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The heritability of hemolysis in stored human red blood cells

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Abstract

Background—The transfusion of red blood cells (RBCs) with maximum therapeutic efficacy is a major goal in transfusion medicine. One of the criteria used in determining stored RBC quality is end of storage hemolysis. Between donors, a wide range of hemolysis is observed under identical storage conditions. Here, a potential mechanism for this wide range is investigated. We hypothesize that the magnitude of hemolysis is a heritable trait. Also, we investigated correlations between hemolysis and RBC metabolites; this will establish pathways influencing hemolysis as future targets for genetic analysis.

Study Design and Methods—Units of RBCs from identical and non-identical twins were collected and stored under standard conditions for 56 days. Hemolysis, ATP, and total glutathione were measured throughout storage. Non-targeted metabolic analyses were performed on RBCs that had been stored for 28 days. Heritability was determined by comparing values between identical and non-identical twins.

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Results—Hemolysis was found to be heritable (mean >45 %) throughout the storage period. Correlations were observed between hemolysis and metabolites from the amino acid, sugar, and purine metabolism, lipid metabolism and transport, and glycolysis pathways. Three metabolites also exhibited heritability (> 20%). No correlation was found with ATP or total glutathione.

Conclusion—The susceptibility of RBCs to lysis during storage is partly determined by inheritance. We have also uncovered several pathways that are candidate targets for future genome wide association studies. These findings will aid in the design of better storage solutions and the development of donor screening tools that minimize hemolysis during storage.

Introduction

The safe and effective transfusion of stored red blood cells (RBCs) has been the centerpiece of transfusion therapy for nearly a century.¹ The creation of the modern blood bank with a reliable inventory of blood products revolutionized medical care. Decades of effort by many investigators have resulted in the development of extended storage solutions and containers that allow storage of RBCs for up to 42 days.¹ In spite of marked advances in RBC storage, the variable quality of stored RBCs remains a major issue in blood banking.²⁻⁴

One of the criteria used to regulate the quality of stored RBCs is to measure the degree of hemolysis during storage.⁵ Hemolysis is considered to be a result of the RBC storage lesion, with greater hemolysis reflecting poorer tolerance for the conditions of storage.⁶ In the US, hemolysis during storage is regulated to be less than 1 %, 95 % of the time, with 95 % confidence. These tight regulations allow typical licensed storage systems to have about 0.35 % hemolysis at the end of 42 storage days.⁵ Due to the wide distribution found in a human population for hemolysis, this need for tight regulations is needed; the mechanism behind this distribution is unknown.⁷ Here we investigate a potential heritable mechanism dictating hemolysis during storage.

Groundbreaking work by Dern and coworkers in the 1960s revealed the heritability of markers of stored RBC quality.^{8,9} In a series of papers, the heritability of post-storage ATP concentration was investigated using parent-sibling studies. These findings, and the heritability of multiple other metabolic pathways, have since been confirmed by our research team in a twin study.¹⁰ Based on our previous results, we hypothesized that hemolysis is a heritable trait. To test this hypothesis, a classic twin study was conducted in which the magnitude of hemolysis was monitored in the RBCs donated by a population of identical and non-identical twins. This report is a continuation of our studies reported previously and utilizes the same participants.¹⁰⁻¹²

Our results indicate that hemolysis is a heritable trait. In addition, hemolysis appears not to be correlated with a decline in the intracellular concentrations of ATP or total GSH (tGSH) even though both traits are heritable. Hemolysis is also correlated with a non-targeted metabolomic scan to pinpoint co-regulated metabolomic pathways. This metabolomic analysis indicates that hemolysis is probably controlled at least partially by a different set of genes than other heritable RBC storage traits.

