

Comparison of multiple red cell volume methods performed concurrently in premature infants following allogeneic transfusion

Demet Nalbant¹, Prasad Bhandary¹, Nell I. Matthews², Robert L. Schmidt¹, Anna Bogusiewicz², Gretchen A. Cress¹, M. Bridget Zimmerman³, Ronald G. Strauss^{1,4}, Donald M. Mock^{2,5} and John A. Widness¹

BACKGROUND: Study of the pathophysiology and treatment of anemia of prematurity is facilitated by direct measurement of red cell volume (RCV) utilizing microliter quantities of blood samples. Our objective was to compare concurrent measurements of multiple direct RCV methods in infants.

METHODS: Eighteen preterm infants receiving clinically indicated transfusions had concurrent flow cytometric determinations of RCV and 24-h red blood cell (RBC) recovery based on donor–recipient differences of biotin-labeled RBCs (BioRBCs), Kidd antigen mismatched RBCs, and fetal hemoglobin–positive (HbF⁺) RBCs. High-performance liquid chromatography (HPLC) was also used for measuring HbF and adult hemoglobin protein concentrations for the determination of RCV.

RESULTS: Concurrent RCV measurements using BioRBCs (18 and 54 $\mu\text{g}/\text{ml}$), Kidd antigen, and HbF flow cytometry were not statistically different compared with RCVs measured using the reference BioRBC density (6 $\mu\text{g}/\text{ml}$). By contrast, the HbF–HPLC method overestimated RCV by 45% compared with the reference method. All the methods demonstrated 100% 24-h post-transfusion RBC recovery (PTR_{24}).

CONCLUSION: Because BioRBC, Kidd antigen, and fetal hemoglobin (HbF) flow cytometry are safe and accurate methods requiring <10 μl of patient blood for determining RCV and PTR_{24} in preterm infants, they can be useful in clinical and research studies of anemia and other conditions.

Anemia is a common and serious clinical problem among critically ill premature infants. Important contributors to anemia of prematurity include low hemoglobin (Hb) at birth, blood loss due to phlebotomy for laboratory testing, and inability of the infant to produce sufficient red blood cells (RBCs) to overcome blood loss and postnatal expansion of blood volume (BV) with rapid growth. Currently, the two most common treatments for anemia in premature infants are allogeneic RBC transfusion and recombinant erythropoietin (1). To assess the effectiveness of these and other therapies, safe,

accurate, and versatile methods for determining circulating red cell volume (RCV), BV, and 24-h posttransfusion recovery (PTR_{24}) of transfused blood are needed. Among ill premature infants, RCV is deemed a better indicator of the need for RBC transfusion than either whole-blood Hb or hematocrit (HCT) levels (2,3).

Flow cytometric enumeration of RBCs can be used in the determination of RCV and BV and requires only a few microliters of blood. This method relies on its ability to discriminate transfused from endogenously produced RBC populations. Flow cytometry has been used for detecting fetomaternal hemorrhage (4,5), for determining RBC phenotype following bone marrow transplant (6), for measuring RCV (7–9), for detecting illicit blood transfusions in athletes (10), and for determining RBC survival (11,12). Recently, our group has, for the first time, demonstrated that RCV can be accurately determined in adult humans and sheep using multiple distinct populations of biotin-labeled RBCs (BioRBCs) enumerated by flow cytometry (8,13).

In this study, we extend the previous work with the objective of comparing concurrent RCV determinations using four different methods in very-low-birth-weight (VLBW) premature infants weighing <1.5 kg at birth: flow cytometric methods, including (i) multidensity BioRBC, (ii) Kidd antigen (Jka and Jkb) mismatches between adult donor and infant RBCs, and (iii) dilution of infant RBCs containing primarily HbF by donor RBCs containing primarily adult hemoglobin (HbA); and a non–flow cytometric method that measures change in proportion of HbA and HbF proteins using high-performance liquid chromatography (HPLC). Only the biotin labeling method requires labeling of donor RBCs before transfusion. Because of extremely limited recovery data for stored donor blood in infants, we also determined the PTR_{24} of transfused RBCs using the same methods.

Based on our previous findings in adults (8,11), we hypothesized that (i) RCV determined using RBCs biotinylated at three high biotin densities (18, 54, and 162 μg of biotinylating

¹Department of Pediatrics, University of Iowa, Iowa City, Iowa; ²Department of Biochemistry and Molecular Biology, University of Arkansas for Medical Sciences, Little Rock, Arkansas; ³Department of Biostatistics, University of Iowa, Iowa City, Iowa; ⁴Department of Pathology, University of Iowa, Iowa City, Iowa; ⁵Department of Pediatrics, University of Arkansas for Medical Sciences, Little Rock, Arkansas. Correspondence: John A. Widness (john-widness@uiowa.edu)

Received 5 February 2013; accepted 5 April 2013; advance online publication 2 October 2013. doi:10.1038/pr.2013.143

reagent per milliliter RBCs), Kidd antigen mismatch, and Hb type differences methods would not differ significantly from RCV determined using a previously validated low BioRBC density (6 µg/ml) as the reference method; (ii) allogeneic RBCs would completely equilibrate within the first 20-min posttransfusion (i.e., there would be no mixing or spleen effect); and (iii) PTR₂₄ assessed by all the methods would not be significantly different than 100%.

RESULTS

Eighteen premature infants with gestational ages at birth between 26 and 30 wks were studied (Table 1). Mean (±SD) birth weight was 0.96 ± 0.24 kg (range: 0.39–1.40 kg). On the day the study transfusion was administered, infants were 18 ± 14 d old (range: 1–45 d) with body weights of 1.21 ± 0.45 kg (range: 0.37–2.21 kg).

Flow Cytometric Identification of RBC Populations

The four discrete BioRBC densities and the unlabeled RBCs demonstrated complete separation, permitting accurate enumeration of each of the four BioRBC populations (Figure 1a). Pre- and posttransfusion histograms based on Kidd Jka and Jkb antigens also exhibited no overlap of peaks (Figure 1b,c). Because the study transfusion was the first transfusion for this specific infant, there was only a single peak (Jkb. RBCs)

originating from the infant in the pretransfusion sample (Figure 1b). After transfusion of Jkb mismatched RBCs, the expected two peaks were present. By contrast, both pre- and posttransfusion samples from an infant who had already received Kidd antigen mismatched blood before the study showed two distinct peaks (Figure 1c). After the transfusion, the proportion of Jka⁻ donor RBCs increased whereas the proportion of Jka⁺ infant RBCs decreased. The histograms for HbF-positive (HbF⁺) RBCs in subjects without and with previous transfusions demonstrated peak patterns similar to those for the two Kidd antigens (Figure 1d, e).

Comparison of RCV Measurement Methods by Regression Analysis

RCV measured at 20-min posttransfusion using the reference BioRBC density agreed well with the RCV measured using the higher BioRBC densities, Kidd antigen, and HbF flow cytometry methods but not with that measured using the HbF-HPLC method (Figure 2). The agreement of the reference BioRBC method at densities 18, 54, 162 µg/ml were greater than for the Kidd antigen and HbF flow cytometry methods as judged by range of the 95% confidence interval (CI) for both the slope and y-intercept (Figure 2a–c; Table 2). Among all the methods, the HbF HPLC method showed the weakest correlation with the reference BioRBC density (R² = 0.869 and the widest 95% CIs; Figure 2f; Table 2).

