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Reticulocyte-Based Estimation of Red Blood Cell Lifespan

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Abstract

We introduce a new, minimally invasive laboratory technique called REticulocyte-Based Estimation of Lifespan (REBEL) of erythrocytes in humans. Its major advantage over existing techniques is its applicability to patients with both changing and steady-state erythropoiesis status. The feasibility of REBEL was tested in five hemodialysis dependent End-Stage Renal Disease patients. The RNA degradation half-life was first determined for each subject on day one by flow cytometry measurement of the decay rate of Thiazole Orange stain. Reticulocyte age distribution was then measured from residual RNA content weekly for two months to estimate the RBC production rate time course. Mean RBC lifespan per subject was estimated by fitting the integrated RBC production rate overtime to the measured RBC count and optimizing the integration limits. All calculations were performed in MATLAB. The mean reticulocyte RNA half-life was 0.71 ± 0.11 days. The small coefficient of variation 15.6% indicated that the degradation rate of RNA did not vary substantially between subjects. The mean RBC lifespan $T_{RBC} = 76.6 \pm 23.8$ days was comparable to the reported values for this patient population.

Keywords

red blood cell lifespan; reticulocyte age distribution; flow cytometry; End-Stage Renal Disease

Introduction

Standard techniques for RBC lifespan estimation use a label agent that attaches to the cell during its circulation within the body and measuring that agent's activity over time. In general, two types of labeling methods have been developed: cohort labeling, where cells of

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a certain age are labeled, and random labeling, where all cells present at one moment in time are labeled irrespective of their age [1, 2]. A cohort labeling method such as heavy nitrogen (^{15}N), or radioactive iron (^{59}Fe), incorporates the label into the RBC precursor cells during their production in the bone marrow, usually as a substrate used in hemoglobin synthesis. However, the incorporation of the label into the precursors does not occur instantly after administration of the label and may be prolonged over several days resulting in labeled RBCs which are not exactly of the same age. In addition, cohort labeling methods suffer from reuse of the label in the production of further RBCs [3]. Random labeling with chromium 51 (^{51}Cr) is a commonly used clinical method for red cell survival analysis. This labeling method is in general conducted ex vivo, which can damage the cells and thus might affect their subsequent survival in the circulation. ^{51}Cr labeling also appears more sensitive to loss of the label via dissociation of the label from the viable RBC as well as radioactive decay. Despite general disadvantages using radioactive tracers and unpredictable values of ^{51}Cr elution, tagging with this radioisotope remains the principal method of red cell labeling. Recently, biotin labeling of RBC has been more frequently used as a nonradioactive, nontoxic alternative for red cell survival studies [4]. Both techniques expose the patient to potential risk factors related to autologous transfusion of a manipulated blood product. Other methods, such as carbon monoxide (CO) exhalation, have been developed to determine RBC turnover without depending on a label [5–7]. This technique is confounded by environmental issues and only gives a measure of RBC turnover at a certain time point assuming stationary production of hemoglobin during the RBC lifespan.

Shortening of RBC lifespan is one of the factors that contribute to the anemia of chronic kidney disease. The toxic uremic environment damages circulating RBC's and decreases their lifespan by as much as 70% [8]. Furthermore, aggressive adjustments of exogenous erythropoietin (EPO) may also affect RBC turnover. Rapid dose decrease or even discontinuation of EPO is known to trigger preferential destruction of newly-formed RBC, a process termed neocytolysis [9]. Furthermore, RBCs produced by patients with chronic renal disease exhibit varying hemoglobin content due to disrupted iron kinetics. As a result, hypochromic RBC's with shortened lifespan are often produced [10]. Blood loss, common in the hemodialysis patient, may also manifest as decreased RBC lifespan. On average, an ESRD patient loses 3 L of blood per year due to processes associated with dialysis [11]. Variability in RBC lifespan confounds the treatment of renal anemia if the treatment is only based on longitudinal measurement of hemoglobin alone. This is because the RBC production rate and the RBC turnover rate are convoluted with each other. Being able to pinpoint inter- and intra-individual variations in RBC lifespan may significantly improve the anemia management practice patterns [12].

