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The Heritability of Glutathione and Related Metabolites in Stored Red Blood Cells

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Abstract

Red blood cells (RBCs) collected for transfusion deteriorate during storage. This deterioration is termed the “RBC storage lesion”. There is increasing concern over the safety, therapeutic efficacy, and toxicity of transfusing longer-stored units of blood. The severity of the RBC storage lesion is dependent on storage-time and varies markedly between individuals. Oxidative damage is considered a significant factor in development of the RBC storage lesion. In this study, the variability during storage and heritability of antioxidants and metabolites central to RBC integrity and function were investigated. In a classic twin study, we determined the heritability of glutathione (GSH), glutathione disulfide (GSSG), the status of the GSSG, 2H⁺/2GSH couple (E_{hc}) and total glutathione (tGSH) in donated RBCs over 56 days of storage. Intracellular GSH and GSSG concentrations both decrease during storage (median net loss of 0.52 ± 0.63 mM (median $\pm$ SD) and 0.032 ± 0.107 mM, respectively over 42 days). Taking into account the decline in pH, E_{hc} became more positive (oxidized) during storage (median net increase of 35 ± 16 mV). In our study

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population heritability estimates for GSH, GSSG, tGSH, and E_{hc} measured over 56 days of storage are 79%, 60%, 67%, and, 75% respectively. We conclude that susceptibility of stored RBCs to oxidative injury due to variations in the GSH-redox buffer are highly variable among individual donors and strongly heritable. Identifying the genes that regulate the storage-related changes in this redox buffer could lead to the development of new methods to minimize the RBC storage lesion.

Keywords

red blood cells; erythrocyte; glutathione; heritability; storage lesion; twin study; transfusion medicine

Introduction

The safe and effective storage of red blood cells (RBCs) for use in transfusion therapy is one of the great achievements in modern medicine. Continuing research is focused on extending the shelf life of RBC units and improving their therapeutic effectiveness. Current additive solutions and refrigerated storage allow the storage of RBCs for a maximum of 42 days in the US. During storage, many biochemical and biophysical alterations occur. The accumulation of these changes has been termed the “storage lesion” and is a major issue in transfusion medicine [1–3]. Transfusion of RBC units stored for extended periods of time are implicated in causing morbidity and complications following transfusion. The biochemistry and biology behind these adverse effects are the focus of many laboratory and clinical studies [4–7].

Many changes take place in the metabolome and proteome of RBCs during storage. One change that occurs in stored RBCs is the accumulation of oxidative damage [8–12]. Several different markers of oxidative damage have been measured in stored RBCs, namely: lipid peroxidation [9, 13], F_2 -isoprostanes [14], protein carbonyls [9, 15], and non-reversible covalent linking of cytosolic peroxiredoxin 2 to RBC membranes [11].

A hypothesis for an increase in oxidative damage is the gradual decrease in the capacity of stored RBCs to neutralize oxidants during extended periods of storage. An important part of the defense against oxidative damage in RBCs is the intracellular redox buffer. The intracellular redox buffer is comprised of glutathione (GSH) and glutathione disulfide (GSSG), a reversible redox couple ($GSSG, 2H^+/2GSH$). Maintaining an excess of GSH is integral in keeping cellular proteins reduced and functioning [9, 13, 16–20]. It has also been shown that increasing the intracellular level of GSH levels leads to healthier RBCs throughout the storage period [12]. This has led to the suggestion that maintaining a high level of GSH is beneficial for the long-term storage of RBCs and could slow the rate of the appearance of the storage lesion.

The severity of the RBC storage lesion is highly variable among donated units of blood. It has long been known that the post-storage 24-hour recovery of stored RBCs from different donors is markedly variable [21]; although there is consistency between repeat measures within an individual [22]. These observations led to studies demonstrating a heritable

component to the RBC storage lesion [23]. There are many inborn errors in the metabolism of GSH that can alter its availability in tissues [24, 25]. In earlier work [26], we established the heritability of the elements of the principal intracellular redox buffer in fresh RBCs, *i.e.* GSSG and GSH. We therefore hypothesized that alterations occurring in the concentrations of GSH and GSSG during storage are also genetically controlled and they are distinct from genes controlling basal levels of GSH.