There was a single notable outlier RCV determined using the Kidd antigen and both HbF methods (Subject 13 shown as a triangle in Figure 2d–f). RCV values for this subject were greater than mean ± 2 SD relative to the other RCV values. We have no explanation for this outlier. This point was excluded from the regression analyses.

Comparison of 20- and 90-min Posttransfusion RCV Determinations

In our previous adult sheep studies, we observed a slowly equilibrating pool of intravascular RBCs that accounted for about one-third of the total circulating RBCs (13). This was likely due to pool of RBCs sequestered in the ovine spleen that equilibrates slowly with the circulating RBCs and BioRBCs. To determine whether a similar phenomenon was present in VLBW infants, we compared paired RCV values at 20- and 90-min posttransfusion using a linear mixed model analysis for repeated measures (Table 3). Differences between mean RCV values at 20 and 90 min were not significant, thus indicating no reservoir of slowly mixing RBCs in the spleen of VLBW infants.

Effect of Individual Method on RCV

Two statistical comparisons of the RCV results from the individual methods were performed: linear mixed model analysis with repeated measures and Bland–Altman plot analysis. In both comparisons, RCV determined by individual methods was expressed as a ratio relative to the reference BioRBC density RCV. Because mean posttransfusion RCV values at 20 and 90 min were not significantly different, the linear mixed

Table 1. Study subject demographics

Subject number	Gestational age at birth (wk)	Birth weight (kg)	Study age (d)	Study weight (kg)
1 ^{a,b,c}	27.9	0.83	22	1.25
2 ^{a,c}	28.7	1.31	40	2.21
3 ^{a,c}	28.0	0.88	23	1.10
4 ^{a,b,d}	28.0	1.09	8	0.96
5 ^{a,c,e}	28.7	1.37	10	1.32
6 ^{a,d,e}	28.0	0.93	1	0.93
7 ^{a,c}	29.9	0.73	3	0.76
8 ^{a,c,e,f}	30.1	1.40	33	1.96
9 ^{a,c,e,f}	27.4	0.91	45	1.76
10 ^{a,c,f}	26.7	0.86	33	1.55
11 ^{a,c,f,g}	27.4	0.83	15	0.98
12 ^{a,d,f}	26.3	0.39	1	0.37
13 ^{a,c,e,f,g,h}	27.4	1.08	15	1.20
14 ^{a,c,e,f,g}	28.9	0.92	34	1.52
15 ^{a,b,c,e,f,g}	27.0	1.00	9	0.87
16 ^{a,b,c,e,f,g}	27.0	0.93	13	1.06
17 ^{a,b,d,e,f,g}	26.3	0.95	5	0.92
18 ^{a,c,e,f,g}	27.4	0.95	13	0.98
Mean	27.8	0.96	18	1.21
SD	1.06	0.24	14	0.45

BioRBC, biotin-labeled RBC; HPLC, high-performance liquid chromatography.
^aBioRBC, n = 18. ^bKidd Jka mismatch, n = 5. ^cPeripheral capillary posttransfusion whole-blood sample, n = 14. ^dCentral arterial posttransfusion whole-blood sample, n = 4. ^eKidd Jkb mismatch, n = 10. ^fHbF flow cytometry, n = 11; ^gHbF HPLC, n = 7. ^hOutlier.

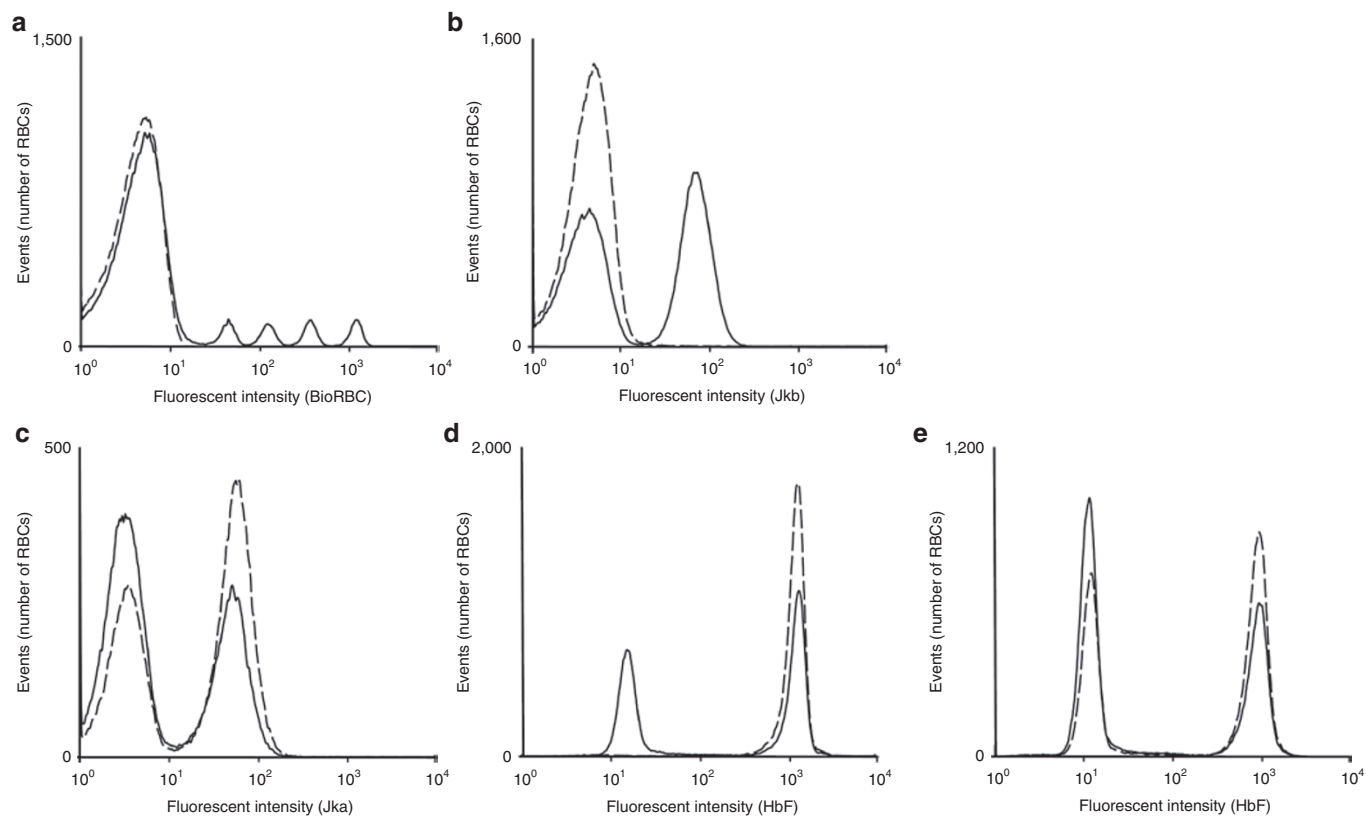


Figure 1. Flow cytometry histograms of pre- and posttransfusion samples showing number of red blood cells (RBCs) enumerated (y-axis) to log of fluorescent intensity (x-axis) showing distinct RBC populations detected by different methods. Dashed lines indicate pretransfusion samples and solid lines indicate posttransfusion sample results. **(a)** Subject 15, BioRBCs detected using Alexa-streptavidin; **(b)** Subject 14 with no previous transfusion, Jkb⁺ RBCs detected using anti-Jkb (infant RBCs are Jkb⁻ and donor RBCs are Jkb⁺); **(c)** Subject 4 with a previous transfusion, Jka⁺ cells detected using anti-Jka (Infant RBCs are Jka⁺ and donor RBCs are Jka⁻); **(d)** Subject 12 with no previous transfusion, HbF⁺RBCs detected using anti-HbF; and **(e)** Subject 15 with a previous transfusion, HbF⁺RBCs detected using anti-HbF. BioRBC, biotin-labeled RBC; HbF, fetal hemoglobin.

model analysis of the methods was based on the RCV values averaged for these two posttransfusion times. In this analysis, results were deemed not different if the $\pm 95\%$ CI ratio included 1.0. Mean RCV ratios were not significantly different among the methods with two exceptions: (i) 162 $\mu\text{g}/\text{ml}$ BioRBC density overestimated the RCV by 4.4%, a difference likely to be unimportant for most clinical and research applications; and (ii) HbF HPLC overestimated RCV by 44.9%, a large difference (Table 4).

