeling [2, 3] is also used to measure the erythrocyte lifespan, however its procedure is very laborious as well, requiring aseptic labeling of the erythrocytes and repeated blood samplings. Breath carbon monoxide (CO) measurement [4, 5] also may be useful to estimate erythrocyte turnover; however, this technique cannot be applied to smokers. We have proposed a method to estimate erythrocyte mean age from HbA1c and average glucose [6]. However, the method needed a glycation constant to be determined by another method. Some indices such as reticulocyte and haptogloblin were not sensitive enough to indicate mild hemolysis. Cases with latent hemolysis were reported which showed normal reticulocyte and normal haptogloblin levels, and yet, they showed shortened erythrocyte lifespan [7–9].

Creatine in the cells is maintained by creatine transporters. Deficiency in these transporters leads to symptoms [10, 11]. Young erythrocytes have adequate transporter activity resulting in intracellular creatine being tens of times higher than in plasma. However, the activity of the transporter gradually diminishes, so that old erythrocytes cannot maintain this concentration gradient.

Erythrocyte creatine (EC) has been demonstrated to be an excellent indicator of hemolysis [12, 13]. Estimation of mean erythrocyte age (M_{RBC}) using EC would be more convenient than the ^{51}Cr method, as it requires only one blood sample. Though an increase in EC value has been correlated with shorter lifespan of erythrocytes, EC value itself has not previously been used for the estimation of M_{RBC} directly. An estimation of M_{RBC} would be more useful for quantitative assessment of patients than simple EC value. Moreover, M_{RBC} derived by EC may be comparable with M_{RBC} derived by other methods.

In this study, we aimed to formulate an equation to obtain M_{RBC} from EC concentration based on a model.

RESULTS

Relationship between M_{RBC} and $\log_e EC$

A significant linear relationship (r = −0.9475, df =19, t = 12.92, $p = 7.368 \times 10^{-11}$) was observed between ^{51}Cr-derived M_{RBC} and $\log_e EC$ (Figure 1). The relationship appears to be mono-exponential which is concurrent with the prediction by our model (Supplement) that the relationship would be bi- or mono-exponential.

A regression line was as follows.

$$\log_e EC = -0.04379 M_{RBC} + 2.882 \quad (1)$$

$$\Leftrightarrow M_{RBC} = -22.84 \log_e EC + 65.83 \quad (2)$$

A standard value of EC of 1.4 µmol/g Hb gives an M_{RBC} of 58.14 days.

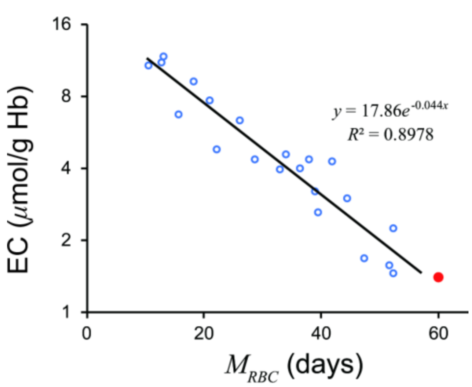

Figure 1. Relationship between M_{RBC} and $\log_e EC$. A significant linear relationship was observed. A *red closed circle* denotes a standard value; M_{RBC} = 60 days, EC = 1.4µmol/g Hb. A *black line* denotes a regression line. *EC*, erythrocyte creatine; M_{RBC}, mean erythrocyte age.

Equation (2) accurately estimated M_{RBC} from EC values (Figure 2).

DISCUSSION

The current study successfully established a reliable method of estimating M_{RBC} from EC based on a creatine model (Supplement). We would be able to determine a glycation constant for the method to estimate erythrocyte mean age from HbA1c and average glucose [14].

Although Fehr et al. [13] divided patients into a severe hemolytic disease group and a group with milder forms of hemolysis, our model suggested that logarithm of EC may combine the two groups (Figure 3). The regression formula passed close to a standard value of EC, 1.4 µmol/g Hb and 60 days of M_{RBC}, which proves the validity of the formula.