Materials and Methods

Twin subject enrollment and sample collection

The study was approved by the Human Subjects office of The University of Iowa Carver College of Medicine. Written informed consent was obtained from all participating subjects. Subjects were qualified for participation by meeting criteria for autologous blood donation according to standard operating procedures of The University of Iowa DeGowin Blood Center. Twin pairs were not required to donate samples at the same time. Standard health history and demographic information was obtained at the time of enrollment and informed consent. Reported height and weight were used to calculate body mass index (BMI). BMI was derived from the formula: $BMI = \text{weight (kg)} / (\text{height(m)})^2$. From these data, the heritability of height, weight, and BMI were calculated as independent assessments of the suitability of our sample population for studies of heritable traits. Each subject donated one unit of whole blood that was processed according to standard operating procedures into a leukocyte-reduced RBC unit in AS-3 extended storage media (Haemonetics Corp, Braintree, MA). During processing, integral leukocyte reduction filters were retained for extraction of DNA.

Sample preparation

Samples of AS-3 preserved RBC units were prepared from the main unit on each day of sampling. The AS-3 preserved RBCs were sampled by sterile docking of tubing to the RBC unit, back-filling the tubing with RBCs and sectioning into segments. This procedure was performed on the first day after donation (day 0), and every 14 days thereafter until day 56.

Segments were drained into 5 mL Eppendorf tubes; after mixing an aliquot was removed for complete blood count (CBC) testing. The remaining sample was centrifuged at 500 *g* for 5 min, after which the storage media (AS-3) was removed. Samples were further processed and used for measurement of ATP, GSH, and GSSG in RBCs as previously described.^{10,11}

Hemolysis assay

The collected storage medium (AS-3) was centrifuged to remove any remaining RBCs and the supernatant diluted with isotonic phosphate buffered saline 4.5-fold (900 μL total volume). The diluted AS-3 solution was analyzed for free hemoglobin with UV/VIS spectroscopy (HP 8453 diode array spectrophotometer, Agilent, Santa Clara, CA) in a 1 cm quartz cuvette using absorbance at 415 nm ($\epsilon_{415} = 128,000 \text{ M}^{-1} \text{ cm}^{-1}$). A single reference wavelength of 700 nm was used to correct for baseline drift. The molar amount was converted to grams of hemoglobin assuming a molecular mass of 64,500 g mol^{-1} . Hematocrit (Hct) and total hemoglobin were obtained using a hematology analyzer (Sysmex XT-2000, Sysmex Corp, Kobe, Japan). To determine the percentage of hemolysis in a sample the following formula was used: $[\text{AS-3 hemoglobin}] \times (1 - \text{Hct}) / \text{total hemoglobin}$. The coefficient of variation for an assay is 5 -10 % which is mostly due to pipetting errors and lysis of residual RBCs.

Zygosity testing

DNA for zygosity testing was obtained from leukocyte reduction filters by rinsing filters with 15 mL DPBS. The rinse volume was centrifuged at 500 *g* for 10 min and the cell pellet was resuspended in 2 mL of DPBS. DNA was extracted from the cell pellet using a nucleic acid extraction instrument (AutoGen QuickGene 610L, AutoGen, Holliston, MA) and kit (Fuji QuickGene DNA Whole Blood Kit, AutoGen, Holliston, MA).

Genotyping was performed using a previously developed panel of 24 single nucleotide polymorphisms (SNPs).¹⁰ SNP genotyping was performed using PCR assays (TaqMan, Applied Biosystems, Foster City, CA) on a Genotyping System (EP1 SNP, Fluidigm, San Francisco, CA) with a Dynamic Array Integrated Fluidic Circuits (GT48.48, Fluidigm, San Francisco, CA). Monozygotic (MZ) twins were identified by 90 % or greater genotype concordance; all other twin pairs were identified as dizygotic (DZ).