In the second statistical comparison, agreement among the methods for measuring RCV at 20-min posttransfusion was assessed by Bland-Altman plots (Figure 3). Using the reference BioRBC density as the denominator, the ratios of the RCV for each method demonstrated agreement among all methods except for HbF HPLC. For the three higher BioRBC densities, the 95% limits of agreement with the reference method were as follows: -3.3 to $+6.5\%$ for 18 $\mu\text{g}/\text{ml}$; -4.4 to $+7.9\%$ for 54 $\mu\text{g}/\text{ml}$; and -7.6 to $+15.6\%$ for 162 $\mu\text{g}/\text{ml}$. Agreement with the reference BioRBC method was not as close for the Kidd antigen (Jkb) or the HbF flow cytometry methods. The limits of agreement were -6.4 to $+19.2\%$ for Jkb and -9.6 to $+20.1\%$ for HbF flow cytometry. The HbF HPLC method significantly overestimated RCV; the limits of agreement were $+6.8$ to $+56.5\%$. As with the regression analyses, Subject 13's outlier data point was

excluded from the Kidd antigen and both HbF analyses. Due to the small sample number ($n = 4$), comparison of RCV values by the Kidd Jka method against the reference BioRBC density was not performed.

Short-Term Recovery of Transfused RBCs

To determine PTR_{24} , we assessed the percentage of transfused RBCs remaining 24 h after transfusion using the four BioRBC densities, Kidd antigen, and both HbF methods. Differences in means between 20 min and 24 h for each method were tested for significance by mixed model analysis for repeated measures. Mean PTR_{24} measured by BioRBC for the 14 study subjects were not different between 20 min and 24 h (Table 5). Indeed, PTR_{24} was not significantly different from 100% for any of the methods (Bonferroni adjusted P values: 0.14 to >0.99), thus providing evidence that the reticuloendothelial system of VLBW infants does not remove any of the transfused RBCs—whether labeled with biotin or differentiated from recipient RBCs by minor antigen or HbF—within the first 24 h posttransfusion. The mean percentage change from 20 min ranged from a decrease of -3.7% (95% CI: -9.8% , 2.8%) for HbF flow cytometry to an increase of 3.7% (95% CI: -2.4% , 10.2%) for Kidd Jkb. Due to the small sample number, data from the Kidd Jka method (two data points) were not analyzed. Because four

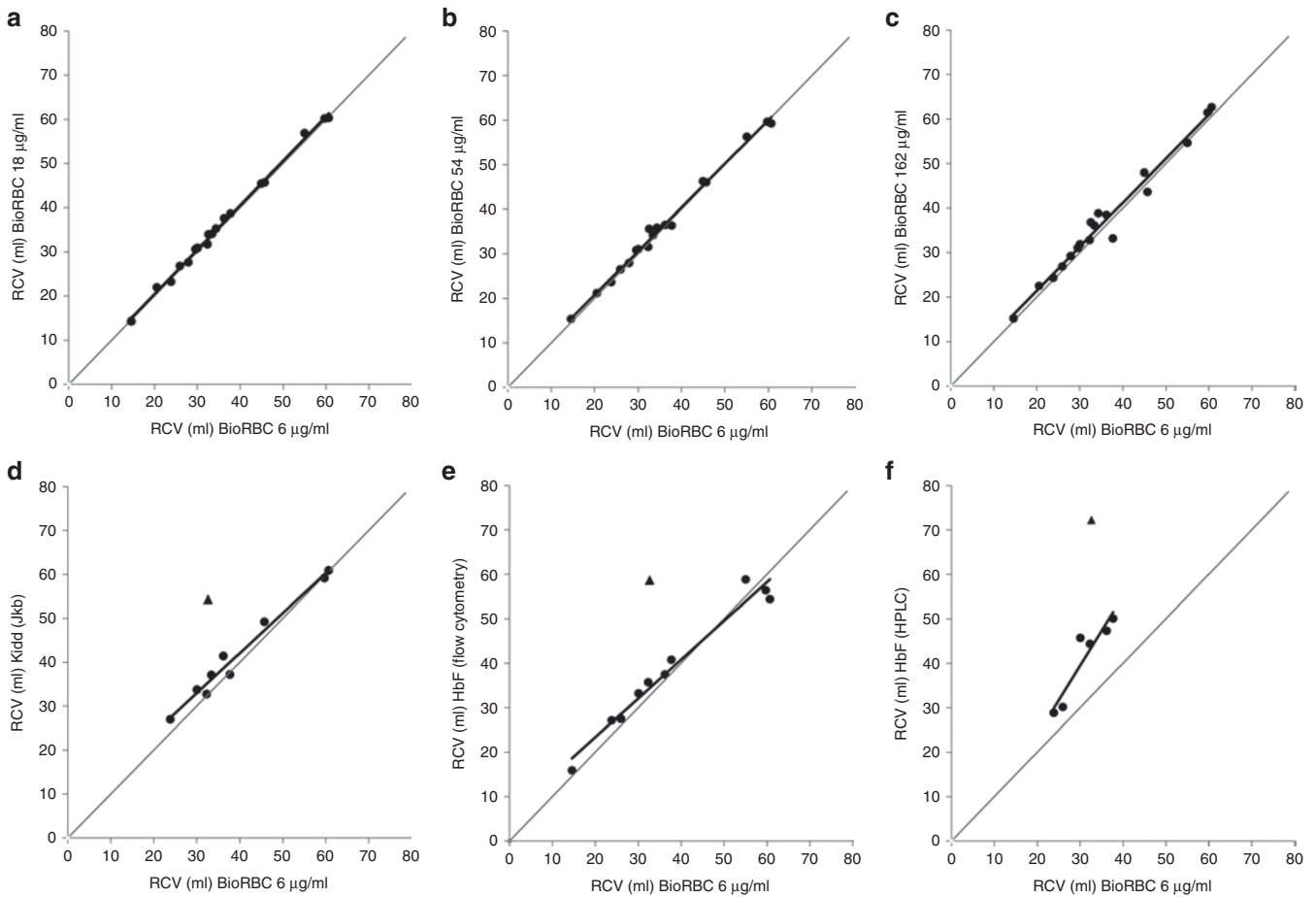


Figure 2. Agreement of red cell volume (RCV) measurements determined at 20 min. Using biotin-labeled red blood cell (BioRBC) densities (a) 18 µg/ml, (b) 54 µg/ml, and (c) 162 µg/ml. (d) Kidd antigen (Jkb). (e) HbF flow cytometry methods plotted vs. RCV measured from the reference BioRBC density. (f) RCVs determined by fetal hemoglobin (HbF) high-performance liquid chromatography (HPLC) method were overestimated compared to the reference BioRBC density. The line of identity is indicated by the thin diagonal gray line. The triangles in panels (e–f) represent the outlier data point.

subjects did not have an appropriate 24-h posttransfusion sample, they were also excluded.