The objective of this study is to introduce a new technique of RBC lifespan estimation from longitudinal reticulocyte measurements. A proof of concept is demonstrated on a small sample data set acquired from End Stage Renal Disease patients undergoing anemia management

Materials and Methods

The fundamental idea of the proposed technique is to deduce the RBC production rate (k_{RBC}) from reticulocyte data and utilize a pharmacodynamic relationship between RBC count, k_{RBC} , and RBC lifespan (T_{RBC}) to back-calculate the latter. The RBC production rate can be obtained from the reticulocyte age distribution obtained by flow cytometry readings provided that the reticulocyte RNA degradation half-life ($t_{RNA1/2}$) is known.

Study Subjects

The feasibility study was performed as a part of Intelligent Control Approach to Anemia Management trial (NCT00572533). The study protocol was approved by the Institutional Review Board of the University of Louisville and conformed to the Declaration of Helsinki. Out of the 62 subjects enrolled in the parent study, 5 subjects were selected and consented for the sub-study. Blood samples (~ 1 mL) were collected pre-dialysis weekly for 8 consecutive weeks and stored at 4°C for flow cytometric analysis that took place within 6 hours of acquiring the sample. RBC count was obtained from the sample collected for the parent study. The sample collected on Day 1 of the sub-study was used to establish degradation rate of the reticulocyte RNA.

Half-life for residual RNA degradation

The sample collected on Day 1 was placed in Dulbecco medium (RPMI Medium 1640, Gibco) and incubated in 37° C and 5% CO₂ atmosphere for 12 h. At 1, 3, 6, and 9 h, a sample was taken and evaluated for reticulocyte count using flow cytometry. The percent reticulocyte content in the blood was measured using Thiazole Orange staining according to the manufacture instructions (Retic-reagent, Becton Dickinson). The absolute reticulocyte count was computed by multiplying the reticulocyte percentage by the RBC count obtained from the CBC reading and plotted vs. time. A monoexponential curve was fitted to these data to determine the exponent λ which was the slope of the line in the semi-logarithmic scale of RET vs. time (see Fig. 1). The half-life of reticulocyte RNA degradation was calculated as

$$t_{RNA1/2} = \frac{\ln 2}{\lambda} \quad (1)$$

Estimation of RBC Production Rate

The weekly blood samples were stained with Thiazole Orange and the distribution of the fluorescent signal (FL1) in reticulocytes was measured by flow cytometry (Becton-Dickinson, FACSCalibur) as described previously [14]. Briefly, 5 μ l of blood was added to tubes containing a staining solution of 1 ml of thiazole orange solution (Retic-reagent, Becton Dickinson). The samples were incubated for 30 min in the dark at room temperature. Then, flow cytometry analysis was performed by recording 500,000 events with the detection of forward scatter (FCS), side scatter (SCS), and green fluorescence (FL1). The debris and platelets were discriminated from RBC based on the size. The gated RBCs were presented as two histograms of FL1 signals emitted by stained and unstained RBC. The unstained part served to find the threshold channel (ch_T) separating mature RBC from RET.

The relationship between the FL1 represented by the channel number ch and a time a reticulocyte needs to convert to a mature RBC defined as a RBC with an RNA content less than ch_T has been derived elsewhere [13]:

$$RT(ch) = \frac{\ln(10)}{256\ln(2)} t_{RNA1/2} (ch - ch_T) \quad (2)$$

Since FL1 data are not informative about the time instance at which reticulocytes are born, but ch_T is well defined, RT can be interpreted as the reticulocyte age relative to its conversion to a mature RBC. Consequently, the oldest reticulocytes correspond to $RT = 0$ at which age they become mature RBCs. The rate of that conversion is the RBC production rate k_{RBC} :

$$k_{RBC} = \frac{\Delta RET}{\Delta RT} = \frac{\# \text{circulating reticulocytes of age between RT and 0}}{RT - 0} \quad (3)$$

where RT is a small relative age defined by the 5th percentile of RT distribution. To determine the 5th percentile one can construct the density function for RT distribution [13]:

$$p(RT_i) = \frac{\# \text{cells in channel } ch_i}{RT(ch_i) - RT(ch_{i-1})} \frac{RBC}{\# \text{cells in all channels}} \quad (4)$$

The RT density function $p(RT)$ is shown in Fig. 2. One can calculate $k_{RBC} = p(0)$. The time course of k_{RBC} is presented in Fig. 3.