Global metabolomics profile analyses

The untargeted metabolic profiling platform employed for this analysis combined three independent platforms: ultrahigh performance liquid chromatography/tandem mass spectrometry (UHLC/MS/MS²) optimized for basic species, UHLC/MS/MS² optimized for acidic species, and gas chromatography/mass spectrometry (GC/MS). Samples were analyzed using procedures described in van 't Erve *et al.*¹⁰

Statistics

Hemolysis was correlated with other metabolites using the Pearson correlation coefficient. Statistical significance was determined using one-way analysis of variance. The differences were considered statistically significant when *p* values were less than 0.1. Calculations were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp. Armonk, NY).

Metabolite identification and data analysis—Metabolites were identified by automated comparison of the ion features in the experimental samples to a reference library of chemical standard entries that included retention time, molecular weight (*m/z*), preferred adducts, and in-source fragments as well as associated MS spectra and curated by visual inspection for quality control using software developed at Metabolon.¹³ Missing values were assumed to be below the limits of detection. For statistical analyses and data display purposes these values were imputed with the compound minimum (minimum value imputation) after normalization to total protein as determined by Bradford assay for each sample.

Heritability calculations—Heritability estimates were calculated for the change in hemolysis during the entire storage period, and for the population at each measured time point. The one-way model of intraclass correlation coefficient (ICC) was used to determine the similarity of a measure in a twin pair: $ICC = (MS_{\text{between}} - MS_{\text{within}}) / (MS_{\text{between}} + MS_{\text{within}})$, where MS_{between} is the estimate of the mean-square variance between all twin-pairs and MS_{within} is the estimate of the mean-square variance within the sets of pairs in that group.¹⁴ The ICC is used to compare the variation within specific pairs to that of the

population as a whole, and falls on a scale of -1 to +1. Higher positive values indicate that there is less variation within the pairs of subjects than there would be within randomly paired subjects. Positive values approaching 0, as well as negative ICC values, indicate that the variation within pairs of subjects is similar to the variation expected within random pairs. A strong heritable trait between MZ twins would be expected to have an intraclass correlation coefficient near +1. From the ICC values heritability was estimated using the method derived by Newman *et al.*, $h^2 = (ICC_{MZ} - ICC_{DZ}) / (1 - ICC_{DZ})$.⁹

Results

Twin subjects and known heritable traits

Among 18 twin pairs, zygosity testing identified 13 MZ and 5 DZ twin pairs. The means of age, weight, and BMI were not significantly different between MZ and DZ twin groups (Table 1). As previously reported, a high degree of estimated heritability for height (96 %), weight (97 %), and BMI (63 %) was observed in this study population.¹¹ The similarity of these results to estimates in previous reports supports the validity of the sample population for determination of heritable traits.¹⁴⁻¹⁶

Hemolysis under storage conditions

The hemolysis of RBCs follows a linear pattern (≈ 0.013 % per day) (Figure 1). On average, at day -42 of storage hemolysis was ≈ 0.6 %. This is below the 1 % hemolysis benchmark established by the Food and Drug Administration in the United States. At day 42 of storage, two individuals had hemolysis values higher than 1.00 %. These individuals were part of two different MZ twin pairs, with their partners having hemolysis values of 0.78 and 0.97 % respectively. At day 56 the number of individuals with hemolysis higher than 1.00 % had risen to 8. Four individuals were part of 2 MZ pairs; two individuals were part of 2 distinct DZ pairs with their twin not exceeding 1.00 %. The remaining two individuals were part of 2 distinct MZ pairs, with their twin having a hemolysis value of 0.87 and 0.56 % respectively. When comparing mean values between MZ and DZ twin groups for hemolysis, no statistical differences were observed (Table 1).

Hemoglobin and MCV under storage conditions

The hemoglobin in RBCs does not significantly change over the storage period (15.3 – 16.7 g dL⁻¹ over 56 days) (Figure 3A). This is in concordance with previous studies. The mean cell volume of the RBCs (MCV) does not significantly change for the whole population. However, in certain units, the MCV increases with storage time (Figure 2B).

