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Measurement of Post-Transfusion Red Cell Survival with the Biotin Label

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Abstract

The goal of this review is to summarize and critically assess information concerning the biotin method to label red blood cells (RBC) for use in studies of RBC and transfusion biology — information that will prove useful to a broad audience of clinicians and scientists. A review of RBC biology, with emphasis on RBC senescence and in vivo survival is included, followed by an analysis of the advantages and disadvantages of biotin labeled RBC (BioRBC) for measuring circulating RBC volume, post-transfusion RBC recovery, RBC lifespan, and RBC age-dependent properties. The advantages of BioRBC over ⁵¹Cr RBC labeling, the current reference method, are discussed. Because the biotin method is straightforward and robust, including the ability to follow the entire lifespans of multiple RBC populations concurrently in the same subject, BioRBC offers distinct advantages for studying RBC biology and physiology, particularly RBC survival. The method for biotin labeling, validation of the method, and application of BioRBCs to studies of

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sickle cell disease, diabetes, and anemia of prematurity are reviewed. Studies documenting the safe use of BioRBC are reviewed; unanswered questions requiring future studies, remaining concerns, and regulatory barriers to broader application of BioRBC including adoption as a new reference method are also presented.

Keywords

Biotin; red cell volume; red cell survival; multiple density; application; non-radioactive; vulnerable population

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I. Biology of Red Blood Cell Survival

Overview of RBC Survival

Every second, about two thousand red blood cells (RBC) are released by the bone marrow; an equal number are removed from the circulation, mostly in the liver and spleen, maintaining a balance. Between these events, individual human RBC circulate an average of 115 days with a range of 80 to 130 days depending on the survival analysis method employed [1]. In healthy humans, RBC removal is not random (unlike the removal of some types of leukocytes), but instead is highly dependent on RBC age. Anemia results when too few RBC are produced, their lifespan is markedly reduced, they are lost by hemorrhage or phlebotomy, or there is a combination of these three processes.

The determination of human RBC survival (RCS) is an old, but still challenging, area of research with both biological and technical complexities. Areas that are still not completely understood include the mechanisms responsible for normal RBC senescence *in vivo* that determine endogenous RCS and the mechanisms responsible for the lesions occurring during RBC storage that influence post-transfusion RCS. Although a large decrease in RCS such as that in sickle cell disease (14–55 d) can result in anemia [2], there are circumstances such as diabetes mellitus in which relatively modest variation in RCS (80–130 d) can be important, whether or not overt anemia results [3].

There are two types of RBC labels. The first type labels or “tags” a representative population of circulating RBC irrespective of RBC ages; both biotin and ^{51}Cr are RBC population labels. The second type labels a cohort of RBCs, all of a similar age; labeling of hemoglobin with oral glycine (a precursor of both globin and heme) containing a radioactive or stable isotope of N or C is an example of a cohort method. The choice of an optimal label depends primarily on the application, but factors such as applicable regulatory policies and available analytical capabilities also play a role. The study of an age cohort using a longstanding stable isotope technique [4] requires no *ex vivo* labeling and is well-suited for studies of endogenous RBC lifespan. Highly purified ^{13}C - and ^{15}N -glycine and precise mass spectroscopic techniques are now available, making this classic technique more feasible.

Studies of donor red cells require an *ex vivo* population label, usually ^{51}Cr or, more recently, biotin. If detailed studies of time-dependent *in vivo* RBC changes, survival determination for the complete RBC lifespan, or the concurrent survival of two RBC

populations is required, then the biotin label is appropriate. If the site of removal of labeled RBC is of primary importance, then a radioactive label is required.

Population labels will be emphasized in this review. When using a population label based on the blood concentration of the label (e.g., ^{51}Cr), blood volume is determined using the dilution principle, and circulating RBC volume (RCV, often referred to as red cell mass) is calculated from an independent measurement of plasma volume or from an assumption about the relation of the venous hematocrit to total body hematocrit. When using a population label based on enumeration of labeled RBC as a proportion of total RBC in a blood sample, the total number of circulating RBC is calculated by observing the “dilution” of a known number of labeled RBC infused into the unknown number of circulating unlabeled RBC. The resultant total number of circulating RBC is multiplied by the MCV (from a routine CBC) to obtain the RCV. RCS is assessed by measuring the decrease in blood concentration of ^{51}Cr or the proportion of BioRBC over time.

Technical uncertainties result from the particular label used to track circulating RBC. The criteria for an ideal label include minimal RBC manipulation required to uniformly attach the label, stability of the label throughout the life of the RBC, absence of RCS shortening due to RBC injury during labeling, and absence of toxicity to the person receiving the labeled RBCs.

Biological Complexity of Red Cell Survival

If a sample of autologous blood containing RBC of all ages is drawn, labeled, and reinfused, then, under normal hematologic steady state conditions, about 0.85 percent of the original pool of labeled RBC will be removed from the circulation each day leading to a linear survival plot (Figure 1). In practice, there are several sources of deviation from this steady state pattern. In this review, the term “RBC survival” encompasses several aspects of survival including immediate recovery, 24 h post-transfusion recovery, and long-term survival.

1. Immediate recovery—Damaged or incompatible transfused RBC may be removed from the circulation during the 5–7 min required for complete mixing in the circulation. This very short-term component of survival cannot be determined without the aid of an independent measure of either blood volume (when using a radioisotope RBC label) or total circulating RBC number (when using BioRBC and flow cytometry). For unstored RBC, immediate recovery is tacitly assumed to be 100% including all of the studies that used the biotin method. At least three lines of evidence indicate that immediate loss of biotin labeled RBC is negligible: a) In normal individuals and sickle cell disease patients, circulating RCV calculated from times within 10 min of infusion agreed with RCV calculated from ^{51}Cr [2, 5]; b) survival of autologous RBC labeled at single density exhibited no detectable RBC loss based on post-transfusion samples at 5, 10, and 20 min [5]; little or no loss of labeled RBC between 5 and 20 min indirectly suggests that there was little immediate loss of BioRBC; and c) survival of allogeneic RBC labeled at multiple densities exhibited no detectable RBC loss in infants based on samples drawn at 20 min [6] and the circulating RCV calculated from these time points agreed with circulating red cell volume calculated from minor

antigen mismatch, which presumably has no immediate loss because none of the RBC are labeled. Thus, while absolute proof is difficult, we conclude that the preponderance of the evidence indicates that immediate post-transfusion BioRBC label loss is likely negligible in comparison to the inherent accuracy of the method.

2. 24 h post-transfusion recovery (PTR₂₄)—After storage under blood bank conditions, RBC will sustain damage—even during approved storage conditions—and severely damaged RBCs will characteristically be removed from the circulation during the first 24 hours after transfusion. At maximum approved storage duration, PTR₂₄ must be greater than 75% according to the Food and Drug Administration (FDA). Remarkably, the RBC that survive the first 24 hours in circulation have a normal or near normal survival [7, 8]. This indicates that either the storage lesion is confined to a subpopulation of RBC or that the lesion is reversible in most RBC. Even without storage, RBC that have been extensively manipulated during *ex vivo* processing may exhibit decreased PTR₂₄ [7, 8].