RBC lifespan

The RBC count at any moment of time $RBC(t)$ is determined by the cell production and elimination processes. The production is represented by $k_{RBC}(t)$ whereas the elimination is defined by the distribution of the RBC lifespan $\ell(\tau)$. The following relationship between these three variables can be derived based on the mass balance principle [14]:

$$RBC(t) = \int_0^\infty \ell(\tau) \int_{t-\tau}^t k_{RBC}(z) dz d\tau \quad (5)$$

In the absence of information about the shape of the RBC lifespan distribution the most simplistic assumption that each red cell has the same lifespan T_{RBC} reduces equation (5) to the following:

$$RBC(t) = \int_{t-T_{RBC}}^t k_{RBC}(z) dz \quad (6)$$

where T_{RBC} is the sole parameter. In the case of constant RBC production rate $k_{RBC}(t)$, $RBC(t)$ becomes constant as well and Equation 6 reduces even further to

$$RBC = k_{RBC} T_{RBC} \quad (7)$$

Consequently, under the stationary assumptions, a single measurement of RBC and k_{RBC} allows one to calculate T_{RBC} as the ratio. However, this theoretical assumption is almost never met in the clinic. Therefore, equation (6) was used to estimate T_{RBC} by integrating the longitudinal k_{RBC} measurements and fitting them to the RBC data. The continuous function $k_{RBC}(t)$ was obtained from discrete weekly k_{RBC} data by linear interpolation. For times before the beginning of the study ($t < 0$) k_{RBC} was set at the initial value at $t = 0$. The curve described by Equation 6 fitted to the RBC data is shown in Fig. 4.

Software

MATLAB 7.13 (Mathworks) with Statistics and Curve Fitting toolboxes was used for all calculations. The integral in Equation 6 was calculated by quadrature using the *quad* function. The curve fitting was performed by minimizing the ordinary sum of squared residuals using *fminsearch* function.

Results

The estimated individual subject RNA half-lives are listed in Table 1. The RNA decay curves shown in Fig. 1 are almost parallel indicating similar $t_{RNA1/2}$ values across all subjects. The mean of 0.71 ± 0.11 days results in the coefficient of variation 15.6%. This indicates that the degradation rate of RNA does not vary substantially between subjects when measured under identical conditions in vitro. We infer that degradation rate of RNA does not vary substantially between subjects.

The time courses of the RBC production rates shown in Fig 3 remain flat with no apparent trends. This observation is consistent with the steady state erythropoiesis the patients were in while receiving constant weekly EPO doses prior to and during the study. Subjects 2, 3, 4, 5 were administered 20, 12, 4, and 4 kU/week, respectively. Subject 1 did not require treatment with EPO due to high Hb. The intra-subject k_{RBC} variability measured as the ratio of the standard deviation of the measured values by their mean were 11, 17, 17, 8, and 26 % for Subjects 1, 2, 3, 4, and 5, respectively. This is in contrasts with the between-subject variability assessed by the k_{RBC} values on day 0 yielding the coefficient of variation 44%. Consequently, applying a regression curve to describe the individual time courses of k_{RBC} to minimize the residual error will not be much different from the simple linear interpolation of the data used for RBC calculations.

The estimates of the T_{RBC} for individual patients were obtained by ordinary least square fitting of the $RBC(t)$ curve described by equation (6) to the patient RBC count data as shown in Fig. 4. The T_{RBC} values are listed in Table 1. Since the T_{RBC} was the only estimated parameter, the estimation was robust with negligible computational time. The initial guesses for T_{RBC} required by the minimization algorithm were calculated from equation (7) assuming constant values for RBC and k_{RBC} prior to the study and equal to their measurements at time 0:

$$T_{\text{RBC0}} = \frac{\text{RBC}(0)}{k_{\text{RBC}}(0)} \quad (8)$$

The mean value of RBC lifespan was 76.3 ± 18.1 days with the coefficient of variation 23.8%.