We explored this hypothesis in a classic twin study where eighteen identical and fraternal twin pairs donated a standard unit of blood. The levels of GSH and GSSG were measured in RBCs stored up to 56 days. Total glutathione (tGSH) levels, and the status of the redox buffer (E_{hc}) were calculated from these measurements. In this study we establish the biological variability among individual donors and a strong heritable component in the redox biology of RBCs during storage

Materials & Methods

Study design and sampling

To investigate the influences of genetics on the storage of RBCs, eighteen monozygotic (MZ) and dizygotic (DZ) twin pairs were recruited over an 8-month period to donate one unit of whole blood each at the DeGowin Blood Center at The University of Iowa. The study was approved by the Human Subjects Office of The University of Iowa. 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. Standard health history and demographic information were obtained at the time of enrollment and informed consent. The information on donors was only that of the standard health history form used to determine eligibility for autologous whole blood donation. Thus, information on ethnicity or lifestyle beyond the standard questionnaire, *e.g.* smoking status, was not collected. Pairs of twins were not required to come in together, nor were there restrictions on the time of day for donations. As a result, individuals within twin pairs donated at different times of the day and sometimes weeks apart. Zygosity of twin pairs was verified using DNA from white blood cells obtained from leuko-reduction filters as described in detail in [26].

Standard 500 mL units of whole blood collected in 70 mL CP2D anticoagulant (Haemonetics Corporation, Braintree, MA) were processed into standard leukocyte-reduced RBC units suspended in 110 mL AS-3 extended storage media (Haemonetics Corporation, Braintree, MA) having hematocrits of 55% to 60%. RBC units were stored at standard refrigerator temperatures. To sample RBCs from the stored RBC units, tubing segments were attached to the RBC units using a sterile docking device and tubing was backfilled with RBCs from the unit. Sampling was conducted on the day following donation (day-0), and on storage days 7, 14, 28, 42, and 56. Although the maximum storage time permitted for RBCs in the US is 42 days, data were also collected at day-56 to determine if values at day-42 were at a nadir or whether further changes would occur after day-42.

Materials

Perchloric acid, glutathione, glutathione disulfide, 1-octanesulfonic acid, sodium monophosphate, and diethylenetriaminetetraacetic acid (DTPA or DETAPAC) were from Sigma Aldrich (St. Louis, MO, USA). CELLPACK™ analyzer solution was obtained from Sysmex Corporation, Kobe, Japan.

Sample preparation

Samples from the stored units were sterilely collected and transferred into 5 mL Eppendorf tubes and centrifuged at 500 *g* for 5 min, after which the RBC-free storage media (AS-3) was removed. The remaining RBC pellet was washed twice by mixing with cold isotonic phosphate buffered saline (pH = 7.4) followed by centrifugation at 500 *g* for 5 min. After washing and thorough mixing to produce a homogenous suspension, an aliquot was taken immediately for complete blood count analysis (CBC) (Sysmex XE-2100™ Automated Hematology System). From the remaining RBC suspension, 300 μ L was removed and extracted with 400 μ L 5% perchloric acid/100 μ M DETAPAC. From each segment three independent samples were made. Samples were centrifuged to pellet the protein (4000 *g*, 5min). The supernatant was stored at -80°C for a minimum of 1 day and a maximum of 1 month.