RCV and BV per Kilogram

Because RCV and BV values at 20-min posttransfusion normalized by body weight are of clinical interest, we calculated the mean pre- and posttransfusion RCV (ml/kg) and BV (ml/kg) using each of the four BioRBC densities, Kidd antigen, and both HbF methods. Mean pretransfusion RCV ranged between 18.9 and 23.6 ml/kg for the flow cytometric methods, but was 30.8 ml/kg for HbF HPLC method (Figure 4a). Following RBC transfusion, infant mean RCV ml/kg increased ~50% for all methods except for RCV determined by HbF HPLC method where the mean increase was only 37%. Mean posttransfusion BV ranged between 73.7 and 85.1 ml/kg when measured using the flow cytometric methods. By contrast, the mean posttransfusion BV measured by the HbF HPLC method was 104.2 ml/kg (Figure 4b).

DISCUSSION

This study determined concurrent RCV and PTR₂₄ measured in VLBW infants undergoing clinically indicated allogeneic RBC

Table 2. Parameters and 95% CIs of the linear regressions for each method relative to the reference BioRBC density

Assay method	Slope	95% CI of slope	Intercept	95% CI of intercept	R ²
BioRBC 18 µg/ml	1.01	0.98 to 1.04	0.3266	-0.83 to 1.48	0.997
BioRBC 54 µg/ml	0.98	0.94 to 1.02	1.1777	-0.45 to 2.81	0.993
BioRBC 162 µg/ml	0.99	0.90 to 1.08	1.6459	-1.65 to 4.95	0.973
Kidd Jkb	0.91	0.78 to 1.04	5.6351	0.28 to 10.99	0.976
HbF flow cytometry	0.87	0.74 to 1.01	5.9154	-0.40 to 11.43	0.965
HbF HPLC	1.55	-0.72 to 2.39	-7.1417	-33.44 to 19.17	0.869

BioRBC, biotin-labeled RBC; CI, confidence interval; HbF, fetal hemoglobin; HPLC, high-performance liquid chromatography.

BioRBC densities, n = 18; Kidd Jkb, n = 9; HbF flow cytometry, n = 10; HbF HPLC, n = 6. Kidd Jka not included because of the small number of available study subjects (n = 5).

transfusions using multiple methods of analysis. These methods included flow cytometric analysis of multidensity-labeled BioRBCs, Kidd antigen and HbF⁺ RBCs, and HbF protein by HPLC. Our study provides evidence that RCV measured using

Table 3. Comparison of mean \pm SEM RCV results for different methods at 20 and 90 min

Assay method	RCV Mean (SEM) (ml)		% Difference (95% CI) (90 min – 20 min) \div 20 min	<i>P</i> value
	20 min	90 min		
BioRBC 6 μ g/ml	33.7 (0.30)	33.4 (0.30)	–0.7% (–2.8%, 1.4%)	0.283
BioRBC 18 μ g/ml	34.2 (0.30)	34.2 (0.30)	–0.2% (–2.6%, 2.3%)	0.821
BioRBC 54 μ g/ml	34.3 (0.30)	34.1 (0.30)	–0.6% (–2.9%, 1.8%)	0.465
BioRBC 162 μ g/ml	35.0 (0.31)	35.1 (0.31)	0.5% (–3.1%, 4.2%)	0.683
Kidd Jka	35.6 (0.32)	36.1 (0.31)	1.5% (–20.0%, 28.8%)	0.520
Kidd Jkb	37.9 (0.35)	37.7 (0.35)	–0.4% (–2.8%, 2.0%)	0.545
HbF flow cytometry	37.4 (0.37)	36.7 (0.36)	–2.0% (–9.4%, 6.1%)	0.388
HbF HPLC	47.6 (0.45)	49.7 (0.46)	4.5% (–6.0%, 16.3%)	0.120

BioRBC, biotin-labeled RBC; CI, confidence interval; HPLC, high-performance liquid chromatography; RCV, red cell volume.

Table 4. Mean RCV method ratio results relative to 6 μ g/ml reference BioRBC density

Assay method	Average of 20- and 90- min ratios			<i>P</i> value
	Mean ratio	95% CI		
		Mean ratio	Mean ratio	
BioRBC 18 μ g/ml	1.018	0.995	1.042	0.14
BioRBC 54 μ g/ml	1.019	0.995	1.043	0.15
BioRBC 162 μ g/ml	1.044	1.007	1.082	0.02
Kidd Jka	1.067	0.995	1.145	0.07
Kidd Jkb	1.126	0.966	1.312	0.15
HbF flow cytometry	1.104	0.920	1.326	0.44
HbF HPLC	1.449	1.103	1.903	<0.01

BioRBC, biotin-labeled RBC; CI, confidence interval; HPLC, high-performance liquid chromatography; RCV, red cell volume.

lowest density reference BioRBC (6 μ g/ml) agreed well with RCV measured using BioRBC densities 18 and 54 μ g/ml, Kidd antigen, and HbF flow cytometry. PTR₂₄ also agreed well among all methods. All methods required only microliter volumes of blood and none were associated with identifiable safety issues.

Since the 1970s, accurate measurement of RCV in infants has not been reported, primarily for ethical concerns related to unnecessary radiation exposure of infants enrolled in research studies. Simultaneous determinations of multiple distinct RBC populations are not possible with ⁵¹Cr or with most other RBC labeling methods. Accordingly, we contend that the multidensity BioRBC method utilizing <10 μ l sample volumes offers substantial potential for advancing our understanding of hematological and cardiorespiratory conditions and disease processes in the smallest, least mature anemic premature infants. Although a large majority of such infants develop significant anemia requiring RBC transfusion, RBC kinetic studies have never before been performed in the smallest, most fragile, high-risk patients being cared for today.

Like the multidensity BioRBC method, the Kidd antigen and both HbF methods utilize microliter volumes of blood. Although the latter methods can only be performed at the time an allogeneic RBC transfusion is administered, neither requires *ex vivo* pretransfusion RBC manipulation (i.e., labeling). This feature of the Kidd antigen and HbF methods negates all safety issues other than those inherently associated with clinically ordered RBC transfusions.

In this study, we first demonstrated the agreement among RCV measurements by the methods mentioned above using regression analysis (Figure 2; Table 2). For these comparisons, the lowest BioRBC density was selected as the reference method. This was based on our previous data-supported hypothesis that labeling with the lowest biotin density would perturb RBCs the least (11,12). RCV determined by the four BioRBC densities demonstrated a very close agreement with one another ($R^2 = 0.973$ – 0.997). This observation is similar to our previous RCV results in adults using the same four BioRBC densities (8). Similar agreement was also observed in our adult sheep multidensity BioRBC RCV study with the exception that the highest BioRBC density studied (96 μ g/ml for sheep) slightly overestimated RCV (7.6%) as compared with the lowest BioRBC density (12 μ g/ml) (13). Although in this study, the non-BioRBC RCV determinations also agreed with the reference BioRBC density, the level of agreement tended not to be as strong. When the inexplicable outlier was excluded from the Kidd antigen and HbF flow cytometry analyses, RCV results of these two methods demonstrated stronger agreement with the reference BioRBC density. The HPLC method of HbF determination demonstrated the weakest correlation with the reference BioRBC density, but HbF HPLC method also had the fewest number of usable data pairs ($n = 6$).