Heritability of hemolysis, hemoglobin, and MCV

The heritability of hemolysis was investigated for both the individual measured time points and the rate of change over the whole storage period. A comparison between the closeness of hemolysis among MZ and DZ twin pairs is provided in Figure 3. Significant heritability (>20 %) was found for all days of storage except for day 0 (Figure 4). In our population, we found significant heritability for hemoglobin content of RBCs and MCV (59 %, and 40 % respectively). These results are concordant with previous studies.¹⁷⁻²¹ The heritability estimates derived from our twin population for these hematological parameters^{20,21}, as well

as for height, weight, and BMI, provide a measure of confidence in our estimates for the heritability of hemolysis.

Correlation of hemolysis with intracellular RBC metabolites

To explore the hypothesis that post-storage ATP influences hemolysis, the Pearson correlation coefficient was calculated from all ATP and hemolysis data points. No statistically significant correlation between intracellular ATP and hemolysis was found (Figure 5, $R^2 = 0.00$, $p = 0.52$). The hypothesis that glutathione levels correlate with hemolysis was also explored (Figure 5). No statistically significant correlation was found between tGSH and hemolysis ($R^2 = 0.05$, $p = 0.43$).

To investigate the relationship between other metabolites and hemolysis, total metabolic scans were performed by HPLC/MS/MS and the results were correlated with hemolysis. Out of 213 identified metabolites, 8 were significantly correlated with hemolysis ($p < 0.05$) and 8 correlated with borderline significance ($0.05 < p < 0.10$) (Table 2). Of the 16 metabolites that correlated ($p < 0.10$) with hemolysis, 7 also demonstrated $\geq 20\%$ heritability (Table 2). Three metabolites correlated with hemolysis and were heritable: adenosine 5'-diphosphate (ADP), glucose-6-phosphate (G6P), and 2-oleoylglycerophosphocholine. Pathways represented by heritable metabolites that may contribute to stored RBC hemolysis include: phenylalanine and tyrosine metabolism, glutathione metabolism, glycolysis, gluconeogenesis, pyruvate metabolism, nucleotide sugars, pentose metabolism, lipid metabolism, carnitine metabolism, inositol metabolism, and adenine/guanine containing purine metabolism. A full list of metabolites identified can be found in Supplementary Table 1.

Discussion

The wide distribution in the degree of hemolysis in stored RBC units suggests that environmental or genetic factors may be responsible for observed differences in this aspect blood storage.⁷ This classic twin study demonstrated that genetic factors contribute substantially to the differences between individual donors in the degree of hemolysis of their stored RBCs. When averaged over all time points of storage, an estimated 43 % of the difference between individual blood donors in RBC hemolysis is due to heritable factors.

Our previous work showed that post-storage RBC ATP and glutathione concentrations are heritable.^{10,12} We therefore hypothesized that, insofar as membrane stability and hemolysis are related to energy metabolism and redox status, hemolysis may correlate with ATP and glutathione concentrations. Surprisingly, hemolysis was not significantly correlated with ATP or glutathione, suggesting that the genetic factors influencing hemolysis may not overlap greatly with those influencing ATP or glutathione levels. Our series of studies leads us to conclude that individual components of the RBC storage lesion (*e.g.* hemolysis, ATP concentration, *etc.*), are all heritable; however, the genes controlling these different aspects of RBC storage are varied. Future research into the precise genetic mechanisms will need to take this into account and individually address the multiple pathways that comprise the RBC storage lesion.

To further investigate metabolic pathways in RBCs that influence hemolysis, we correlated results of an untargeted metabolic screen performed on RBCs stored 28 days with hemolysis at day 28 of storage. Significant correlations with hemolysis were observed for 16 RBC metabolites. The correlated metabolites are primarily in the pathways involving amino acid, sugar, and purine metabolism, lipid metabolism and transport, and glycolysis. A number of metabolites in these pathways are also heritable, suggesting the possibility that genetic variations within these pathways may contribute to the heritability of hemolysis.