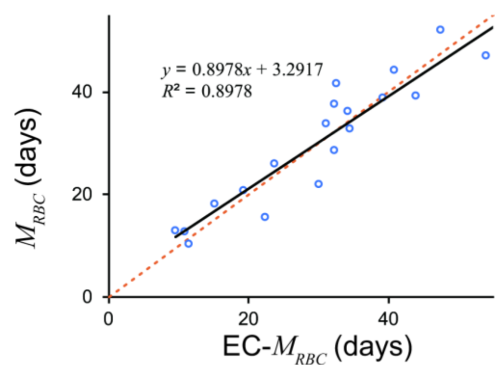

Figure 2. M_{RBC} estimated by EC and ^{51}Cr. EC derived M_{RBC} showed excellent estimation. An *orange dotted line* denotes line of identification ($y = x$). A *black line* denotes a regression line. *EC*, erythrocyte creatine; M_{RBC}, mean erythrocyte age.

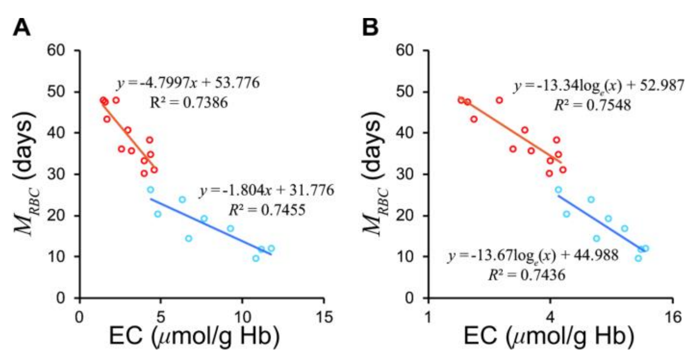

Figure 3. Relationship between EC and M_{RBC} in the groups with severe and mild hemolytic disease. (**A**) The two groups show differing regression lines on a normal scale. (**B**) The two groups are unified on a semi-logarithmic scale. The *Red circles* represent mild group, *sky blue* the severe group according to Fehr et al. [13]. *EC*, erythrocyte creatine; M_{RBC}, mean erythrocyte age.

It cannot be determined which wing of the two lines (Supplement) the observed line of the $\log_e EC - M_{RBC}$ relationship is on; *i.e.* whether the slope of the graph represents the rate constant for creatine diffusion (λ_1) or the rate constant for decline in creatine transporter (λ_2). Another equation may need to be developed for value ranges not explored in this study.

The devised method was formulated entirely based on the previously presented data from only 21 patients

This method should be verified by further study with various hematological diseases including thalassemia and hereditary spherocytosis. Estimation of M_{RBC} from ^{51}Cr half-life may not be optimal, although we believe that it would be tolerable. The EC transporter activity function, $Be^{-\lambda_2 t}$ relies solely on the assumption that the number of transporters reduces overtime randomly due to erythrocytes' lack of nucleus. However, the linear relationship between $\log_e EC$ and M_{RBC} confirms the assumption. The EC measuring method of Fehr et al. [13] used a diacetyl-*l*-naphthol chemical reaction, which is less sensitive than the recently developed *N*-methylcarbamoyl derivative of methylene blue, 10-*N*-methylcarbamoyl-3,7-*bis*(dimethylamino)phenothiazine (MCDP) enzyme method [15]. Further study on the validity of our proposed formula would be best done in a country where ^{51}Cr is available.

CONCLUSIONS

Our equation does allow calculation of M_{RBC} based on EC, which has practical applications such as the diagnosis of anemia.

MATERIALS AND METHODS

Patients

Data from 21 patients with hemolytic anemia, that was published by Fehr et al. [13], was examined. As this is a re-analysis study, approval by the institutional review board was not required.