In addition, we demonstrated that 20 min was sufficiently long for complete equilibration of transfused RBCs with the infant's RBCs. RCV determinations can be perturbed by changes in the enrichment of labeled or mismatched RBCs in the immediate (minutes) posttransfusion period as a result of incomplete mixing or equilibration with body distribution pools. As an example of the latter, in sheep, we reported an apparent 37% increase in RCV during the first 60 min posttransfusion as the circulating transfused RBCs equilibrated with the noncirculating pool of RBCs in sheep (13). In this study, we observed no significant change in RCV determinations for any of the RCV methods between 20- and 90-min posttransfusion (Table 3). These RCV observations are consistent with our previous observations of no change in RCV after 20 min in adult humans (8). They are also consistent with results reported by Bratteby in term infants in which ⁵¹Cr-labeled autologous RBCs reached steady-state levels after an initial 10-min mixing phase (14).

We also showed that different BioRBC densities, Kidd antigen mismatch, and HbF flow cytometric methods can be interchangeably used for determining RCV in infants undergoing RBC transfusions. Although statistical analyses indicated a significant ($P = 0.02$), but clinically unimportant, 4.4% overestimate of mean RCV (95% CI: 0.7–8.2%) for the

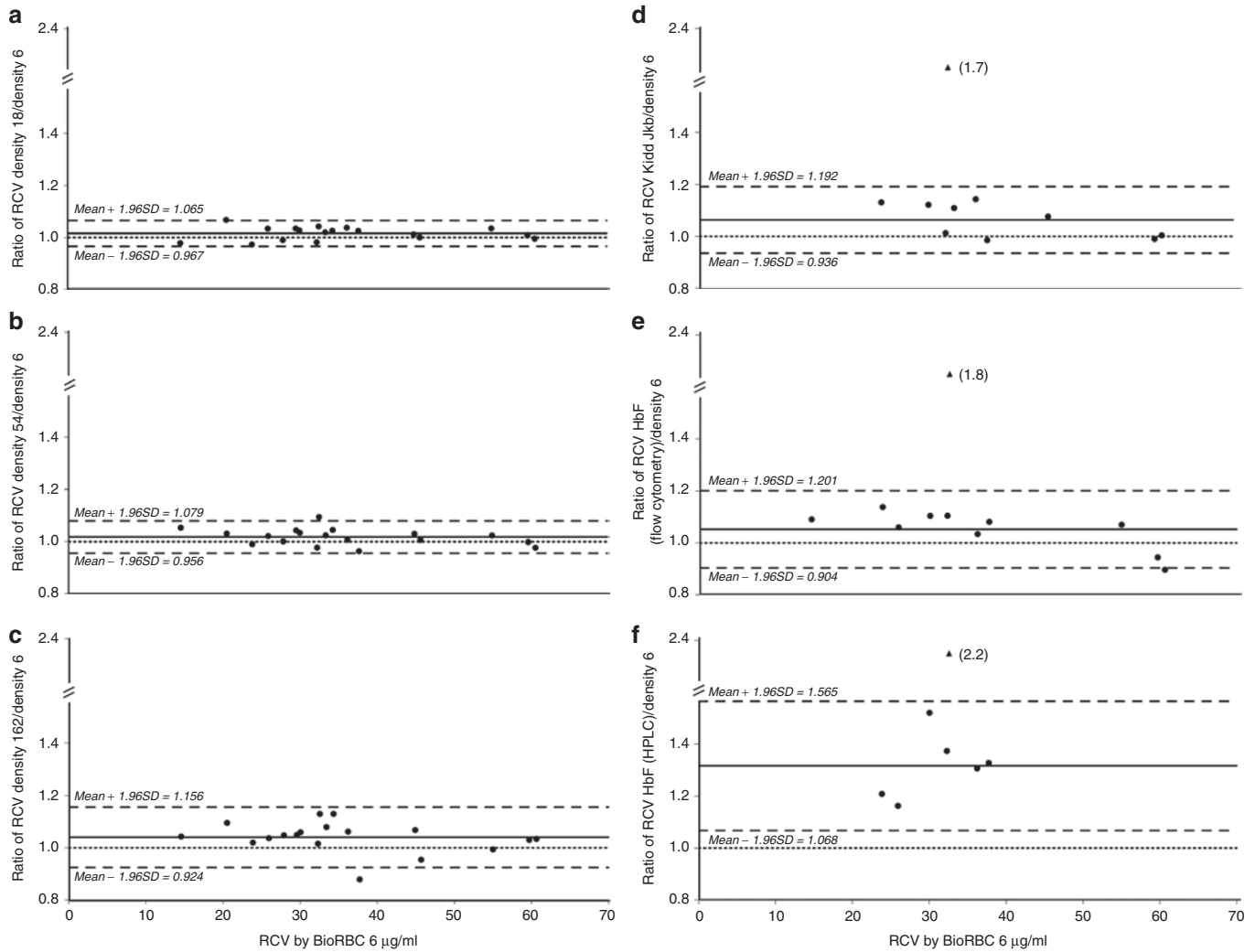


Figure 3. Bland–Altman plots of red cell volume (RCV) relative to the reference biotin-labeled RBC (BioRBC) density compared with (a) BioRBC 18 µg/ml, (b) BioRBC 54 µg/ml, (c) BioRBC 162 µg/ml, (d) Kidd Jkb, (e) fetal hemoglobin (HbF) flow cytometry, and (f) HbF high-performance liquid chromatography (HPLC). The mean ratio of RCVs (solid line), 95% confidence interval (dashed line), and ratio = 1.0 (dotted line) are shown. With the exception of (f) in which HbF HPLC significantly overestimated the limits of agreement for RCV (i.e., the area between the two dashed horizontal lines), all other plots demonstrated good agreement relative to the reference BioRBC density. The triangle data points in panels (d–f) indicate the single outlier data point.

Table 5. Short-term PTR₂₄ survival of transfused RBCs

Assay method	PTR ₂₄ Mean ± SD (%)	Difference (95% CI)	Range of CI	P value
BioRBC 6 µg/ml	102.5 ± 3.5	2.50%	−0.5 to 5.5%	0.142
BioRBC 18 µg/ml	101.0 ± 3.4	0.90%	−2.0 to 3.9%	>0.99
BioRBC 54 µg/ml	101.1 ± 3.1	1.10%	−1.5 to 3.8%	>0.99
BioRBC 162 µg/ml	100.4 ± 5.1	0.40%	−4.7 to 5.8%	>0.99
Kidd Jkb	104.2 ± 4.5	3.70%	−2.4 to 10.2%	0.377
HbF flow cytometry	96.4 ± 4.2	−3.70%	−9.8 to 2.8%	0.424
HbF HPLC	99.8 ± 6.9	−0.30%	−20.6 to 25.2%	>0.99

BioRBC, biotin-labeled RBC; CI, confidence interval; HbF, fetal hemoglobin; HPLC, high-performance liquid chromatography; RCV, red cell volume; PTR₂₄, 24-h posttransfusion RBC recovery.