GSH and GSSG determination with HPLC-BDD

To determine the amount of GSH and GSSG in RBCs, HPLC (ESA Coul Array, Dionex/Thermo Scientific, Sunnyvale, CA) with electrochemical detection (ECD) was used following the protocol outlined by Park *et al.* [27]. The method is based on an ECD system using a boron-doped diamond disc (BDD) electrode (Model 5040, ESA Biosciences, Chelmsford, MA, USA). Samples (65 μ L) were loaded into auto sampler vials with a 100 μ L glass insert. Samples were stored in the temperature controlled (4°C) auto sampler until analysis. At the time of analysis the sample (10 μ L) was loaded on the column and eluted isocratically with 94% 25 mM sodium phosphate pH 2.65, 1.4 mM 1-octane sulfonic acid/3% acetonitrile for 60 min (number of RBCs on column = 10 – 40 million). With each set of samples, four standards containing GSH (500 – 8000 pmol on column) and GSSG (200 – 2000 pmol on column) were included; standard curves generated with each run were used to quantify the analytes for each sample set. Quantitation was performed by integrating the GSH and GSSG peaks in the BDD electrode channel with ESA Coullarray® for Windows version 1.12.

Calculation of intracellular concentration

For each sample, the amount of GSH and GSSG per RBC (mol cell^{-1}), was determined *via* the standard curve specific to each run. First, the quantity (moles) of GSH was divided by the number of RBCs (obtained from CBC) associated with each sample, giving a value in units of mol cell^{-1} . The mol cell^{-1} value was then divided by the mean cell volume (obtained from CBC) resulting in an intracellular concentration of mol L^{-1} for GSH and GSSG. Total GSH (tGSH) is the sum of GSH equivalents, $[\text{tGSH}] = [\text{GSH}] + 2[\text{GSSG}]$. The median of three independently processed samples was taken to reflect the intracellular GSH and GSSG concentration of an individual [28]. From these molar concentrations the status of

the GSSG, $2\text{H}^+/2\text{GSH}$ couple was calculated using the Nernst equation, $E_{\text{hc}} = E^\circ - 27.5 \log ([\text{GSH}]^2/([\text{GSSG}][\text{H}^+]^2)$ in mV [29], and storage temperature of 4 °C [30]. Concentrations are mole per liter; E° (pH 7) taken to be -240 mV and E° (pH 0) = +180 mV for the GSSG, $2\text{H}^+/2\text{GSH}$ couple [31, 32].

Determining intra and extracellular pH

Extracellular pH was measured in the RBC-free AS-3 medium removed from the samples; see above. pH was determined immediately after collection of samples using a Broadley-James Corp pH meter with a VWR International model 14002 Calomel electrode calibrated with VWR standards of pH 4.00, 7.00 and 10.00, as appropriate, taking into account the solution temperature. Intracellular pH was measured in RBC lysates as in [33]. Briefly, 100 μL of the packed RBC pellet was added to 900 μL of Nanopure®water (18.0M Ωcm , pH $\approx$ 6); after lysis the resulting pH was immediately determined. The intrinsic (non- $\text{CO}_2/\text{HCO}_3^-$ -dependent) buffering capacity of RBCs is substantial, having been estimated as 85 mmol L^{-1} (pH unit) $^{-1}$ [30]. Even though this was diluted by a factor of ten in the assay, the minuscule buffering capacity of the purified water, due to the dissolved $\text{CO}_2/\text{HCO}_3^-$ from the astonishing 400 ppm in the atmosphere [34], will be overwhelmed, yielding a good estimate of intracellular pH.

Calculation of ICC and heritability

For twin studies the one-way model of intraclass correlation (ICC) was used to determine the similarity of a measure in a twin pair. $\text{ICC} = (\text{MS}_{\text{between}} - \text{MS}_{\text{within}}) / (\text{MS}_{\text{between}} + \text{MS}_{\text{within}})$, where $\text{MS}_{\text{between}}$ is the estimate of the mean-square variance between all twin-pairs and $\text{MS}_{\text{within}}$ is the estimate of the mean-square variance within the sets of pairs in that group [35]. The ICC is a variant of ANOVA analysis and results in a value on a scale of +1 to -1. An ICC approaching +1 is expected in MZ twins for a strongly heritable trait. The ICC of MZ and DZ pairs for GSH, GSSG and E_{hc} were calculated using IBM SPSS Statistics for Windows, Version20.0 (IBM Corp. Armonk, NY).