Data conversion

We estimated M_{RBC} by multiplying the half life of ^{51}Cr by 2.61. As human erythrocytes do not obey the Poisson process [16], the term "half-life" is not entirely suitable for erythrocytes. Fehr et al. [13] determined ^{51}Cr half-life, the elution-corrected ^{51}Cr half-life, and the mean cell lifespan. The mean cell lifespan was not recorded in their table. The elution-corrected ^{51}Cr half-life would provide an estimate of M_{RBC}, considering that normal erythrocytes in a human have a similar lifespan [16]. However, their elution-corrected ^{51}Cr half-life seems less concordant with EC rank. Complicated procedures sometimes reduce the stability of the system. Therefore, we chose the simple uncorrected ^{51}Cr half-life in the same way as Fehr et al. [13]. Considering that M_{RBC} for normal erythrocytes is about 60 days, and the normal range of ^{51}Cr half-life was 23 – 27 days, multiplying ^{51}Cr half-life by 2.61 (= 60/23) provides a good estimation of M_{RBC} in practice.

The units for erythrocyte creatine concentration used by Fehr et al. [13] were mg/dL of red cells. We converted these into µmol/g Hb by the following equation, assuming mean cell hemoglobin concentration (MCHC) is 33g/dL. The molecular weight of creatine is 131.15. While MCHC varies naturally and decreases in iron deficiency anemia, variability in MCHC is generally low.

$$x \text{mg/dL} = x \times \frac{10^3}{131.15 \times 33} \mu\text{mol/g Hb} = \frac{x}{4.328} \mu\text{mol/g Hb} \quad (3)$$

Data analysis

Data on EC and M_{RBC} were analyzed with a spreadsheet software, Excel® 365 (Microsoft Corporation, Redmond, WA, USA).

Logarithms of EC and M_{RBC} were plotted based on our model (Supplement).

AUTHOR CONTRIBUTIONS

M. Kameyama contributed to theory, the analysis of the data, writing the original draft, and funding acquisition. M. Koga contributed to conceptualization, the analysis of the data, and supervision. T.O. contributed to advise on the nature of EC. All the authors have read and approved the final manuscript.

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CONFLICTS OF INTEREST

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SUPPLEMENTARY MATERIALS

Supplementary Methods

Theory

The terms in the equations are summarized in Table 1.

Creatine in a single erythrocyte

The rate of diffusion of creatine would be proportional to the concentration of creatine in the cell. We assume that the transporter activity obeys an exponential function; *i.e.* transporters diminish randomly (proportional to the number of the transporter) and are not renewed due to lack of nucleus.

$$\frac{dCr}{dt} = -\lambda_1 Cr(t) + Be^{-\lambda_2 t} \quad (1)$$

where $Cr(t)$ denotes creatine concentration in a *t*-day-old erythrocyte, λ_1 denotes rate constant of creatine diffusion. $Be^{-\lambda_2 t}$ ($B > 0$) is creatine transporter activity. This differential equation can be solved analytically if $\lambda_1 \neq \lambda_2$.

$$\frac{d(Cr - \frac{B}{\lambda_1-\lambda_2}e^{-\lambda_2 t})}{dt} = -\lambda_1 Cr(t) + \frac{B\lambda_1}{\lambda_1-\lambda_2}e^{-\lambda_2 t} \quad (2)$$

$$Cr(t) = Ae^{-\lambda_1 t} + \frac{B}{\lambda_1-\lambda_2}e^{-\lambda_2 t} \quad (3)$$

where *A* denotes an integral constant.

$Cr(t)$ is a sum of two exponential functions, it can be treated as a bi-exponential function or a monoexponential function within a range of interest.

The fate of $Cr(t)$ is dependent on whether $\lambda_1 - \lambda_2 > 0$ or not. If $\lambda_1 - \lambda_2 < 0$ and $C'(0) > 0$, $Cr(t)$ has a peak when $t > 0$ (Figure 1 *orange line*). It can be treated as a mono-exponential function after the second term is negligible. However, as EC monotonically and rapidly decreases after birth of the erythrocytes [1], we can exclude this condition.