3. Long-term survival—Long-term survival (lifespan) is defined only for those RBC that remain in the circulation for at least one day after infusion. Depending on the application, long-term survival may be defined in several ways. For RBC produced during steady state erythropoiesis that exhibit linear removal for a substantial fraction of their lifespan, the linear portion of the survival curve may be extrapolated to the x-axis to determine the RBC lifespan (“mean potential lifespan”, MPL). Reduced, but still linear, survival may be seen in some types of storage lesions where the overall population of RBC is affected enough to decrease survival [9, 10]. For RBC with nonlinear removal (e.g., RBC in sickle cell disease, other severe hemolytic states, and even in some normal individuals [3]), a half-time for removal (denoted T₅₀) is useful, as reviewed in [1]. The analysis of RBC lifespan is discussed further in the following sections.

RBCs from normal humans are commonly assumed to have a similar lifespan of approximately 115 d. In fact, studies show that survival of the RBC from one individual to another varies by approximately 15% [3]. Nevertheless, after leaving the bone marrow, essentially all RBC normally remain in the circulation for a long period. After about 80 d in circulation, the RBC are somehow marked for removal by the reticuloendothelial system. Whether senescent marker(s) appear at 80 days on all RBC leading to their gradual removal, or whether the marker(s) appear at different times on individual RBC leading to rapid removal is not clear [11]. The nature of the “clock” that keeps track of age and the identity of the marker(s) that leads to removal have been the subject of intense interest for many years but are still not well understood.

RBC Senescence

The study of the mechanisms for RBC senescence and removal is complex for a number of reasons. First, there may be significant species differences, as shown by variation in RBC lifespan and the importance of cell age in determining removal from the circulation. Second, there is no simple identifier for old/senescent RBC. RBC density has been used extensively as a surrogate for age, and a human study using BioRBC has confirmed that the mean density of older RBC is increased. However, that study also provides evidence that many old

RBC are not dense [12]. Third, changes that occur as RBC age may not be related to RBC senescence or provide the mechanism for removal — just as gray hair that appears with age does not provide the mechanism for death in the elderly. Fourth, factors that lead to early RBC removal under abnormal conditions, such as oxidation or phosphatidylserine (PS) exposure, may or may not be part of normal senescence. Fifth, RBC changes that lead to very rapid removal from the circulation will no longer be apparent because these RBC are removed from the circulation and are no longer present on blood samples collected for study. Given these difficulties, studies of the normal senescence mechanism may require the use of specific inhibitors of proposed pathways to determine whether RBC lifespan is prolonged. Because BioRBC can be isolated *ex vivo* using magnetic beads [12, 13], they can be utilized in such studies.

Despite these difficulties in evaluating senescence, a number of potential mechanisms have been proposed and studied. These pathways may act alone, but more likely interact with one another in complex ways. RCS is remarkably consistent in most normal subjects, implying that these pathways are well regulated. The following are among the areas that have been examined for their importance in RBC senescence:

1. Physical Properties—As RBC age they become smaller, denser, and less deformable. Size decreases as a result of loss of surface membrane area and intracellular hemoglobin. Accordingly older RBC have both lower MCV and MCH [14–16]. However, the decrease in MCV is proportionally greater than the decrease in MCH, so MCHC increases and, thus, density increases. MCV may also decrease due to loss of K^+ (the major intracellular monovalent cation) through Ca^{++} -dependent and/or Ca^{++} -independent pathways (see below). A study using BioRBC has provided evidence that most density changes occur in younger RBC and that some, but not all, older cells are denser than normal [12]. That study also showed that very old RBC have abnormal morphology and tend to aggregate.

2. Neoantigens and Naturally Occurring Antibodies—There is extensive evidence that senescent RBC display a neoantigen that is recognized by naturally occurring antibodies (NABs), leading to phagocytosis [17, 18]. NABs are germ line encoded antibodies produced without need of specific antigen stimulation. The target appears to be an aging-modified form of Band 3 (anion exchanger 1, AE1), which is the most abundant RBC surface protein. The proposed modifications include oxidation [19], proteolysis [20], aggregation [21], denaturation [22], and/or crosslinking of molecules on the RBC surface [23]. Using the biotin RBC labeling method, the presence of increased immunoglobulin (consistent with NAB binding) on old RBC from dogs [24] and humans [12] has been confirmed; both species exhibited the predicted age-dependent removal of RBC from the circulation, but this was not observed in rabbits [25].

3. Phosphatidylserine (PS) Externalization—As in other cell types, RBC membrane PS is normally confined to the inner leaflet of the membrane bilayer. This asymmetrical distribution is maintained by aminophospholipid transferase, also known as flippase. The asymmetrical distribution is degraded by scramblase, a nonspecific phospholipid transmembrane transporter. When flippase is inactive and scramblase is active, PS is externalized, leading to phagocytosis. In rodents, external PS increases in older RBC [26–

28], and flippase activity is decreased [29]. In studies of older human RBC using BioRBC, flippase was found to be decreased but without evidence for increased PS externalization [12].

4. Calcium Accumulation—RBCs normally maintain a low intracellular $[Ca^{++}]$ by means of a very active Ca^{++} pump. If Ca^{++} accumulates, many harmful results occur, including activation of the protease calpain, activation of a K^{+} efflux channel (the Gardos channel), inhibition of flippase/activation of scramblase, vesicle formation, transglutaminase activation [30], and decreased deformability. Ca^{++} -dependent pathways are important for RBC in some disease states such as sickle cell disease and may contribute to RBC senescence [31]. Although this mechanism has yet to be thoroughly investigated, it is amenable to studies utilizing BioRBC to isolate RBC.

5. CD47—Finally, there is evidence to suggest that CD47 plays an important and complex role in RBC clearance. Through interaction with macrophage signal-regulatory protein alpha and the resultant propagation of inhibitory signals, CD47 prevents erythrophagocytosis [32]. This is illustrated by the short lifespan of RBC from CD47 knockout mice [33]. However, a recent *in vitro* study [34] provided convincing evidence that modified (most likely oxidized) CD47 on experimentally aged RBC leads to increased erythrophagocytosis. This activity required the presence of thrombospondin-1 (TSP-1) or a specific TSP-1 peptide. Additional *in vivo* studies—perhaps including BioRBC—are needed to confirm these findings.

II. Advantages and Disadvantages of BioRBC in Clinical and Biological Studies

The first accurate measurements of RBC lifespan were made by Winifred Ashby in 1919 using a differential agglutination approach based on the ABO blood group system [35]. In the late 1940's, methods based on *ex vivo* radioisotope labeling emerged, and ^{51}Cr became the standard label for the measurement of RCS [36]. More recently, *ex vivo* RBC labeling with biotin has been used with increasing frequency to study the three types of RCS. To our knowledge, there have been 14 reports of BioRBC used in adults (Table 1) and another 10 reports in infants (Table 2).