Two approaches were taken to analyze heritability: 1) an overall heritability was determined. ICC values were calculated for MZ and DZ twin populations for each day sampled during storage (days 0, 7, 14, 28, 42, and 56). The mean values of all ICCs in MZ and DZ twin populations were used to estimate an overall heritability of analyte levels; and 2) the heritability of changes in analyte levels was calculated. ICC values for changes in analyte levels were calculated for each twin pair over the entire storage period. The mean of the ICC values of MZ and DZ twin populations were used to estimate the heritability of changes in analyte levels during storage. Heritability estimates were calculated using the method derived by Newman *et al.*, $h^2 = (\text{ICC}_{\text{mz}} - \text{ICC}_{\text{DZ}}) / (1 - \text{ICC}_{\text{DZ}})$ [36].

Statistics

One-way analysis of variance was used for statistical analysis. Results are expressed as the mean $\pm$ SD. The differences were considered statistically significant when p values were less than 0.05. Calculations were performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp. Armonk, NY).

Results

Characterization of study population

Eighteen pairs of adult twins (aged 18 – 48 y) were enrolled in the study, Table 1. The study population included 13 MZ twin pairs (median age 25 ± 7 y; range, 18 – 48 y) and 5 DZ twin pairs (median age 26 ± 6 y; range, 18 – 33 y). There were no statistically significant differences in the mean height, weight, or age between the MZ and DZ groups, Table 2. These groups are the same or very similar to the groups of the studies reported in [26, 37].

GSH, GSSG, and tGSH levels during storage of RBCs

Under standard processing and storage conditions, the median value for the intracellular concentrations of the whole population for GSH, GSSG, and tGSH in RBCs decreased over the 56-day sampling period, Figure 1. The median loss of intracellular [GSH] was 0.52 ± 0.63 mM (median $\pm$ SD) over 42 days; this trend continued with an additional decrease of 0.16 mM in the mean GSH levels between day-42 and day-56. These results indicated there is large variability in the temporal profiles of individuals. RBCs from some individuals lost a substantial fraction of the intracellular GSH during storage whereas others were able to maintain GSH levels throughout the storage period. The rate of loss appears not to be linear over the storage period with suggestions of a slower rate in the first week or so of storage; however, these data do not allow any firm conclusions on this. In the population, large variability was observed for [GSH] (0.3 – 3.0 mM, 10-fold at day-0) between the individuals. This variability decreased with time in storage (0.4 – 1.6 mM, 4.2-fold at day-56). [GSSG] also declined under storage conditions, Figure 1. A median decrease of 32 ± 107 μ M was observed for GSSG over 42 days of storage. GSSG levels decreased by an additional 4.3 μ M between day-42 and day-56 of storage. The levels of GSSG between individuals are highly variable prior to the start of the storage period (16.6 – 553 μ M, 33-fold at day-0). This variability remained high at the end of storage (6 – 236 μ M, 40-fold at day-56). The loss of GSH and GSSG leads to a median decrease in the [tGSH] of 0.57 ± 0.77 mM over 42 days, Figure 1. An additional 0.21 ± 0.52 mM of tGSH equivalents are lost between day-42 and 56. Variability between individuals behaves similarly to the variability in GSH concentration (0.5 – 4.0 mM, 12-fold at day-0; 0.5 – 2.1 mM, 4.2-fold at day-56). No statistically significant differences between MZ and DZ twin groups are observed in mean values for GSH, GSSG, and tGSH concentrations, Table 2.

Distinct genes control basal levels of GSH and rates of decrease

From previous work, we established the heritability of GSH and related metabolites in fresh RBCs. To investigate the correlation between basal GSH levels and the changes in GSH levels during storage, correlation analyses between the various time points were performed. No correlation was observed between GSH levels at the beginning of storage and the end ($p = 0.08$). This suggests that different genes control basal GSH levels and the changes in the GSH buffer throughout storage.