If $\lambda_1 - \lambda_2 < 0$ and $C'(0) \leq 0$, $Cr(t)$ decreases monotonically. As $\lambda_2 > \lambda_1$, $e^{-\lambda_2 t}$ decreases more rapidly. Moreover, the second negative term must be small, considering that $C'(0) < 0$ yields $\frac{B}{\lambda_2-\lambda_1} < \frac{\lambda_1}{\lambda_2}A$. Therefore, the second negative term can be considered negligible comparing the first term.

Table 1. Terms used in the text.

Term	Definition	Representative value
$Cr(t)$	creatine concentration in a *t*-day-old erythrocyte	
λ_1	rate constant for creatine diffusion	
λ_2	rate constant for decline in creatine transporter	
λ	substitute for λ_1 or λ_2	
A, *B*, *C*, *D*	constants	
EC	mean erythrocyte creatine concentration	1.4 µmol/g Hb
α	a parameter of gamma distribution	25.59
β	a parameter of gamma distribution	5.59
$p(t)$	probability density function of RBC death	
R_0	erythrocyte production rate	– /day
$R(t)$	the number of erythrocytes at *t* days after birth	
M_{RBC}	mean red blood cell age	60 days
RBC	number of erythrocytes	–

If $\lambda_1 - \lambda_2 > 0$, $Cr(t)$ is a bi-exponential function. The logarithm of a bi-exponential function can be expressed by a bent line (Figure 1B *blue line*), because a large *t* makes one term negligible, while small *t* (→−∞) makes the other term negligible.

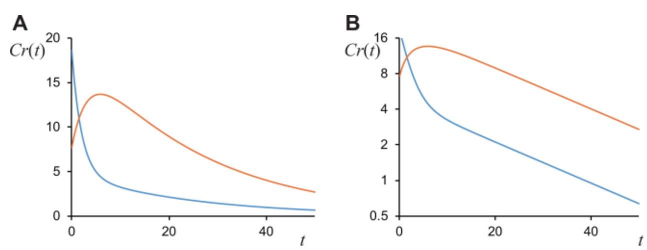

Figure 1. Examples of $Cr(t)$. (**A**) normal scale; (**B**) logarithmic scale. *Orange line:* $\lambda_1 - \lambda_2 < 0$ and $C'(0) > 0$, the function has a peak. *Blue line:* $\lambda_1 - \lambda_2 > 0$, $Cr(t)$ is a bi-exponential function. The logarithm of a bi-exponential function can be expressed by a bent line.

Problem applying single cell model to erythrocyte population

Equation (3) itself would not be suitable to obtain mean erythrocyte age, because EC is not measured from a single cell.

$$EC = \left(\sum_i^n Cr(t_i)\right)/n = A\left(\sum_i^n e^{-\lambda_1 t_i}\right)/n + \frac{B}{\lambda_1-\lambda_2}\left(\sum_i^n e^{-\lambda_2 t_i}\right)/n \quad (4)$$

$y=e^{-\lambda x}$ is downward convex. The centroid of n-polygonal, $(t_i, e^{-\lambda t_i})$ is over the curve of $y=e^{-\lambda x}$.

$$\left(\sum_i^n e^{-\lambda t_i}\right)/n \geq \exp\left(-\lambda\frac{\sum_i^n t_i}{n}\right) \quad (5)$$

Therefore, when $\lambda_1-\lambda_2>0$,

$$EC \geq Ae^{-\lambda_1 M_{RBC}} + \frac{B}{\lambda_1-\lambda_2}e^{-\lambda_2 M_{RBC}} \quad (6)$$

Erythrocyte lifespan

Kameyama et al. [2] have recently calculated RBC lifespan based on the probability density function $p(t)$ of RBC death proposed by Shrestha et al. [3].

$$p(t) = \frac{1}{\Gamma(\alpha)\beta^\alpha}t^{\alpha-1}e^{-t/\beta} \quad (7)$$

Γ denotes the Euler gamma function.

$$\Gamma(\alpha) = \int_0^\infty x^{\alpha-1}e^{-x}dx \quad (8)$$

The number of erythrocytes (RBC) and mean erythrocyte age (M_{RBC}) was calculated. (See Kameyama et al. [2] for details.)