Both the reference ^{51}Cr method and the biotin method measure survival of a single representative population of circulating RBC that includes older RBC for which removal is imminent and younger RBC recently released from the bone marrow. Compared to the ^{51}Cr method [37] and other RBC labeling methods, the BioRBC method has important advantages, but also a few disadvantages (Table 3). First and foremost, the BioRBC method, unlike ^{51}Cr and other radioactive labeling methods, poses no radiation risks. Accordingly, the biotin method is more acceptable - particularly when studying vulnerable populations that include pregnant women, fetuses, infants and children. From a regulatory standpoint, BioRBC are acceptable for studying RCS in countries that exclude use of all radioisotopes, including many in Western Europe. In addition, the expense of label disposal and the potential requirement to treat subjects' blood samples as radioactive material are eliminated.

Moreover, with radioisotopes, once the labeled RBC are infused their continued presence in the circulation can only be assessed by measuring the concentration of radioactivity in blood and thus cannot distinguish separate RBC populations when only a single isotope such as ^{51}Cr is used.

A unique advantage of BioRBC is that multiple populations of RBC can each be biotinylated at discretely different biotin densities and have RCS for each population determined using the same methodology. Doing so permits concurrent flow cytometric tracking of two or more separate RBC populations in individual study subjects (Figure 2). Although concurrent RBC labeling with more two or more isotopes or a combination of one isotope with an immunologic method for an allogeneic transfusion have been utilized in humans to measure RCS for a variety of purposes [38–43], all require by two separate analytic methodologies. Thus, relative to multi-density biotin RBC labeling, this increases the technical complexity, cost, and difficulty. In the case of multiple radioactive labels, safety concerns are added that are not appropriate for research studies in the vulnerable study populations noted above. Because the biotin method can independently track multiple populations, RBC preserved in different storage media or RBC stored for different time intervals can be compared concurrently in the same individual, i.e., rather than sequentially after a “wash-out” period. Sequential studies have the disadvantage of requiring additional time, expense, and effort and cannot account for changes in RCS that occurred between sequential studies.

BioRBC enumeration by flow cytometry is not susceptible to artifacts resulting from partial label loss, thus avoiding some of the imprecision seen with other labels. To illustrate, about 4% of ^{51}Cr elutes from RBC during the first 24 h [44]; investigators reporting ^{51}Cr survival studies compensate for this loss by referencing the Day 1 (24 h post-infusion) sample as the 100% value, i.e., rather than time of transfusion of the labeled RBC. In addition, approximately 1% of ^{51}Cr subsequently elutes from RBC per day [44, 45]. Unfortunately, this rate varies substantially (from 0.56 to 2.04%), is not predictable from individual to individual, and cannot be inferred from the RBC disappearance curves for a given individual [46, 47]. Analysis of biotin label by flow cytometric enumeration provides the number of RBC for which fluorescent intensity falls within a given range; thus, each cell is classified as belonging (or not belonging) to a specific RBC population (Figure 2). Even the partial loss of label from a BioRBC during its lifespan and the resulting decrease in fluorescence signal has no effect on enumeration as long as the fluorescent intensity still falls within the same flow analysis range. Finally, unlike ^{51}Cr , accuracy of the BioRBC enumeration is affected by neither hemoconcentration nor dilution.

Tracking of labeled RBC over their entire lifespan is important for accurate estimation of the distribution width of parameters of long-term survival such as mean remaining life span and mean potential lifespan (see Supplementary Appendix A). The biotin method permits accurate tracking of RCS well beyond the T_{50} . In contrast, ^{51}Cr radioactivity per mL of blood at Day 50 is typically reduced to only 12% of the value at Day 1 due to a combination of elution and an isotopic decay (^{51}Cr half-life is 28 d). Elution and decay make accurate measurement of blood radioactivity problematic after about 30 d. Finally, for the purpose of RBC labeling in preclinical studies, the ^{51}Cr rapidly elutes from the RBC in some species

(e.g., sheep) rendering the ^{51}Cr method unsuitable for measuring RCS. The ability to identify individual biotin-labeled RBC for their entire cell lifespan also makes possible two powerful analytical capabilities. The first is multicolor flow cytometric analysis, which allows many cellular properties discussed below to be followed as the cells age *in vivo* [2, 12]. The second is magnetic isolation of labeled RBC after a period of time in the circulation, yielding RBC with a known age range for analysis [2, 3, 12].

With the BioRBC method, RCV and RCS can be quantified using the small volumes of blood that can be safely sampled from extremely small fetuses and infants. Because flow cytometric enumeration requires very small blood volumes ($\sim 200,000$ RBC/ $0.05\ \mu\text{L}$ of blood), quantification of BioRBC enrichment of single blood samples used in RCV and RCS studies can be performed on $15\ \mu\text{L}$ of blood. In contrast, the ^{51}Cr method requires $5\text{--}10\ \text{mL}$ of blood unless special microsampling and counting techniques are applied [48]. The biotinylation reagents are readily available from commercial sources at modest cost, and flow cytometers with adequate technical characteristics are readily available in most medical centers and hospital laboratories. The reagents for ^{51}Cr labeling are more costly. However, biotin labeling is more complex and has some additional disadvantages compared to radioisotopes. For example, in contrast to body scans of short-lived isotopes, determination of the anatomic sites of BioRBC removal has not been reported.

Biotinylation also requires additional wash steps relative to radioisotope labeling that could result in hemolysis, even though this has not been reported or observed by us (unpublished observations). Furthermore, these wash steps require careful removal of the wash solution to avoid the removal of RBC from the top of the column of RBC that could change the RBC age distribution and thus survival behavior of the RBC being studied. A theoretical disadvantage of biotinylation of RBC is that biotinylation could lead to the covalent modification of multiple exposed amino acid residues of proteins on the cell membrane surface, not just the epsilon amino groups of lysine residues. Such modification could increase immunogenicity of RBC that were biotinylated (relative to unlabeled RBC and to those that are radiolabeled with ^{51}Cr).

III. Method for Biotin RBC Labeling

The method described below was developed jointly by the Iowa, Arkansas, and Cincinnati groups and is being applied in all three locations with minor variations [5, 6, 49, 50]. All are adaptations of the method of Cavill et al. who reported the first human application of the biotin RBC labeling method to measure RCV in adults [51].