Loss of tGSH during RBC storage

There are several mechanisms by which the tGSH concentration in RBCs could be decreased. One potential pathway investigated in this study was the export of GSSG from RBCs. The export of GSSG by RBCs is a well-described, ATP-dependent mechanism; rate = $0.6 \text{ zmol cell}^{-1} \text{ s}^{-1}$ (z is zepto, 10^{-21}) using the data in [38, 39]. The supernatant AS-3 medium after 56 days in storage was analyzed for GSH and GSSG using HPLC-BDD. Both GSH and GSSG were below the limit of detection ($<1 \text{ }\mu\text{M}$ and $<0.5 \text{ }\mu\text{M}$, respectively) in the three samples analyzed. These three samples were chosen as they had the greatest loss of tGSH over the 56-day storage period ($1.5 \pm 0.1 \text{ mM}$). Theoretically, this would translate to $68 \pm 0.9 \text{ attomol cell}^{-1}$ of GSSG released (theoretical rate = $0.01 \text{ zmol cell}^{-1} \text{ s}^{-1}$, much less than the projected typical rate *in vivo*; see above.). With approximately 2.3×10^{12} RBCs in one 500 mL unit (obtained from CBC), this would result in a maximum of $300 \pm 3 \text{ }\mu\text{M}$ of GSSG in the storage solution of these samples; thus, had significant GSH or GSSG accumulated in the storage solution it would have been easily detected. Others have observed similar results and investigated other aspects of the decrease in tGSH levels; see Discussion.

The half-cell reduction potential of the GSSG, $2\text{H}^+/2\text{GSH}$ couple during storage of RBCs

The measured GSH and GSSG intracellular concentrations together with the intracellular pH (Table 3) were used to determine E_{hc} of the GSSG, $2\text{H}^+/2\text{GSH}$ couple throughout the storage period. Even though the intracellular concentrations of both GSH and GSSG decreased over time, E_{hc} within the RBC became more oxidized (more positive) during the entire storage period (median increase of $35 \pm 16 \text{ mV}$ over 42 days), Figure 2. Between day-42 and day-56 a median increase of 3.9 mV was observed. Values for E_{hc} range from -143 to -196 mV at day-0 and -119 to -173 mV for day-56 between individuals. There is no statistical difference between MZ and DZ twin groups in the mean values of E_{hc} , Table 2.

Heritability of analytes during RBC storage

Meanheritability over entire storage period—The ICC values were calculated for the MZ and DZ twin populations for each day sampled. Because the rates of change in the concentrations of GSH and GSSG from day-42 to day-56 were very similar to the rates observed from day-14 to day-42 all six time points were used for the estimates of heritability. The median value of the ICC for the six time points in the MZ and DZ twin populations was used to calculate an overall heritability estimate for each analyte. A strong concordance between the values of MZ twins at all time-points resulted in a high mean ICC for GSH, GSSG, tGSH, and E_{hc} (0.85 ± 0.10 , 0.61 ± 0.13 , 0.62 ± 0.12 , and 0.63 ± 0.12 , respectively); the correlation is weaker in DZ twins (0.27 ± 0.69 , 0.02 ± 0.12 , -0.17 ± 0.53 , and -0.50 ± 0.48 , respectively). The mean of MZ and DZ ICCs at all time points resulted in estimated mean overall heritabilities for GSH, GSSG, tGSH, and E_{hc} of 79%, 60%, 67% and 75%, respectively, Figure 3.