The estimate of T_{RBC} is most accurate if the k_{RBC} and RBC time courses span over the time interval comparable to T_{RBC} . If $k_{\text{RBC}}(t)$ curve is shorter, then it needs to be extrapolated for times before the beginning of measurements ($t < 0$). In the presented approach the constant extrapolation using $k_{\text{RBC}}(0)$ value was applied. In principle, this allows us to obtain a T_{RBC} estimate from a single measurement at time 0 that has to be equal to T_{RBC0} given by equation (8). Table 1 contains individual T_{RBC0} values for comparison. The T_{RBC0} were very close to T_{RBC} values with the difference not exceeding 8%. The mean of T_{RBC0} 73.3 ± 17.2 days were not statistically significant from the mean of T_{RBC} ($p = 0.068$, paired T-test). This is expected for patients at steady-state erythropoiesis. However, if the erythropoietic status of patients changes prior to measurements (e.g. due to treatment with EPO or disease progression), then the T_{RBC0} might be different from T_{RBC} .

Discussion

The reticulocyte-based estimation of lifespan of RBC (REBEL) technique utilizes the relationship between the production, elimination, and erythrocyte count in order to obtain T_{RBC} . The key step in REBEL is the determination of the RBC production rate from the reticulocyte age distribution. To do that, the half-life of reticulocyte RNA must be known. Our results indicate a relatively small inter-subject variability in RNA half-life which facilitates the use of a typical population-based value as a time scale for reticulocyte age distribution. As a result, we expect that the individual determination of RNA half-life for every patient will not be necessary, thereby simplifying our technique to obtaining only weekly reticulocyte and RBC counts. An adequately powered study will be required to assess the RNA half-life variability. The accuracy of the REBEL RBC lifespan estimate is dependent of the assumption that the in vitro measurements of $t_{\text{RNA1/2}}$ are similar to the in vivo values. To the best of our knowledge, no reports on the in-vivo reticulocyte RNA degradation half-life have been published to date. The major experimental hurdle to overcome is a small percentage of circulating reticulocytes relative to RBCs that would require labeling with an RNA-specific dye a large volume of blood in order to obtain sufficiently high Signal-to-Noise Ratio. An additional assumption of the REBEL method for the study presented here is that the in vivo RNA degradation in each individual is time invariant and equal to 9 h, as measured at the beginning of the study. Both of these assumptions should be verified in future better powered studies.

The mean RBC lifespan 76.3 days obtained by REBEL is comparable to the mean lifespan obtained by the traditional ^{52}Cr random labeling RBC survival studies in chronic kidney disease patients. Vos et al. [8] reported the median RBC lifespan of 58.1 days with the interquartile range 54.6–71.2 days. The mean for that population obtained in a subsequent study using a modeling approach was 62.8 days with the between subject variability of

11.2% [15]. The latter corresponds to the CV of the mean for our sample. In another study in hemodialysis patients, modeling RBC data yielded a mean RBC lifespan of 64 days with 30% between-subject variability [16]. Our reported value of T_{RBC} may be greater than 64 days due to a small number of subjects in our study. However, the 95% CI for the REBEL estimate is (72.3, 79.3) implying a significant difference from the reported means. A more precise answer will require a study involving a larger number of subjects.

Compared to random labeling-based measurement of RBC lifespan, REBEL does not require stationary conditions prior to the study. This method is valid for patients receiving treatment with ESA's, suffering disease progression, or experiencing other events that may change the production of RBCs, as long as the resulting RBC population originates from maturing reticulocytes. This condition excludes individuals receiving blood transfusions. However, such an assumption is not limited to CKD patients, but can be applied to any patient population that allows repeated blood sampling. If a subject is at steady-state erythropoiesis (constant EPO dose), REBEL only requires a single blood sample to determine T_{RBC} .