After washing RBC to remove loosely adherent proteins that would otherwise be biotinylated in the subsequent step and then lost *in vivo*, RBC at a hematocrit of 25% are incubated with a biotinylating reagent. The water-soluble sulfo-succinimido-biotin (abbreviated as s-NHS-biotin) is currently used in human studies at a typical concentration of $18\ \mu\text{g}/\text{mL}$ RBC. Membrane proteins on the outward facing bilayer are covalently labeled with biotin; specifically, a covalent amido bond is formed between the carboxyl group of biotin and the epsilon amino group of lysine residues in the membrane proteins [3, 52]. BioRBC are infused, and blood samples are obtained at timed intervals thereafter. For

determination of RCV, the number of BioRBC infused must be known, but RCS can be determined without knowing the exact number of BioRBCs infused. As little as 15 μ L of venous or capillary blood is stained with avidin (or streptavidin) conjugated with one of several commercially available fluorescent labels. The binding (staining) reaction is stopped by several washes that remove the (strept)avidin. In the initial washes, the wash solution contains biotin, which is added to prevent cross-linking of the BioRBC by the tetravalent (strept)avidin.

The BioRBC (as a proportion of total RBC) are enumerated by flow cytometry and commonly depicted as a histogram of the “events” present in a “gate” specific for the RBC biotin densities of interest (Figure 2). WBC and platelets are excluded by the gate. Over approximately the first two weeks in circulation, about one-third of the biotin labels per RBC are lost [53]. Based on studies conducted with isolated pure proteins labeled with a variety of biotinylation reagents, *in vivo* cleavage of the biotin label occurs by both enzymatic and non-enzymatic mechanisms [54]. One manifestation of biotin label loss from the RBC surface is a small leftward migration of the BioRBC peak position on the flow cytometric population histogram [5, 53]. As long as the peaks representing the fluorescent-labeled BioRBC population(s) remains separate from the unlabeled RBC and from each other, enumeration of the proportion of BioRBC can be accurately determined. Consequently, loss of label does not limit accuracy in determining RCV [5, 6, 55] or RCS under conditions of either normal survival [53] or accelerated removal [50].

Because RBC can easily be biotinylated at several discrete densities per RBC and enumerated as separate RBC populations (Figure 2), RBC lifespan can be determined for multiple populations of RBC concurrently. Similarly, different discretely labeled populations of BioRBC can be used to repetitively determine RCV. For RBC biotinylated at multiple densities, with time the peaks move leftward in parallel as a result of label loss without merging. Theoretically, increasing the fluorescence per BioRBC could be further increased (e.g., by using a brighter fluorescent probe or by employing a “sandwich” strategy for the tetravalent streptavidin) thereby allowing more densities of BioRBC that survive normally and lengthening the time until the lowest densities BioRBC peak approaches the unlabeled peak.

IV. *In vivo* validation of the biotin method

Validation of the BioRBC method demonstrating that BioRBC accurately reflect *in vivo* RCS in humans has been demonstrated in studies that assessed 24 h recovery and long-term survival.

Validation for determination of RCV has been performed in both adults and infants by confirmation that BioRBC survival is equivalent to a reference label. In adults, ^{51}Cr has been used as the reference method to show equivalency using either one or two biotin densities of BioRBC [5, 53]. Results using four densities of BioRBC (6, 18, 54, and 162 $\mu\text{g/mL}$) further validate the method by demonstrating that the four densities of BioRBC concurrently administered yield equivalent RCV results [49]; if the density of biotin labeling had significantly reduced immediate BioRBC survival, one would have predicted RCV

differences among the four labels. Moreover, in infants, clinical transfusion with allogeneic RBC with both minor RBC antigen differences and differences in hemoglobin species (HbA in adult donor blood and HbF in infant autologous blood) allowed comparison to two reference methods; in very low birth weight (VLBW) infants weighing <1.5 kg at birth, equivalent RCV values were observed [6, 56]. Moreover, one of these infant studies used four densities of BioRBC and observed equivalent RCV results for the four BioRBC densities [6].

Similar validation of short-term recovery in determining PTR₂₄ has also been performed. The results confirmed that BioRBC survival is equivalent to reference comparison labels in both adults [5, 53, 57] and infants [6]. In the adult and infant studies in which the two to four, discrete biotin densities were compared among one another, equivalent PTR₂₄ results were observed [6, 50, 57]. Concurrent validation of long-term *in vivo* survival of BioRBC vs. reference methods is challenging because multiple measurements of labeled RBC survival must be over weeks to months. Yet the long observation periods offer the greatest sensitivity in detecting subtle survival differences. In adults, long-term RCS of BioRBC agrees well with that of ⁵¹Cr labeled RBC [53]. Survival of BioRBC has also been shown to accurately track ⁵¹Cr RCS *in vivo* under conditions of accelerated removal [50]. In a more recent study of eight adults that used several densities of autologous BioRBC [57], T₅₀ and mean potential lifespan data were similar for the two lowest densities (6 and 18 µg/mL) and agreed with the literature [58]. Survival as assessed by T₅₀ and mean potential lifespan of the next lowest biotin density (54 µg/mL) was slightly shorter, and survival of the highest biotin density (162 µg/mL) was considerably shortened. Differences in the pattern of survival for the four BioRBC densities illustrate the magnitude of progressive shortening of RCS with increasing BioRBC density (Figure 3 Panel A). This shortening of long-term RCS for the higher density BioRBC likely reflects subtle alteration of the BioRBC by biotinylation per se.

In critically ill, VLBW infants, long-term RCS has been measured using four densities of allogeneic BioRBC [59]. Similar to adults, the three lowest BioRBC densities tracked well with Kidd antigen difference and with each other (Figure 3 Panel B); however, the highest density exhibited shorter survival. Of note, the perturbing effects of the loss of BioRBC due to laboratory phlebotomy and dilution of BioRBC as a result of body growth and subsequent RBC transfusions did not permit accurate estimation of either T₅₀ or mean potential lifespan in these infants. This problem can be overcome by applying modeling that adjusts for these perturbing factors [60].

V. Mathematical modeling of RCS

The quantity of RBC in the blood stream depends on both their production and their survival. Accordingly, determination of RCS is only possible by eliminating the confounding effect of RBC production. As indicated above, two approaches exist for determining RCS in the circulation: population labeling and cohort labeling. With the population approach, a representative sample of all circulating RBC is selectively followed. In practice, population labeling is accomplished in two ways.

The first and most common way is by labeling a representative sample of the entire population of RBC while ensuring quantification without interference from the non-labeled cells. To assure validity of the results, the survival of the labeled RBC must have the same survival properties as unlabeled cells. The biotin method for labeling of RBC approaches that ideal. Data generated with the biotin method under erythropoietic steady state conditions are particularly suitable for mathematical modeling because flow cytometric enumeration of BioRBC is acceptably accurate (i.e., $\pm 5\%$) throughout 95% of RBC lifespan.

A second, less common approach for deriving population RCS data is to quantify differences between donor and recipient RBC. In practice, this approach includes immunologic differences in minor or major RBC surface antigens [58, 61] or differences in the predominant type of hemoglobin protein (e.g., HbA vs. HbF) [6]. These approaches are limited to situations in which antigen differences exist and in which antibodies specific for the antigens of interest are capable of cleanly separating donor from recipient RBC. Obviously, this approach does not permit RCS determination of autologous RBC. Application of population labeling methods yields numerous descriptive survival parameters. Most are obtained by mathematical modeling, but there are two notable exceptions: post-transfusion RBC recovery at 24 h (PTR₂₄) and the time to disappearance of 50% of the RBC label or of 50% of the labeled RBC (T₅₀). PTR₂₄ is most commonly applied short-term RCS parameter and is required by the FDA for evaluating RBC storage media and RBC treatments as discussed below [62, 63].