BioRBC densities 6, 18, and 54 µg/ml, n = 14; BioRBC 162 µg/ml, n = 11; Kidd Jka, n = 2; Kidd Jkb, n = 7; HbF flow cytometry, n = 7; HbF HPLC, n = 4.

162 µg/ml BioRBC density (Table 4), we concluded that 162 µg/ml BioRBC density can be used for RCV determination. By contrast, the HbF HPLC method overestimated RCV by 45%; clinically important discrepancy makes the HPLC method unsuitable for RCV determination.

As hypothesized, based on our previous report in infants (15), PTR₂₄ of stored allogeneic RBCs with all methods was not significantly different from 100%, i.e., relative to the 20-min posttransfusion sample. In addition, PTR₂₄ values of 100% as determined by methods that do not require *ex vivo* RBC modification, i.e., the Kidd antigen and both HbF methods, supports our hypothesis that biotinylation of RBCs does not cause a change in PTR₂₄ in infants. These findings stand in contrast with stored RBC PTR₂₄ data in adults in which PTR₂₄ data show a significant decline relative to immediate posttransfusion enrichment (16).

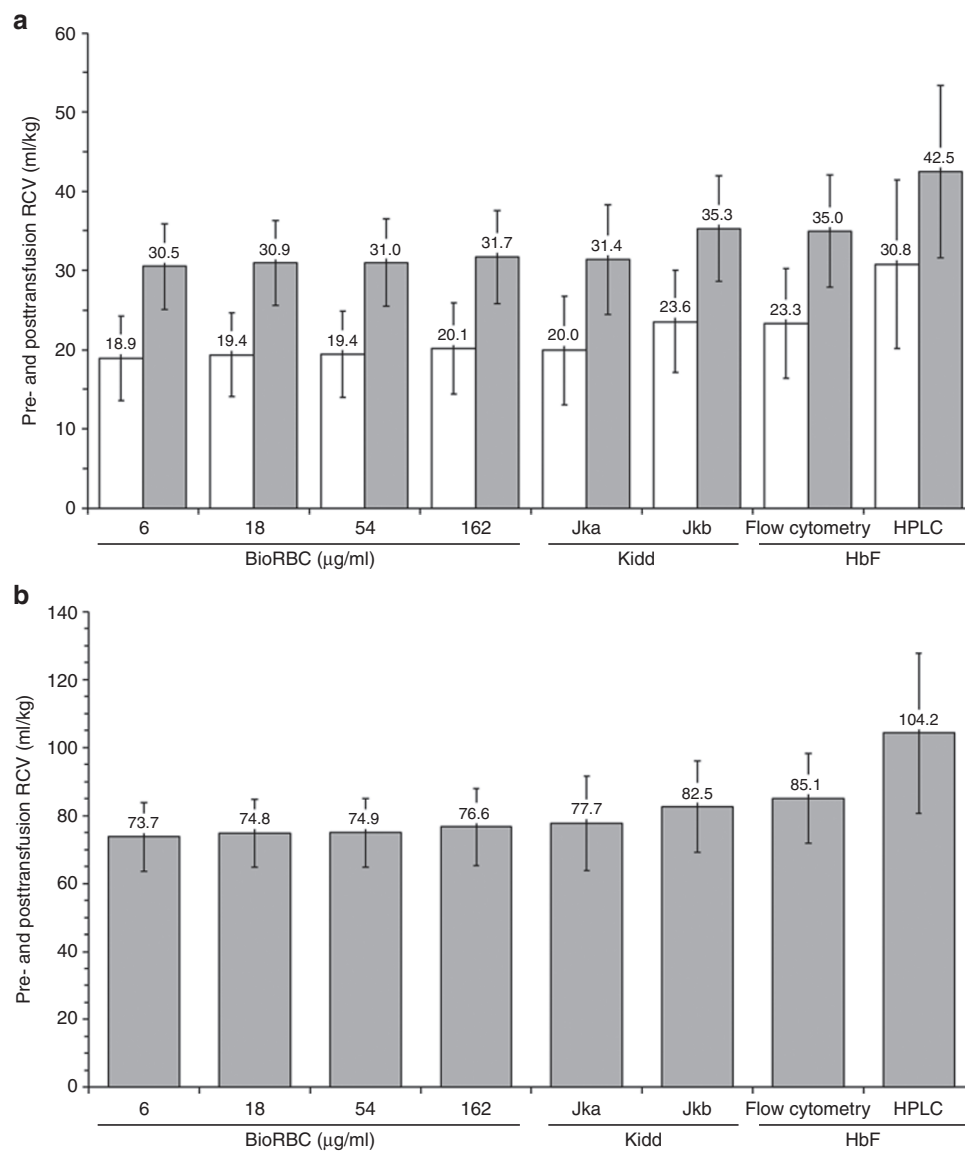


Figure 4. Results of mean (\pm SD) red cell volume (RCV) and blood volume (BV) determinations by each method. **(a)** Pretransfusion RCV in ml/kg (white bars) and posttransfusion RCV in ml/kg (gray bars); **(b)** posttransfusion BV in ml/kg. BioRBC, biotin-labeled RBC; HbF, fetal hemoglobin.

Using the RCV data determined in this study, BV per kg was derived by dividing RCV by simultaneously determined HCT measurements. Mean posttransfusion BV derived in this manner ranged from 73.7 to 85.1 ml/kg for the three flow cytometric methods (Figure 4). Because accurate HCT values immediately before transfusion were not available, it was not possible to determine pretransfusion BV. The close agreement among the posttransfusion BV determined among the flow cytometry methods and the lack of close agreement in the measurement of RCV using HbF protein by HPLC method (104.2 ml/kg) indicates that the latter method is associated with one or more unidentified systematic errors. While the derived BV values observed are within the ranges previously reported (17–20), reports of BV are highly variable because some—as in this study—are derived from only RCV and HCT, whereas others are based on direct measurement of both RCV

and plasma volume, or plasma volume alone. Studies using the latter approach report larger BVs because of the larger distribution space of the plasma (18,21). In considering infant BV measurements derived using only RCV and HCT values, it must also be recognized that HCT determinations in infants are not as reproducible as those in older children and adults. This is because of discrepancies in HCT with capillary vs. venous sampling (22).

In conclusion, this study demonstrates that RCV can be safely measured in premature VLBW infants using $<10 \mu\text{l}$ of blood without exposure to radioactivity. Evidence of strong agreement in the concurrent RCV determinations among the flow cytometric methods suggests that these methods provide true and accurate direct measurement of infant RCV. Because of the high sensitivity, safety, and reproducibility of the multidensity BioRBC method and because BioRBC studies can

be performed using either autologous or allogeneic RBCs (or both simultaneously), we contend that RCV measurement using biotin-labeled RBCs is the most useful and versatile in infants. Kidd antigen and HbF flow cytometry methods also perform well in the evaluation of the kinetics of allogeneic donor blood and have the advantage of not requiring *ex vivo* labeling. The application of these newer flow cytometric methods in measuring RCV in the smallest, least mature, and critically ill infants are useful tools in advancing our understanding of their hematological and cardiorespiratory conditions and disease processes.

METHODS

Institutional Review Boards at the University of Iowa (performance site) and the University of Arkansas for Medical Sciences (analysis site) approved the study. This included approval for administering Kidd mismatched RBCs. Informed written parental consent was obtained.