Heritability of changes over the 56-day storage period—There was marked variability between individual subjects in the rates of change in the levels of analytes during storage. This observation suggested that changes in analyte levels during storage were

heritable. To explore this observation further, the heritability of the change in analyte values during the storage period was estimated by calculating the ICC for each twin pair over the six time points. The mean ICC values for the change in analyte concentrations of all MZ twins were 0.64 ± 0.39 , 0.56 ± 0.36 , 0.70 ± 0.32 , and 0.65 ± 0.48 for GSH, GSSG, tGSH, and E_{hc} , respectively. The mean values for ICC were weaker in DZ twins (-0.26 ± 0.47 , 0.15 ± 0.41 , -0.26 ± 0.32 , and 0.04 ± 0.35 , respectively). Comparing the mean of all MZ and the mean of all DZ ICCs calculated over the storage period resulted in estimated heritabilities for changes in GSH, GSSG, tGSH, and E_{hc} of 71%, 48%, 64%, and 75%, respectively, Figure 3.

Known heritable traits in this study population

To determine if this was an informative population for a twin-study, the heritability of three well-established heritable traits height, weight, and BMI, were analyzed [37]. The ICC values for height, weight, and BMI in our study population were: MZ twins = 0.94, 0.98, and 0.97; and ICC values for DZ twins = -0.37 , 0.31, and 0.92, respectively. These ICCs yield estimated heritabilities of 96% for height, 97% for weight, and 63% for BMI, Figure 3. These heritabilities are remarkably consistent with values previously reported for heritability of these traits: heritability of height (87 – 93 %) in the affluent societies of Western Europe [40]; heritability of 81% for weight [41]; and heritability of 62% for BMI [42]; providing confidence that our study population was informative with respect to estimates of heritability.

Discussion

In this study we observed strong evidence for the heritability of the intracellular concentration of GSH and related metabolites in stored RBCs. The concentration and stability of these metabolites during the storage of RBCs has been associated with the RBC storage lesion [13, 17, 19, 43, 44]. Supplementation of stored RBCs with GSH has been shown to partially protect from free radical and oxidant-induced injury, thereby maintaining more normal cellular function [12, 16]. Recently, methods have been explored for storing RBC units anaerobically with the goal of reducing oxidative damage throughout the storage period [43]. Efforts such as these to improve RBC storage by preventing oxidative damage may benefit from identifying and understanding the genetic modifiers that influence GSH and related metabolites during storage. Among other applications, knowledge of the genetic determinants of the redox buffer during RBC storage could be used to select donors based on their genetic propensity to maintain GSH levels during storage. This information can be used to provide optimal personalized storage conditions, and ideal donor/recipient combination. Screening of RBC donors based on genetic traits that affect the storage lesion could improve RBC quality and thus patient care. Other anti-oxidants and heritability throughout RBC storage

Other anti-oxidants and heritability throughout RBC storage

Glutathione and related metabolites are of great importance to the redox status of cells and tissues. However, there are many additional pathways that contribute to the antioxidant network to defend against oxidative stress. In RBCs routes for the removal of H_2O_2 include

the peroxiredoxins (Prdx, especially Prdx 2), catalase (CAT), and glutathione peroxidase (GPx) [45–47]. The roles of GPx and CAT have been well studied; GPx, was discovered as the defender of hemoglobin against oxidative stress [48]; GPx requires GSH as a cofactor for removal of hydroperoxides; low levels of GSH can limit the efficiency of GPx to do so [48]. CAT is now believed to account for nearly half of the removal of H₂O₂ in RBCs [46]. CAT is highly efficient; it has a high rate constant for removal of H₂O₂ and does not require cofactors to perform this catalytic function. It is now believed that the Prdx family of proteins, in particular Prdx 2 is responsible for removing a significant portion of H₂O₂ in RBCs [45, 46, 49]. There has been work by Chakraborty and Chaudhuri on the heritability of GPx and CAT in fresh RBCs [37]. In their study population these redox enzymes have significant heritabilities, 72% and 60%, respectively. How these heritabilities influence storage needs to be investigated. To our knowledge there has been no work on the heritability of Prdx's.