The most commonly applied long-term RCS parameters include mean lifespan, mean potential lifespan, mean remaining lifespan [64], and mean age [65]. A detailed discussion of these three survival parameters with comparison to one another is included in Supplementary Appendix A.

VI. Clinical and Biological Applications of the Biotin Method

Sickle Cell Disease

Sickle cell disease causes a severe hemolytic anemia. Until recently, the vasocclusive component of sickle cell disease has been given most of the clinical emphasis. Solid evidence now indicates that an important link exists between hemolysis and vasocclusion and that this link is mediated by the ability of free hemoglobin to bind to and reduce the availability of NO, thereby reducing NO's vasodilating and platelet inhibiting effects [66–68]. Hemolysis, especially intravascular hemolysis in sickle cell disease, has been postulated as an important factor in the development of pulmonary hypertension and early death. The measurement of RCS in sickle cell disease is complicated by the presence of two types of RBC: 1) F cells that contain about 25% fetal hemoglobin (HbF); and 2) non-F cells that contain nearly 100% sickle hemoglobin (HbS) and no detectable HbF. Even though F cells contain about 75% HbS, they are resistant to sickling and thus have a longer lifespan. Patients with higher levels of HbF tend to have lesser morbidity and lower mortality. In many sickle cell disease patients, HbF can be increased by administration of hydroxyurea, the only FDA-approved treatment.

The biotin method is well suited to the study of RCS in sickle cell disease because it allows measurement of RBC properties as they age. Moreover, in combination with a commercially available fluorescent antibody to HbF, the biotin method can quantitate the following: 1) lifespans of RBC that do or do not contain HbF [69], 2) the lifespan of dehydrated sickle RBC [70], 3) the rate of *in vivo* dehydration [2], and 4) the age-dependent extent of phosphatidylserine externalization [71].

Unanswered questions related to hemolysis in sickle cell disease likely to be facilitated by application of the biotin method include the following:

1. How strong is the correlation between hemolysis and pulmonary hypertension?
Studies to date have relied on surrogate measures of hemolysis rather than direct measurements of RCS.
2. How important is the degree of intravascular hemolysis that occurs?
3. How does transfusion modify the survival of endogenous sickle RBC?
4. Is the survival of heterozygous sickle trait, i.e., Hb (AS) RBC decreased during periods of athletic and military training?

Diabetes

RCS in people with diabetes is of particular interest because RBC hemoglobin A_{1c} (HbA_{1c}) content, a key biological marker of blood glucose (glycemic) control, is dependent not only on mean blood glucose concentration but also on the duration over which Hb is exposed to the ambient concentration of glucose. In people without diabetes, HbA_{1c} ranges from 4.8–6.0% of total Hb. Recent clinical criteria define diabetes by a HbA_{1c} at or above 6.5% and pre-diabetes as 5.7–6.4%. Although HbA_{1c} can range up to ~15% in diabetes, the American Diabetes Association has set 7.0% as the therapeutic target and 8.0% as the level at which therapy should be intensified. However, a study of normal subjects and those with diabetes under stable glycemic control, both of whom have normal blood counts and RBC indices, indicated that there are circumstances in which HbA_{1c} may be misleading [3]. In these groups, autologous BioRBC were used to show that the synthesis rate of HbA_{1c} in RBC *in vivo* was linear with time as the RBC aged. The mean RBC age ranged from 38 to 59 d and averaged 49 d. Based on the linear increase, people with a mean RBC age 20% greater than the mean would have HbA_{1c} 20% greater than the average person at the same glucose; likewise, people with a mean RBC age 20% less than the mean would have HbA_{1c} 20% lower. This level of subject-to-subject variation in HbA_{1c} not dependent on blood glucose concentration predicts that some people with diabetes will have HbA_{1c} values with a level of glycemic control that differs significantly from those normally expected. If unrecognized, this could result in otherwise avoidable hypo- or hyperglycemic episodes and the resulting diabetic complications. Thus, reliance on specific HbA_{1c} cut points for clinical decisions makes the implications of small differences in RCS highly relevant to improving diabetic care [13]. One of the important current controversies in the field of diabetes is the mechanism for observed differences in HbA_{1c} between racial groups despite equivalent degrees of glucose tolerance; differences in RCS is one candidate [72].

Unanswered questions regarding RCS and HbA_{1c} likely to be facilitated by application of the biotin method include the following:

1. How prevalent are variations in RCS that alter clinical decisions?
2. Is there variation in RCS as a consequence of either diabetes itself or the forms of anemia commonly associated with diabetes?
3. How can people with variant RCS most readily be identified and the variation taken into account in guiding diabetes therapy?

Chronic Disease

The role of hepcidin, a hormone produced in the liver, in the regulation of iron metabolism has only recently been elucidated. Hepcidin inhibits iron transport across the gut mucosa, thereby preventing excess iron absorption and maintaining normal somatic iron levels. Hepcidin regulation differs between iron deficiency anemia and the anemia of chronic disease (increasingly referred to as the anemia of chronic inflammation). Plasma hepcidin levels are decreased in individuals with iron deficiency anemia and increased in those with anemia of chronic disease [73, 74]. Hepcidin is an acute phase inflammatory marker, regulated by IL-6. Among children with iron deficiency anemia, those who are not obese have decreased serum hepcidin levels as expected and mount a good erythropoietic response to iron supplementation; in contrast, those who are obese have increased hepcidin levels similar to anemia of chronic disease and mount a considerably smaller erythropoietic response to iron [75]. This suggests a link between obesity and iron metabolism that parallels recent speculation that inflammation may be the initiating pathophysiologic mechanism leading to obesity, insulin resistance, and diabetes.

There is limited literature on RCS in anemia of chronic disease; none of the studies used BioRBC. The literature is mixed concerning whether there are alterations in RCS [76, 77]. One study that based assessment of RCS on exhaled carbon monoxide (a metabolic product that is unique to heme catabolism) demonstrated shorter RCS in patients with rheumatoid arthritis than in either controls [78] or patients with osteoarthritis (a degenerative rather than a primarily inflammatory condition).

Unanswered questions regarding RCS in anemia of chronic disease and in chronic inflammation that are likely to be facilitated by application of the biotin method include the following:

1. How prevalent are variations in RCS and what is the extent of the variations?
2. What is the mechanism for altered RCS?
3. Do therapies directed at the inflammatory process reverse defects both in RCS and in iron availability for RBC production?