Study Subjects

A prospective, convenience sample of 18 newborn infants eligible for study included those being treated with the expectation of survival who were <31 wks gestation and expected to receive a clinically indicated RBC transfusion. Infants with congenital anomalies were excluded. Infants who had received previous RBC transfusions were not excluded. Of the 18 subjects, 8 had received one or more previous allogeneic RBC transfusions.

BioRBC Transfusion

When a clinical RBC transfusion (15 ml/kg packed RBCs with HCT of ~75%) was ordered, equal aliquots of allogeneic RBC (1.0 ml/kg packed RBCs for each density) were labeled at four discrete biotin densities as described below. Each BioRBC population transfused was calculated to produce a final enrichment of ~2% of the total number of circulating RBCs. Immediately following the infusion of the 11 ml/kg of the unlabeled clinical transfusion, the remaining 4 ml/kg containing the four BioRBC densities were infused for a period of 10 min.

Kidd Antigen Mismatch

Potential donor blood units were screened for identifying Kidd antigen mismatches with infant study subjects when possible. Donor blood units identified as mismatches were then used when a clinical transfusion was ordered. Some infants received mismatched RBCs by chance, i.e., the donor was not prescreened and selected for that infant.

Blood Sampling

Pre- and post-RBC transfusion blood samples were collected. Posttransfusion infant samples included 150 μ l volumes drawn within 20–90 min following the completion of the BioRBC transfusion. Two of the 18 infants did not have 20-min posttransfusion samples, so we used 70- and 90-min samples for those two infants as earliest posttransfusion samples. A complete blood count analysis was performed using an automated hematology analyzer (Sysmex XE-2100; Sysmex, Kobe, Japan) for determining RBC count and HCT on the first posttransfusion sample.

Biotinylation of RBCs and Analysis of BioRBCs by Flow Cytometry

The method for biotin labeling of RBCs at distinct densities in adult humans was used and aliquots of posttransfusion blood samples were processed for flow cytometric analysis of BioRBCs as described previously (8). Each distinct BioRBC population was detected as a separate peak on the posttransfusion histogram and the number of events counted under each peak was used for determining the enrichment of each BioRBC population.

Analysis of Kidd Antigens by Flow Cytometry

For Jka⁺ or Jkb⁺ RBC flow cytometry analysis, triplicate 3 μ l aliquots of the pre- and posttransfusion samples were washed to remove plasma

proteins following the same procedures as for BioRBCs (13,23). The RBCs were resuspended in 0.3 ml of wash buffer with 2% bovine serum albumin (A30075; RPI, Mount Prospect, IL) and incubated with 5 μ l of anti-Jka or anti-Jkb primary antibody (Immucor, Norcross, GA) overnight at room temperature with continuous mixing on a rotating wheel. These RBCs were washed twice and were incubated at 37 °C for 1 h with 5 μ l of Alexa Fluor 488 conjugated secondary antibody (H10120; Invitrogen, Carlsbad, CA). RBCs were then washed three times and resuspended in wash buffer with 2% bovine serum albumin (RPI) that had been filtered through a 0.2- μ m filter to a final volume of 0.5 ml. Jka⁺ or Jkb⁺ RBCs were enumerated as described above for BioRBCs.

Analysis of HbF⁺RBCs by Flow Cytometry

The method for flow cytometric analysis of HbF⁺ RBCs used for detecting HbF⁺ cells following fetomaternal hemorrhage (4) was adapted for determining the RCV in premature infants undergoing adult donor RBC transfusion. The percentage HbF⁺ RBCs was determined using Invitrogen Fetal Hemoglobin Test (Invitrogen) with fluorescein isothiocyanate-conjugated monoclonal antibody directed against HbF. The manufacturer's manual was followed with the following minor modifications. Duplicate 1.5 μ l whole-blood samples (~5 \times 10⁶ cells) were fixed in 0.25 ml of 0.05% glutaraldehyde. RBCs were permeabilized in 0.2 ml of 0.1% Triton X-100. Washed and pelleted RBCs were resuspended in 80 μ l of phosphate-buffered saline/bovine serum albumin (Invitrogen/RPI) and incubated with fluorescein isothiocyanate-conjugated HbF monoclonal antibody for 30 min at room temperature. RBCs were washed and resuspended in 0.4 ml of 2% paraformaldehyde before flow cytometry analysis. HbF⁺ RBCs were enumerated as described above for BioRBCs.

Analysis of HbF and HbA protein by HPLC

Analysis of HbA and HbF proteins by HPLC was performed using the method described by Weitzhandler *et al.* (24). Briefly, analysis of stored blood samples maintained at –80 °C was performed using a Dionex ProPac SCX-10 column (Thermo Scientific, Sunnyvale, CA) interfaced with a Waters 600E pump controller (GenTech Scientific, Arcade, NY), a SPD-10AV UV detector (Shimadzu Scientific Instruments, Columbia, MD), and an auto sampler (Perkin Elmer, San Jose, CA). Replicate hemolysate samples were prepared by mixing 5 μ l of blood in 1 ml of eluent solution (50 mmol/l sodium phosphate and 2 mmol/l potassium cyanide, pH 6.0). Ten microliters of sample hemolysate was injected and the relative percentages of HbF and HbA proteins derived by using area under the peak analysis using Shimadzu data management software. With this method, acetylated HbF elutes as a separate peak that is added to the larger nonacetylated HbF for obtaining the total percentage of HbF peak protein in the sample.

RCV and BV Calculations

Posttransfusion RCV was calculated for all methods using the dilution principle as follows:

$$\text{RCV (ml)} = \text{Total number of RBCs}_{\text{Tx}} \times \frac{\%A_{(\text{recipient pre-Tx})} - \%A_{(\text{Tx})}}{\%A_{(\text{recipient pre-Tx})} - \%A_{(\text{recipient post-Tx})}} \times \text{MCV}_{(\text{post-Tx})}$$

where the total number of RBC_(Tx) = Σ [volume of individual infusate \times RBC concentration]. The volume of infusate for individual BioRBC densities was determined gravimetrically (11) using a specific gravity for transfused RBCs of 1.05 (g/ml); %A = percentage of individual BioRBC densities (6, 18, 54, or 162 μ g/ml), Jka⁺, Jkb⁺, or HbF⁺RBCs (for HPLC, %HbF protein); Tx = transfusion; pre-Tx = pretransfusion sample; post-Tx = posttransfusion sample; and MCV = mean corpuscular volume.

Although all study subjects had RCV measurements by BioRBC performed at all four densities, only 14 had Kidd antigen mismatches. For two infants, technical difficulties precluded inclusion of the Kidd antigen results, leaving 12 RCV determinations. For the HbF flow

cytometry and HbF HPLC determinations, these methods had not been fully developed in our laboratory at the time the study was initiated: only 11 had RCV determined by HbF flow cytometry, and only 7 had RCV determined by HbF HPLC.

The posttransfusion BV ml/kg of each study subject was calculated using the following formula:

$$\text{BV (ml/kg)} = \text{RCV (ml/kg)} / \text{posttransfusion HCT}$$

Because pretransfusion HCT was not measured, pretransfusion RCV was determined by subtracting the total volume of RBCs transfused from posttransfusion RCV.