Loss of total tGSH equivalents

During episodic oxidative stress produced by exposure of RBCs to a bolus of oxidant, analogous to species generated during prolonged storage, the oxidation of GSH is transient. Exposure of RBCs to oxidants such as H₂O₂ and *tert*-butylhydroperoxide (*t*-BuOOH) results in the oxidation of GSH to GSSG which then reacts with protein sulfhydryl's (PSH) forming PSSG (protein mixed disulfides), returning one GSH (Figure 4, reaction 1 & 5) [39, 50]. RBCs rapidly remove these oxidants in a matter of seconds [51]. After the oxidant is removed GSSG and PSSG are both reduced back to GSH and PSH in a few minutes without loss of tGSH equivalents [52]. This is in stark contrast to our observation of the loss of tGSH equivalents during storage of RBCs. A possible explanation for not detecting GSSG or GSH in the extracellular milieu is that these species are further degraded by enzymatic or non-enzymatic processes (Figure 4, reaction 4). Recent studies have performed comprehensive metabolite analyses of stored RBCs [20]. Parallel to our study, losses in both GSH and GSSG are observed leading to a loss in tGSH. Interestingly, increases are observed for glycine and cysteine, both of which are building blocks of GSH. This supports the hypothesis that GSH and/or GSSG are degraded. Concomitantly, GSH synthesis could be impaired during the storage process leading to a decrease in tGSH levels [19]. Since, GSH synthesis is dependent on ATP; impaired synthesis is a real possibility. However, no correlation was found between GSH levels and ATP levels in our study ($p = 0.24$) [37].

The status of the intracellular redox buffer of RBCs becomes more positive upon storage

During the storage of RBCs, both the storage solution and the cytosol of RBCs become more acidic with time. Using the Nernst equation and accounting for this change in the intracellular pH of RBCs, E_{hc} of the GSSG, 2H⁺/2GSH couple becomes more positive during storage, indicating an overall oxidation of the buffer. Because there is a loss of tGSH with storage, the capacity of the buffer to respond to oxidative stress is also diminished. This combination “weakens” the buffer and could contribute to the development of the storage lesion.

We have shown that during the storage of RBCs the variability in the levels and the change in levels of components of the intracellular redox buffer are strongly heritable. Heritability

estimates of components of the redox buffer ranged from 48% to 79%. Heritability was demonstrated both in the aggregated data of all time points, and for the individual variability in the change of levels during storage. Because oxidative injury is widely considered to be a critical component of the RBC storage lesion, identification of the genetic determinants of the 3- to 5-fold differences in tGSH observed between study subjects is an important subject for further investigation. Understanding the genetic modifiers of RBC storage could lead to innovations that markedly improve transfusion therapy.

Limitations

The estimates of heritability found here may not be reflected in all population groups. In addition, our estimates have been made on a relatively small sample size. However, estimates of heritability for two well-studied traits match exceptionally well published estimates: height (estimated heritability of 96% in this study compared to the published range of 87–93 % [37] and BMI (63% in this study, published range 39 – 93 % with a weighted mean of 62% [38,39]). Both papers present analyses of many studies that were carried out across many different populations, which included over 100,000 individuals. The high degree of similarity between these published values and our values for these two well-established heritable traits engenders considerable confidence in our estimates on the heritability of GSH, GSSG, E_{hc} , and tGSH in RBCs. It is unknown from our study if environment would be more prominent in situations of malnourishment. Also, lifestyle factors such as smoking could negatively impact GSH levels throughout storage; this was not controlled for in our study, yet considerable heritability was established.

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Highlights

- Total glutathione equivalents decline during storage of RBCs; median net loss of 0.57 mM over 42 days.
- The GSSG, $2H^+/2GSH$ intracellular redox buffer becomes more oxidized during storage of RBCs, median net increase of 35.3 mV.
- High biological variability exists among individual donors for the levels of intracellular GSH and related metabolites and their behavior during storage.
- The intracellular concentration of glutathione during storage of RBCs is a heritable trait (79%).

Figure 1. GSH, GSSG, and tGSH concentrations in RBCs decline during storage

Concentrations of GSH and GSSG were measured using HPLC-BDD in stored RBC samples collected from 36 twin subjects. The concentration of tGSH was calculated as $[GSH] + 2[GSSG]$. The line through each set of points represents the median value for that set.