Infants

Determination of RCV and short-term and long-term survival are critically important in understanding causes of anemia during early development. For premature infants, this understanding is particularly important because anemia is universally prevalent, with an

estimated one million RBC transfusions administered to infants annually in the U.S. [79]. There has been a dearth of RCS studies since 1970 in infants and other vulnerable populations (pregnant women, fetuses and children) because of concern about radiation exposure [80], and this has impeded progress in understanding the pathophysiology of neonatal anemia and in developing effective therapies. In anemic premature infants, circulating RCV and blood volume is particularly critical because RCV is an important determinant of adequate tissue oxygen delivery and because blood volume is an important factor in determining drug dosing schedules. Similarly, whether anemia is driven by physiological senescence or by non-physiological mechanisms such as iatrogenic blood sampling and hemorrhage, long-term RCS is a key determinant of both RCV and its more easily measured surrogate, the blood Hb concentration. The BioRBC method, which avoids radiation exposure and is able to utilize extremely small blood sampling volumes (e.g., 15 μ L) has enabled RCS studies in VLBW infants and other vulnerable study subjects. Ten relatively recent publications report the use of BioRBC studies in infants, including eight since 2000 (Table 2). Seven of these studies focused on RCV; three focused on PTR₂₄ and long-term RCS.

Infant RCV studies using BioRBC: New, clinically relevant information has come from several of the RCV studies. For example, contrary to previous understanding and similar to adult studies [49], blood volume normalized by body weight is about 70 mL/kg [6]. Also, the post-transfusion time during which a sample for determination of RCV is accurate ranges from as little as 20 min to at least 90 min. However, unlike adults and term infants, hematocrit and hemoglobin concentration exhibit a much poorer correlation with RCV in VLBW infants [6, 56, 81, 82]. Moreover, delayed clamping of the umbilical cord importantly increases RCV and circulating Hb despite minimal effects on hematocrit and blood hemoglobin concentration - the parameters previously used to judge effectiveness of delayed cord clamping [83–85]. The mechanisms underlying the discrepancy between RCV and hematocrit/hemoglobin measurements are unclear; further, for clinical practices, definitive evidence that RCV is superior to blood hematocrit or Hb concentration in the management of anemia in these VLBW infants is still lacking [81]. The application of the BioRBC method will allow these questions to be examined further.

Infant long-term RCS studies: Common clinical events (e.g., loss of BioRBC due to laboratory phlebotomy; dilution of BioRBC as a result of body growth and subsequent RBC transfusions) characteristically prevent direct estimation of T₅₀ and mean potential lifespan, the traditional long-term RCS parameters commonly reported in adult steady state studies (Figure 1). Nonetheless, mathematical modeling can account for these confounding factors and provide estimates of these parameters. For example, a recent study of critically ill, VLBW infants used allogeneic, adult RBC labeled at one biotin density and autologous, neonatal RBC labeled at a different biotin density. Contrary to previous studies, RCS for the allogeneic adult BioRBC was similar to that of the autologous infant BioRBC; mean allogeneic RCS ($\pm$ 1 SD) was 74 ± 19 days, and mean autologous RCS was 63 ± 12 days [60]. Further, adult donor RBC survived in the infants approximately half as long as they would have in adult recipients [60]; these observations open a new way of thinking about RBC biology in neonates/infants.

With more RCS studies in infants in progress, unanswered questions that will be facilitated by application of the biotin method include the following:

1. What is the relationship of gestational age (or weight) at birth to RCS?
2. What are the mechanisms responsible for survival of adult allogeneic RBC being shorter when transfused into infants versus adult recipients?
3. Do environmental factors (e.g., oxygen therapy and specific disease processes) shorten RCS?

Transfusion Medicine

In addition to the studies in infants discussed above, the biotin method has other potential applications in transfusion medicine, including comparison of RBC storage media/conditions and comparison of RBC exposed to specific treatments (e.g., pathogen inactivation). The capability to study multiple RBC populations simultaneously is extremely useful in these applications because this capability allows the use of a concurrent control, thus increasing precision while reducing the required number of experiments by half. In addition, the biotin method is able to measure changes in long term RCS that cannot be easily determined with the chromium method.

For the licensing of RBC storage media and RBC treatment procedures to be applied clinically, the FDA requires *in vivo* survival data. These requirements are needed because changes in RBC (storage “lesions”) occur between collection from donors and the time they are transfused; this suggests that transfusion with fresh RBC may be safer and/or more effective than transfusion with stored RBC. For RBC storage media licensure in the United States, post-transfusion RBC recovery and survival analysis is a FDA requirement. FDA licensing of RBC storage media requires that mean PTR₂₄ be ≥ 75% with a standard deviation ≤ 9% and a 95% CI lower limit ≥ 70% after transfusion of labeled autologous RBC [86]. More recently, the FDA is also requiring manufacturers to determine the long-term survival of RBC *in vivo* [9]. The current methods applied in FDA licensure are ⁵¹Cr radiolabeling for RBC recovery and survival and ⁹⁹Tc(m) for blood volume determinations. Although these methods have been considered the gold standard for RCS analysis, the level of imprecision for survival analysis beyond day 28 post-transfusion and the necessity of using radioisotopes in healthy subjects have provided an impetus for developing alternative labeling methods. The biotin method avoids radiation exposure, permits accurate determination of both short- and long-term survival of fresh and stored autologous and allogeneic RBC. Accordingly, the biotin method is an excellent alternative to radiolabeling.

VII. Safety of the Biotin Method

The administration of BioRBC in human subjects carries several potential, but manageable, safety issues (Figure 4). These issues include maintenance of sterility throughout the biotinylation and transfusion procedures, toxicity of the biotinylating reagent, and immunogenicity of RBC surface proteins altered by biotinylation. Thus far, no safety issues have been reported among any of the 89 adult and 154 infant study subjects who received BioRBC (Tables 1 and 2). Surveillance for safety is paramount when performing the four

steps required in all human applications of the biotin method: 1) washing RBC prior to biotinylation; 2) biotinylation itself; 3) washing BioRBC to stop the biotinylation reaction with removal of excess biotinylating reagent and reaction byproducts; and 4) post-transfusion determination of RBC recovery and survival. The FDA requires that these issues be addressed in an IND application.

Sterility of BioRBC

The administration of any blood product is associated with the risk of transmitting an infectious agent (viral, bacterial, or protozoal). To avoid this, the initial blood sample must be drawn sterilely, and biotinylation must be performed using sterile technique under a Class 2 Hood. Ideally transfusion of the BioRBC occurs within 3 to 4 h after labeling. In our studies, the incidence of positive cultures of the BioRBC infusates was similar to that encountered in handling other blood products (cumulative unpublished experience of the authors).

The transfusion of allogeneic blood products, including BioRBC, also confers additional infectious risks from the donor blood. To reduce these risks, AABB (formerly known as the American Association of Blood Banks) guidelines and FDA-mandated blood bank regulations have established strict standards of quality and safety.