$$RBC = R_0\int_0^\infty tp(t)dt = R_0\alpha\beta \quad (9)$$

$$M_{RBC} = \frac{(\alpha+1)\beta}{2} \quad (10)$$

Creatine model

The number of t-day-old erythrocyte is $R(t)$. Each RBC has $Ae^{-\lambda_1 t}+\frac{B}{\lambda_1-\lambda_2}e^{-\lambda_2 t}$ creatine. Therefore, mean creatine concentration, EC can be described as follows:

$$EC = \int_0^\infty R(t) \times\left(Ae^{-\lambda_1 t}+\frac{B}{\lambda_1-\lambda_2}e^{-\lambda_2 t}\right)dt/RBC \quad (11)$$

$$\int_0^\infty R(t)\times e^{-\lambda t}dt = \left[R(t)\frac{e^{-\lambda t}}{-\lambda}\right]_0^\infty - \int_0^\infty R'(t)\frac{e^{-\lambda t}}{-\lambda}dt = \frac{R_0}{\lambda} - \frac{R_0}{\lambda}\int_0^\infty p(t)e^{-\lambda t}dt \quad (12)$$

$$\int_0^\infty p(t)e^{-\lambda t}dt = \frac{1}{\Gamma(\alpha)\beta^\alpha}\int_0^\infty t^{\alpha-1}e^{-(1/\beta+\lambda)t}dt = \frac{1}{\Gamma(\alpha)\beta^\alpha}\frac{\Gamma(\alpha)}{(1/\beta+\lambda)^\alpha} = \frac{1}{(1+\beta\lambda)^\alpha} \quad (13)$$

Hence, EC can be expressed as follows:

$$EC = \frac{A}{\lambda_1\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda_1)^\alpha}\right) + \frac{B}{\lambda_1-\lambda_2}\frac{1}{\lambda_2\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda_2)^\alpha}\right) \quad (14)$$

Approximation of the derived relationship

The Taylor expansion provides the following equation:

$$(1+\beta\lambda)^{-\alpha} \approx 1-\alpha\beta\lambda+\frac{\alpha(\alpha+1)}{2}(\beta\lambda)^2 - \frac{\alpha(\alpha+1)(\alpha+2)}{6}(\beta\lambda)^3+\ldots \quad (15)$$

$$\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^\alpha}\right) \approx \frac{1}{\lambda\alpha\beta}\left(\alpha\beta\lambda-\frac{\alpha(\alpha+1)}{2}(\beta\lambda)^2 + \frac{\alpha(\alpha+1)(\alpha+2)}{6}(\beta\lambda)^3-\ldots\right) = 1-\frac{\beta(\alpha+1)}{2}\lambda+\frac{(\alpha+1)(\alpha+2)}{6}(\beta\lambda)^2-\ldots \quad (16)$$

As $\alpha >> 1$,

$$\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^\alpha}\right) \approx 1-\lambda M_{RBC}+\frac{2}{3}\lambda^2 M_{RBC}^2-\ldots \quad (17)$$

Thus, $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$ can be described approximately as a function of M_{RBC}. Although how α and β vary when M_{RBC} decreases or increases cannot be determined, this implies that the function $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$ would not be greatly affected by α and β if $M_{RBC}=(\alpha+1)\beta/2$ is satisfied. This can be confirmed numerically (Figure 2A). Calculations based on the assumption that β was constant and β was proportionate to α showed similar results. Therefore, β can be considered a constant.

Below, $\frac{1-e^{-x}}{x}$ was approximated to be De^{-Cx}, as the shape of the graphs were similar (Figure 2B).

$$C=\frac{-x_0e^{-x_0}+(1-e^{-x_0})}{x_0(1-e^{-x_0})},\qquad D=\frac{1-e^{-x_0}}{x_0e^{-Cx_0}} \tag{18}$$

provides the same value of the two function and the differential function at $x=x_0$. Figure 2B visualizes the approximation.