The T_{RBC} estimated by REBEL can be considered an average value over the time period of the study. The assumption of a single point lifespan distribution seems to be overly simplistic. More realistic distributions can be tested using equation (5). However, the steady-state data are unlikely to enable the identification of more than one parameter characterizing the lifespan distribution. To allow for more complex probability density functions describing the lifespan distribution, time varying RBC data augmented with mechanistic understanding of the cause of changes should be used. The impact of such modifications on the estimates of the mean (or median) T_{RBC} is yet to be determined.

REBEL determines the RBC lifespan based on the production of RBCs rather than elimination. In this regards, REBEL is similar to the estimation of red blood cell lifespan from alveolar carbon monoxide measurements. Neither technique requires lengthy and invasive labeling studies. The carbon monoxide exhalation method has been validated in healthy subjects [6] and its applications grow to various patient populations including patients with kidney failure [17]. However, like the other available techniques, this method is only valid under steady state erythropoiesis conditions. The CO exhalation also requires gas chromatography equipment for analysis of hemoglobin breakdown products. Such equipment is expensive and not readily available in many health care centers. On the other hand flow cytometry is considered as a standard methodology in most clinical laboratories. Because of this, REBEL might be more easily applicable in clinical practice.

In summary, we introduce a new technique called REBEL, for estimation of RBC lifespan from longitudinal reticulocyte data. REBEL can be used in subjects with non-stationary erythropoietic conditions. At steady-state, the RBC lifespan can be estimated by REBEL from a single blood sample. The estimates of the RBC lifespans obtained for five ESRD patients undergoing anemia treatment yielded a mean value that was comparable to the RBC lifespan values in ESRD patients reported by other researchers. To establish statistical consistency, REBEL should be validated through an adequately powered study against one of the established methods.

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Fig. 1.

Individual subject reticulocyte RNA degradation rates. Blood samples from each subject were incubated in medium over 9 h. At selected time points were stained with thiazole orange to determine the residual RNA content differentiating RET from mature RBCs. The slopes indicate the RNA degradation rate that is used to calculate the RNA degradation half-life. Symbols represent the measurements and lines are fitted exponential curves.

Fig. 2.

Histogram of FL1 signal distribution (upper panel) and density function of RT distribution for Subject 1 (lower panel). 500,000 events representing RBCs of Subject 1 were recorded by a flow cytometer and fluorescent signal corresponding to the filter band width F1 emitted by Thiazole Orange staining residual RNA and auto-fluorescent molecules and displayed as an event count histogram. The FL1 = 220 is the threshold between reticulocytes and mature cells determined from a non-stained blood sample. Since the $t_{\text{RNA}1/2}$ is known for Subject 1, the FL1 signal can be converted to the time to become RBC (RT) with RT=0 corresponding to FL1 = 220 [14], and the resulting RT distribution function $m(\text{RT})$ is shown in the lower panel. $m(0)$ can be approximated by the height of the 0 bar which is the 5th percentile of the distribution. $m(0)$ is equal k_{RBC} at the time of blood drawing.

Fig. 3.
Time courses of the mature RBC production $k_{RBC}(t)$ for individual patients evaluated weekly from the RT distribution density.

Fig. 4.

Estimation of T_{RBC} from fitting Equation 6. (bold line) into the individual subject RBC time course data (symbols). The T_{RBC} values for all subjects are shown in Table 1.

Table 1

Estimates of the individual values for the reticulocyte RNA half-life ($t_{RNA1/2}$) and RBC lifespan (T_{RBC}) obtained by the least squares fitting of the monoexponential curve and Equation 6 to RET and RBC data, respectively. T_{RBC0} was calculated from a single blood sample on day 0 using Equation 7.

Subject	$t_{RNA1/2}$ (days)	T_{RBC} (days)	T_{RBC0} (days)
1	0.71	72.2	71.5
2	0.83	61.3	57.3
3	0.74	107.5	102.2
4	0.73	74.0	74.4
5	0.55	66.5	61.3