In searching the literature, no examples were found of amino acid metabolism abnormalities causing hemolysis. Since most of these metabolites correlated negatively with hemolysis and heritability was not significant for this group of analytes, we hypothesize that these amino acids were produced during proteolysis of intracellular RBC proteins. Noting the consistently negative correlations with hemolysis, high levels of these metabolites could reflect functioning proteolytic enzymes and healthier RBCs. During storage, proteolytic enzymes may gradually become nonfunctional leading to a decrease in free amino acids and an increase in defective proteins, leading to hemolysis.

It is not surprising to find a correlation between hemolysis and lipid metabolism and transport. The ideal composition and stability of the plasma membrane, which is comprised mostly of phospholipids, is crucial to maintain RBC integrity.²² The lipids that correlate positively with hemolysis in our study are mostly saturated lipids. Due to their structure, saturated lipids increase the rigidity of RBC membranes in which they are incorporated. This could lead to an increase in the susceptibility of RBCs to lysis. An exception to this is stearamide, which is negatively correlated with hemolysis. Stearamide is an unsaturated lipid which is much more flexible and we hypothesize that a certain percentage is needed to maintain healthy lipid membranes. Decreasing the amount of stearamide may result in a more rigid membrane, leading to greater susceptibility to hemolysis. On the other hand, the presence of an overabundance of highly unsaturated fatty acids could make the membrane more vulnerable to excessive lipid peroxidation.²³ Lipid peroxidation is known to be a prelude to hemolysis.²⁴ Therefore, the mechanisms we hypothesize would be lipid specific and not generalizable to all unsaturated lipids.

The detected metabolite most strongly correlated with hemolysis is ADP, which is part of the purine metabolism pathway ($R = 0.51, p = 0.002$). Glucose-6-phosphate (part of the glycolysis pathway) is also significantly negatively correlated with hemolysis ($R = -0.41, p = 0.01$). The positive correlation between hemolysis and ADP and the negative correlation with G6P, both of which are produced in the first step of glycolysis, suggest that reduced glycolysis and reduced flux through the pentose phosphate pathway resulting in an accumulation of ADP and reduction in G6P levels may contribute to hemolysis. From the negative correlation analysis with ATP, the accumulation of ADP in RBCs seems more important to hemolysis than the depletion of ATP which is a novel interpretation compared to historic data. One hypothesis explaining this data is that an increase in ADP antagonizes the function of sodium and potassium pumps which are required to maintain osmotic balance. Even in the presence of sufficient ATP, the pumps would cease to function which leads to hemolysis. Genetic variability in the regulation and metabolism of ADP and G6P

may therefore contribute to storage related hemolysis, with less emphasis being placed on the maintenance of ATP.

A positive correlation was observed with ophthalmate, which is part of the glutathione metabolic pathway. This is consistent with previous reports of oxidative stress increasing hemolysis. Also, an increase in ophthalmate could indicate a decrease in GSH synthesis; GSH has been implicated in RBC health and is also highly heritable.¹² An increase in oxidative stress could be the result of decreased pentose phosphate pathway products such as NADPH as there is a decrease in G6P; this compromises the ability of RBCs to handle oxidants such as hydrogen peroxide. Ophthalmate only showed minimal heritability in this population. Ophthalmate is considered a marker of oxidative stress; the finding of non-heritability is consistent with other markers of oxidative stress (*e.g.* F-2 isoprostanes and 8-oxodeoxyguanosine lacking heritability).²⁵

In conclusion, this study revealed that hemolysis in stored RBCs is a heritable trait, and that the heritable metabolites ATP and glutathione do not correlate significantly with hemolysis. These observations suggest that there are distinct genetic modifiers of different aspects of the RBC storage lesion. Understanding the genetic underpinnings of hemolysis and other aspects of the RBC storage lesion will be important in the development of improved RBC storage systems.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hemolysis increases linearly over a 56-day storage period

Lines show most and least observed hemolysis, black and white boxes represent first and third quartile; intersection of boxes is median hemolysis. Mean hemolysis at day 42 is $0.6 \pm 0.3\%$, which is concordant with literature values and regulatory criteria.