Figure 2. The half-cell reduction potential (E_{hc}) of the GSSG, $2H^+/2GSH$ couple in RBCs becomes more oxidized during storage

Concentrations of GSH and GSSG were measured using HPLC-BDD in stored samples of RBCs collected from 36 twin subjects. The half-cell reduction potential (E_{hc}) of the GSSG, $2H^+/2GSH$ couple was calculated from [GSH] and [GSSG] using the Nernst equation. The pH value for each day was measured and used to determine the value of E_{hc} . The line through each set of points represents the median value for that set.

Figure 3. Levels of GSH, GSSG, tGSH, and E_{hc} are heritable in stored RBCs

Heritability was estimated using the method of Newman *et al.* from the mean intra-class correlation coefficients [36]. The estimated overall (population) heritability for all time points for GSH is 79%, for GSSG 60%, for tGSH 67%, and for E_{hc} 75%. The estimated heritability of changes in these values during storage (temporal heritability) for GSH is 71%, for GSSG 48%, tGSH 76%, and E_{hc} 64%. The heritability of the well-established heritable traits, BMI (63%), height (96%), and weight (97%) in our study subjects are concordant with reported values [40, 42], providing confidence that our study populations are informative with respect to estimates of heritability.

Figure 4. Possible mechanisms of GSH and GSSG loss during storage of RBCs

There are several reactions that could change the tGSH concentration in RBCs: (1) reaction of GSSG with protein sulfhydryl groups (PSH) leading to formation of protein mixed disulfides (PSSG) and free GSH; (2) export of GSSG from RBCs through ATP-dependent transporters or micro vesicle formation; (3) degradation of GSH or GSSG by enzymatic or non-enzymatic process in the RBC cytoplasm. Once GSSG is exported from the RBC, several reactions could account for its removal: (4) degradation of exported GSSG or GSH in the storage medium by enzymatic or non-enzymatic reactions; (5) reaction of GSSG with sulfhydryl groups at the outer RBC membrane or on proteins in the storage solution.

Table 1

Gender distribution of final study participants

	MZ twins	DZ twins
Female pairs	11	2
Male pairs	2	2
Male/female pairs	-	1
Total pairs	13	5

Table 2

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

Trait	Monozygotic (MZ)	Dizygotic (DZ)	<i>p</i> -value ^b
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	0.2
BMI	24 ± 4.3	22 ± 2.7	0.3
[GSH] / mM	1.2 ± 0.5 ^a	1.1 ± 0.5 ^a	0.3
[GSSG] / μM	126 ± 84 ^a	119 ± 62 ^a	0.6
<i>E</i> _{hc} / mV	−262 ± 15 ^a	−266 ± 13 ^a	0.1
[tGSH] / mM	1.4 ± 0.6 ^a	1.3 ± 0.5 ^a	0.3

^aMedian over entire storage period ± SD^bOne way ANOVA, DZ vs. MZ

Table 3Measured pH values of stored RBCs^a

Day	pH extracellular ^{b,c}	pH intracellular ^{b,d}
0	7.43 ± 0.17 (7.20–7.93)	6.85 ± 0.13 (6.67–6.97)
7	7.51 ± 0.13 (7.33–7.74)	6.69 ± 0.14 (6.5–6.83)
14	7.42 ± 0.13 (7.20–7.7)	6.57 ± 0.09 (6.5–6.63)
28	7.27 ± 0.07 (7.12–7.33)	6.52 ± 0.16 (6.38–6.75)
42	7.01 ± 0.07 (6.89–7.1)	6.43 ± 0.25 (6.21–6.70)
56	6.83 ± 0.09 (6.75–6.95)	6.35 ± 0.18 (6.21–6.29)

^a RBCs were stored at 4 °C, pH was determined at room temperature.^b Mean ± SD (range)^c Mean of sixteen independent samples^d Mean of four independent samples, each sample analyzed in triplicate.