Statistical Analysis

Test for differences in mean RCV among the methods was performed by linear mixed model analysis for repeated measures. The natural log transformation of RCV was used for normalizing the data distribution. The mixed model included method, time, and method \times time interactions. In addition to the estimation of the fixed effects in the mixed model, this method of analysis allows for selection of the covariance structure that best fits the variance-covariance of the RCVs for the different methods and times in the same subject. From the linear mixed model analysis, the test for method by time interaction and the test for the posttransfusion time effect were examined. To report mean RCV in the original scale, the method $\ln(\text{RCV})$ means from the fitted linear mixed model were back transformed and the standard error was computed using the delta method. The differences in $\ln(\text{RCV})$ between the BioRBC densities were also back-transformed. This provided estimates for the mean RCV ratio relative to reference BioRBC density.

Bland-Altman plots of the observed RCV ratio for a given density, Kidd antigen (Jkb), or both HbF methods relative to reference BioRBC method were included to display the distribution of ratios. The plots also show the mean ratio and the 95% limits of agreement for the mean ratio (i.e., mean \pm 1.96 SD).

Descriptive data were expressed as the mean \pm SD. Bonferroni correction for multiple comparisons was used. All the statistical analyses were performed with computer software (SAS, Version 9.3, 2002–2008; SAS Institute, Cary, NC). A *P* value <0.05 was considered significant.

ACKNOWLEDGMENTS

We thank Robert S. Franco and Peter Veng-Pedersen for their helpful discussions with us regarding technical and theoretical aspects of this study. We appreciate the many contributions of the clinical laboratory staff led by Mitchell J. Owen and overseen by Matthew D. Krasowski, without which this work would not have been possible. We thank the many outstanding clinical research contributions of Iowa's neonatal nurse research team (Karen Johnson, Laura Knosp, Nancy Krutzfeld, Ruthann Schrock Sara Scott, and Jin Zhou) and the research laboratory team (Earl Gingerich and Jessica Goehring). We thank S. Isil Cevik for her assistance with preparation of figures and Mark Hart for his valuable secretarial assistance. The Sysmex XE-2100 automatic hematology analyzer used in this study was generously provided on an on-loan basis from Sysmex, Kobe, Japan. Finally, we appreciate the willingness of study subject families in allowing their infants to participate.

STATEMENT OF FINANCIAL SUPPORT

This publication was supported in part by the U.S. National Institutes of Health (NIH) grant P01 HL046925, by the Thrasher Research Fund 0285-3 (Salt Lake City, UT), and by grant UL1RR024979 from the National Center for Research Resources (NCRR). The paper's contents are solely the responsibility of the authors and do not represent the official views of the NIH, the Thrasher Research Fund, or the NCRR.

REFERENCES

1. Strauss RG. Anaemia of prematurity: pathophysiology and treatment. *Blood Rev* 2010;24:221–5.
2. Jones JG, Holland BM, Hudson IR, Wardrop CA. Total circulating red cells versus haematocrit as the primary descriptor of oxygen transport by the blood. *Br J Haematol* 1990;76:288–94.
3. Mock DM, Bell EF, Lankford GL, Widness JA. Hematocrit correlates well with circulating red blood cell volume in very low birth weight infants. *Pediatr Res* 2001;50:525–31.
4. Davis BH, Olsen S, Bigelow NC, Chen JC. Detection of fetal red cells in fetomaternal hemorrhage using a fetal hemoglobin monoclonal antibody by flow cytometry. *Transfusion* 1998;38:749–56.
5. Dziegiel MH, Nielsen LK, Berkowicz A. Detecting fetomaternal hemorrhage by flow cytometry. *Curr Opin Hematol* 2006;13:490–5.
6. Blanchard D, Bruneau V, Bernard D, et al. Flow cytometry analysis of dual red blood cell populations after bone marrow transplantation. *Br J Haematol* 1995;89:741–7.
7. Hudson IR, Cavilli IA, Cooke A, et al. Biotin labeling of red cells in the measurement of red cell volume in preterm infants. *Pediatr Res* 1990;28:199–202.
8. Mock DM, Matthews NI, Zhu S, et al. Red blood cell (RBC) volume can be independently determined *in vivo* in humans using RBCs labeled at different densities of biotin. *Transfusion* 2011;51:148–57.
9. Wynn R, Dixon S, al-Ismaail SA, et al. Flow cytometric determination of pre-transfusion red cell volume in fetuses and neonates requiring transfusion based on RhD+ dilution by transfused D- red cells. *Br J Haematol* 1995;89:620–2.
10. Arndt PA, Kumpel BM. Blood doping in athletes—detection of allogeneic blood transfusions by flow cytofluorometry. *Am J Hematol* 2008;83:657–67.
11. Mock DM, Matthews NI, Zhu S, et al. Red blood cell (RBC) survival determined in humans using RBCs labeled at multiple biotin densities. *Transfusion* 2011;51:1047–57.
12. Mock DM, Matthews NI, Zhu S, et al. Comparison of red blood cell survival in sheep determined using red blood cells labeled with either biotin at multiple densities or [14C]cyanate: validation of a model to study human physiology and disease. *Transfusion* 2012;52:963–73.
13. Mock DM, Matthews NI, Zhu S, et al. Red blood cell (RBC) volume can be independently determined *in vivo* in the sheep using ovine RBCs labeled at different densities of biotin. *Transfusion* 2010;50:2553–64.
14. Bratteby LE. Studies on erythro-kinetics in infancy. 8. Mixing, disappearance rates and distribution volume of labelled erythrocytes and plasma proteins in early infancy. *Acta Soc Med Ups* 1967;72:249–71.
15. Strauss RG, Mock DM, Widness JA, Johnson K, Cress G, Schmidt RL. Posttransfusion 24-hour recovery and subsequent survival of allogeneic red blood cells in the bloodstream of newborn infants. *Transfusion* 2004;44:871–6.
16. Hess JR, Hill HR, Oliver CK, et al. Twelve-week RBC storage. *Transfusion* 2003;43:867–72.
17. Aladangady N, Aitchison TC, Beckett C, Holland BM, Kyle BM, Wardrop CA. Is it possible to predict the blood volume of a sick preterm infant? *Arch Dis Child Fetal Neonatal Ed* 2004;89:F344–7.
18. Bratteby LE. Studies on erythro-kinetics in infancy. XI. The change in circulating red cell volume during the first five months of life. *Acta Paediatr Scand* 1968;57:215–24.
19. Phillips HM, Holland BM, Abdel-Moiz A, et al. Determination of red-cell mass in assessment and management of anaemia in babies needing blood transfusion. *Lancet* 1986;1:882–4.
20. Riley AA, Arakawa Y, Worley S, Duncan BW, Fukamachi K. Circulating blood volumes: a review of measurement techniques and a meta-analysis in children. *ASAIO J* 2010;56:260–4.
21. Mollison PL, Veall N, Cutbush M. Red cell and plasma volume in newborn infants. *Arch Dis Child* 1950;25:242–53.
22. Rivera LM, Rudolph N. Postnatal persistence of capillary-venous differences in hematocrit and hemoglobin values in low-birth-weight and term infants. *Pediatrics* 1982;70:956–7.
23. Mock DM, Matthews NI, Strauss RG, Burmeister LF, Schmidt R, Widness JA. Red blood cell volume can be independently determined *in vitro* using sheep and human red blood cells labeled at different densities of biotin. *Transfusion* 2009;49:1178–85.
24. Weitzhandler M, Farnan D, Horvath J, et al. Protein variant separations by cation-exchange chromatography on tentacle-type polymeric stationary phases. *J Chromatogr A* 1998;828:365–72.