Figure 2. Intracellular hemoglobin and MCV do not significantly change over the 56-day storage period

Lines show most and least observed hemolysis, black and white boxes represent first and third quartile; intersection of boxes is median hemolysis.

Figure 3. Snapshot of hemolysis values among twin pairs at day 28 of storage
Graph shows monozygotic (MZ) pairs on the left and dizygotic (DZ) pairs on the right. From the graph it is evident that MZ pairs have values much closer together compared to DZ pairs which is a hallmark of heritability. Individual lines represent twin pairs.

Figure 4. Hemolysis is a heritable trait with the exception of day 0; hemoglobin and MCV are heritable in this population

Heritability was estimated using the method of Newman *et al.* from the mean intra-class correlation coefficients.²⁶ The heritability of the well-established heritable traits, BMI (63%), height (96%), and weight (97%) in our study subjects are concordant with reported values^{14,15} providing confidence that our study population is informative with respect to estimates of heritability. NH = not heritable.

Figure 5. ATP and total GSH (tGSH) levels at the day of measure do not correlate with hemolysis at the day of measure throughout the 56-day storage period
Data points are from all individuals spanning all measured time points.

Table 1
Comparison between the mono- and di-zygotic twin populations in this study

Trait	Monozygotic (MZ) [†]	Dizygotic (DZ) [†]	<i>p</i> value [*]
Female pairs	11	2	
Male pairs	2	1	
Male/female pairs	-	1	
Total pairs	13	4	
Age / Years	25 ± 7	26 ± 9	0.7
Weight / kg	68 ± 14	66 ± 8.6	0.6
Height / m	1.68 ± 0.07	1.74 ± 0.06	0.02
BMI	24 ± 4.3	22 ± 2.7	0.11
Hemolysis / % [‡]	0.59 ± 0.32	0.56 ± 0.22	0.85

* One way ANOVA DZ versus MZ.

[†] Mean ± SEM

[‡] Hemolysis at day 42 is compared.

Table 2
Correlation of day-28 metabolites with hemolysis levels at day-28

Pathway	Metabolite	Corr coef.	Q value	Heritability (%) [‡]
Glycolysis, gluconeogenesis, pyruvate metabolism	glucose-6-phosphate	-0.41 [†]	0.75	30
Lysolipid	2-oleoylglycerophosphocholine	0.36 [†]	0.98	53
Purine metabolism, adenine containing	adenosine 5'-diphosphate	0.51 [†]	0.34	74
Phenylalanine & tyrosine metabolism	phenol sulfate	-0.30 [*]	0.98	40
Glutathione metabolism	ophthalmate	0.20 [†]	0.75	4
	glycylglycine	-0.37 [†]	0.97	NH
	alanylglycine	-0.40 [†]	0.75	NH
Dipeptide	lysylglycine	-0.35 [†]	0.98	NH
	prolylasparagine	-0.43 [†]	0.75	NH
Nucleotide sugars, pentose metabolism	xylitol	-0.28 [*]	0.98	6
Long chain fatty acid	cis-vaccenate (18:1n7)	0.30 [*]	0.98	NH
Fatty acid, amide	stearamide	-0.29 [*]	0.98	NH
Carnitine metabolism	carnitine	0.33 [*]	0.98	24
Inositol metabolism	chiro-inositol	0.32 [*]	0.98	47
Monoacylglycerol	1-linoleoylglycerol (1-monolinolein)	0.29 [*]	0.98	NH
Purine metabolism, guanine containing	guanosine 5'-diphospho-fucose	-0.30 [*]	0.98	20

* 0.05 p 0.10

[†] p 0.05

[‡] NH = heritability was not observed for this trait