Toxicity of Biotinylation Reagent

Cytotoxicity was assessed using a sensitive hematopoietic stem cell assay (HALO-96 suspension expansion kit, HemoGenix, Colorado Springs, CO; <http://hemogenix.com>). Cultured human marrow mononuclear cells were exposed to the supernatant from the biotinylation reaction mixture and the supernatants of the washes after biotinylation at either 9 µg/mL (low) or 243 µg/mL (high) of biotinylation reagent per mL of RBC. Adenosine triphosphate (ATP) concentrations were measured by bioluminescence after 5 days in cell culture. No significant decreases in ATP concentration were observed indicating that the RBC biotinylation reaction products had no toxic effect on either intracellular energy status or cellular proliferation.

Antibodies to BioRBC

Alteration of the protein rich surface of the RBC by covalent attachment of biotin to exposed lysine residues of RBC surface proteins may elicit an immune response in some individuals. Indeed, in one of our studies, antibodies to BioRBC developed in 3 of 20 adults transfused with autologous BioRBC [87]. To date a total of 4 adult study subjects have developed antibodies to BioRBC 3 to 4 months post-transfusion [57, 88]. None had evidence of hemolysis, change in the BioRBC survival, abnormalities of their peripheral blood count or RBC or reticulocyte indices, or untoward clinical signs or symptoms. However, we recently re-challenged a healthy adult who had produced detectable anti-BioRBC antibodies on initial exposure to a mixture of four densities of BioRBCs. We observed that BioRBCs were removed at an accelerated rate ($162 > 54 > 18 > 6$ µg/mL). This person had no other evidence of hemolysis; peripheral blood count, RBC morphology, and reticulocyte indices were normal. Moreover, the subject's plasma concentration of biotin and urinary excretion of biotin remained at or modestly greater than normal during the study and at follow up four

months after the second BioRBC re-challenge. There were no untoward clinical signs or symptoms (unpublished observations). We infer that an amnestic response was elicited rendering estimation of true *in vivo* RCS inaccurate. None of 29 newborn infants who received BioRBC had an antibody response (unpublished data). This is expected because infants rarely develop antibodies to RBC antigens post-transfusion [89]. The extremely low immunogenicity of allogeneic RBC in infants provided the rationale for the AABB standard that, during the first four months of life, infants undergo RBC antibody screening only at the time of their first RBC transfusion [89, 90].

To date, only a minority of the adults and infants who received BioRBC (Tables 1 and 2) have been tested for antibodies to BioRBC. In addition, the best method for detecting antibodies to BioRBC has yet to be determined. Our groups initially used an assay for BioRBC antibodies based on agglutination in test tubes [88]. However, we have recently developed a more sensitive anti-IgG antibody method using gel cards (manuscript in preparation).

In summary, the safety issues raised above related to biotinylation of RBC for *in vivo* use are concerns that fall under FDA jurisdiction. Although only a small subset have been tested for the development of antibodies to BioRBC, and only a small subset of those have detectable antibodies to BioRBC, the small number justifies continued FDA surveillance and the FDA requirement for an IND to use BioRBC in humans.

Unanswered questions regarding the method for detecting antibodies to BioRBC, the immunogenicity of BioRBC, and the characteristics of the antibodies produced include the following:

1. What is the mechanism of antibody production?
2. Can immunogenicity be decreased by using “low dose” biotin RBC labeling thereby reducing (or even eliminating) antibody production?
3. Do the antibody epitopes include the biotin moieties, other fragments of the biotin label, and/or other RBC surface proteins that are structurally altered in response to the biotinylation procedure?

VIII. Summary and Conclusions

The use of BioRBC in *in vivo* studies of RCS offers many advantages — with few disadvantages — over the current reference method, ⁵¹Cr RBC labeling. Chief among the advantages are avoidance of radioisotopes, more accurate measurements of RCS and age-dependent cellular properties for the entire RBC lifespan, and the ability to concurrently follow multiple RBC populations in the same study subject. While the occasional development of antibodies to BioRBC remains a legitimate concern, current data indicate that safety may not be a significant clinical issue. As new knowledge is acquired through application of BioRBCs, our anticipation is that safety issues will continue to be few and clinically insignificant and that improvements in the biotin method can further reduce safety concerns. Use of BioRBCs has the potential for increasing knowledge in a wide range of clinically important areas that can lead to safer, more effective therapies in hematology and

transfusion medicine. We anticipate that an increasingly stronger case can be made for adoption of the biotin method as a new reference method for evaluation of RBC storage media and blood banking practices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Survival of autologous BioRBCs data from a healthy adult in erythropoietic steady state illustrating PTR₂₄, T₅₀, and mean potential lifespan as defined in the accompanying text [57]. Depicted is the decline enrichment RBC biotinylated at a single low density (6 µg of biotinylating reagent per mL of RBC).

Figure 2.

Representative flow cytometric histogram of multidensity BioRBC. In a histogram of the number of RBC versus fluorescent intensity per RBC, five discrete RBC populations (four BioRBC and one unlabeled RBC) are observed in a venous blood sample after infusion of a mixture of BioRBCs labeled individually at 4 discrete biotin densities.

Figure 3.

Post-transfusion RCS decreases with increasing BioRBC densities. Comparison of RCS concurrently studied with four separate 3-fold increasing densities of BioRBC. Panel A: Mean data for autologous RBC of eight normal adults as reported by Mock et al. [57]. The two highest densities yielded significant progressive shortening of long-term RCS. Panel B: Mean data of allogeneic BioRBC for nine very low birth weight infants are compared to non-manipulated Kidd antigen mismatched RBC as reported by Widness et al. [59]. The three lowest BioRBC densities track well with Kidd antigen, but the highest density BioRBC resulted in a significantly accelerated decline in tracking.

Figure 4.

Potential safety issues encountered in the preparation and administration of BioRBC. Bold font indicates actions to be taken to improve safety.

Table 1

Adult studies utilizing BioRBC labeling for RBC survival measurement.