As $\frac{1-e^{-x}}{x}$ can be treated as an exponential function, it follows that $\frac{1}{\lambda\beta x}\left(1-\frac{1}{(1+\beta\lambda)^{x}}\right)$ can also be treated as an exponential function, because

$$\begin{aligned}&\frac{1}{\lambda\beta\alpha}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)\\&=\frac{\log_e(1+\beta\lambda)}{\lambda\beta}\frac{1-\exp(-\log_e(1+\beta\lambda)\alpha)}{\log_e(1+\beta\lambda)\alpha}\\&\approx\frac{\log_e(1+\beta\lambda)}{\lambda\beta}D\exp\left(-C\log_e(1+\beta\lambda)\left(\frac{2M_{RBC}}{\beta}-1\right)\right)\end{aligned} \tag{19}$$

C, D can be estimated by equation (18). To obtain an approximation around M_{RBC0}, x_0 should be as following:

$$x_0=\log_e(1+\beta\lambda)\left(\frac{2M_{RBC0}}{\beta}-1\right) \tag{20}$$

In conclusion, EC can be expressed approximately as a (bi-)exponential function of M_{RBC}.

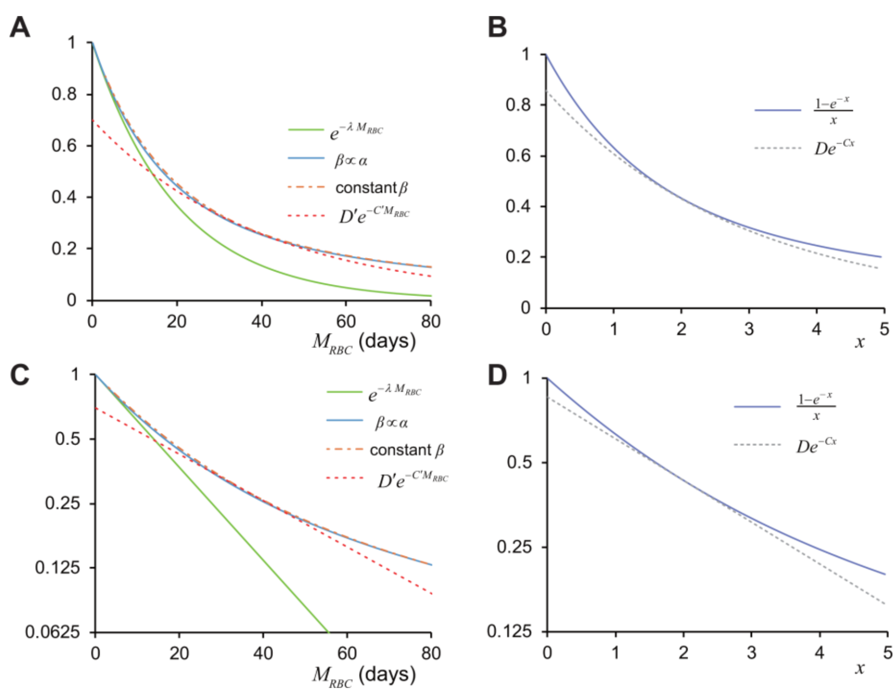

Figure 2. (**A**) Relatiohship between M_{RBC} and $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$. The two condition of $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$ (constant β and $\beta\propto\alpha$) showed similar results. Note that $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$ is consistently larger than $e^{-\lambda M_{RBC}}$. $D'e^{-C'M_{RBC}}$ is an approximation at $M_{RBC}=36.33$ with exponential function by equation (19). (**B**) $\frac{1-e^{-x}}{x}$ and its approximation, De^{-Cx} (equation (18)) when $x_0=2$. (**C**, **D**) Semi-log scales of (**A**, **B**) show that $\frac{1-e^{-x}}{x}$ and $\frac{1}{\lambda\alpha\beta}\left(1-\frac{1}{(1+\beta\lambda)^{\alpha}}\right)$ can be treated as an exponential function.

Supplementary References

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