Author(s) (y)	N	Autologous vs. Allogeneic	Gender (M/F)	Age (y)	Normal Controls Included	Disease State	Primary Purpose of Study
A. RBC volume							
Cavill et al., 1988 [51]	22	Autologous	ND	ND	No	Patients referred for RCV measurement	Validation of biotin labeled RBCs compared to ^{51}Cr labeled
Franco et al., 1998 [2]	7	Autologous	ND	ND	No	SS Disease	Validated biotin RBC labeling by compared to ^{51}Cr RBC labeling
Mock et al., 1999 [5]	10	Autologous	5/5	ND	Yes	None	Validate RCV by biotin RBC labeling measured by flow cytometry & ^{125}I -SA total biotin label by compared to ^{51}Cr RBC labeling
Mock et al., 2011 [49]	8*	Autologous	3/5	ND	Yes	None	Comparison of RCV results among 4 different BioRBC densities
Mock et al., 2004 [55]	10 [†] *	Autologous	5/5	ND	Yes	None	Compare RCV determined independently using 2 BioRBC populations with ^{51}Cr -labeled RBCs
B. Post-transfusion recovery (PTR₂₄)							
Franco et al., 1998 [2]	7 [†]	Autologous	ND	ND	No	SS Disease	Validated biotin RBC labeling by compared to ^{51}Cr RBC labeling
C. Long-term RBC survival, i.e., T₅₀ and mean potential lifespan							
Franco et al., 1998 [2]	7 [†]	Autologous	ND	ND	No	SS Disease	Validated biotin RBC labeling by compared to ^{51}Cr RBC labeling
Mock et al., 1999 [53]	10 [†]	Autologous	5/5	ND	Yes	None	Validate RCS by biotin RBC labeling measured by flow cytometry & ^{125}I -SA total biotin label by compared to ^{51}Cr RBC labeling
Franco et al., 2000 [70]	3	Autologous	ND	ND	No	SS Disease	To test the high-density-enriched (HDE) population of sickle cells for survival
Yassin et al., 2003 [71]	6 [†]	Autologous	ND	ND	No	SS Disease	Determine the relationships among PS externalization, HbF content, hydration state, and cell age in SS patients
Buchta et al., 2005 [91]	7	Autologous	2/5	[52–81]	No	Patients with post-op blood loss	Evaluate efficacy and safety of transfusion of blood collected via drains following orthopedic surgery
Franco et al., 2006 [69]	10	Autologous	ND	ND	No	SS Disease	Determine overall BioRBC survival, non-F- and F-cell survival in SS disease
Cohen et al., 2008 [3]	12	Autologous	5/7	49 ± 8 [36–62]	Yes	Type 1 diabetes (n=6)	Explained discordance between HbA _{1c} in diabetics attributable to red cell survival

Author(s) (y)	N	Autologous vs. Allogeneic	Gender (M/F)	Age (y)	Normal Controls Included	Disease State	Primary Purpose of Study
Mock et al., 2011 [57]	8 ^{†*}	Autologous	3/5	ND	Yes	None	Comparison of RCS results among 4 different BioRBC densities
Mock et al., 2012 [50]	10	Autologous	5/5	ND	Yes	None	Documents the ability of BioRBCs to measure RBC survival of red cells coated with RhD antibodies
Franco et al., 2013 [12]	6 [†]	Autologous	2/4	50 ± 10 [36–62]	Yes	None	Cell sorting of BioRBCs to investigate mechanism of human RBC senescence
Total Studied	89						

Mean ± SD (Median) [Range]

* Multidensity BioRBC study

[†] Same study subjects as in another study

Table 2

Infant studies utilizing BioRBC labeling for RBC survival measurement.

First Author (y)	N	Autologous vs. Allogeneic	Birth Weight (g)	Birth Gest Age (wk)	Post-Natal Age at Start of Study (d)	Primary Purpose of Study
A. RBC volume						
Hudson et al., 1990a [82]	20 [†]	Allogeneic	[620–2,400]	[25 to 34]	25 [2–40]	Determine circulating RCV in anemic preterm infants
Hudson et al., 1990b [56]	24	Allogeneic	1,050 ± 26	[25 to 34]	26 [2–40]	To investigate the value of measurements of RCV in predicting the need for, & benefit from, RBC transfusion defined by a fall in cardiac output
Mock et al., 2001 [81]	26	Autologous	974 ± 191 [551–1,300]	28.0 ± 1.8 [24–31]	35.7 ± 16.4 [7–79]	Examined the correlation between HCT and circulating RBC volume in VLBW infants
Strauss et al., 2003 [84]	31	Autologous	1,990 & 1,810	36	1	Test hypothesis that delayed cord clamping at delivery will expand neonatal circulating blood and RCV to favorably impact clinical & laboratory endpoints
Aladangady et al., 2004 [83]	32	Allogeneic	1,220 g [480–2,060]	(30) [24–32] 6	1	Investigate the relation between the measured intravascular BV & current methods of indirectly assessing BV status in sick preterm infants
Aladangady et al., 2006 [92]	23	Allogeneic	ND	[24 to 33]	1	Evaluate placental blood transfer to preterm infants at delivery by delayed clamping, position of infant relative to placenta & with oxytocin
B. Short-term post-transfusion recovery (PTR₂₄)						
Strauss et al., 2004 [93]	15 [†]	Allogeneic	948	37	14 [16–54]	Examine the feasibility, efficacy, and safety of transfusing stored allogeneic RBCs on RBC recovery & survival
Nalbant et al., 2013 [6]	18	Allogeneic *	950 ± 230 [390–1,400]	27.8 ± 1.1 [26.3–30.1]	17.9 ± 13.8 [1.0–45.0]	Comparison of results using different direct RCV measurement techniques
C. Long-term RBC survival, i.e., T 50 and mean potential lifespan						
Strauss et al., 2004 [93]	15 [†]	Allogeneic	948	37	14 [16–54]	Examine the feasibility, efficacy, and safety of transfusing stored allogeneic RBCs on RBC recovery & survival
Widness et al., 2013 [59]	17 ^{†*}	Allogeneic *	950 ± 230 [390–1,400]	27.7 ± 1.1 [26.3–30.1]	15.3 ± 12.3 [1–45]	Comparison of concurrent tracking survival of 4 different BioRBC densities & 1 minor RBC antigen mismatch
Strauss et al., 2013 [60]	8 [†]	Autologous & Allogeneic *	808 ± 64 [590–1916]	25.7 ± 0.7 [24.4–31.0]	2.5 ± 1.7 [0.5–13.6]	Comparison of concurrent tracking survival of 2 different BioRBC densities
Total Studied	154					

Mean ± SD (Median) [Range]

* Multidensity BioRBC study

† Same study subjects as in previous study

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Table 3 Comparison of Methodological Features of Biotin vs. ^{51}Cr RBC Labeling

<u>Methodological Features</u>	<u>BioRBC</u>	<u>^{51}Cr</u>	<u>Comment</u>
Ability to use in vulnerable study populations?	Yes	No	Ethical considerations may prohibit radiation use in research.
Radioactive waste disposal?	No	Yes	^{51}Cr disposal is a hazard and an expense.
Ability to follow >1 RBC population concurrently?	Yes	No	BioRBC method shortens time if >1 RBC label is required.
Correction required for label elution?	No	Yes	ICSH correction for average ^{51}Cr elution is only valid for 30 d [36]
Artifact from hemoconcentration or hemodilution?	No	Yes	Capillary blood sampling is possible for BioRBC method.
Limitation due to counting errors	>120+ d	30 d	
Ability to do multichannel analysis?	Yes	No	
Ability to recover labeled RBCs for analysis?	Yes	No	
Required sample volume (mL)	0.01	0.1–1	BioRBC volumes are suitable for infant studies.
Complexity of labeling procedure	4–6 wash steps	No wash steps	Biotinylation requires 2 sets of wash steps and a sterile hood.
Suitability for pre-clinical animal testing	High	Moderate	<i>In vivo</i> biotinylation is possible.
Ascertain the anatomic location of RBC removal?	No	Yes	
Development of antibodies?	Yes	No	With no reported adverse clinical outcomes