

Methylglyoxal concentrations differ in standard and washed neonatal packed red blood cells

Autumn S. Kiefer¹, Thomas Fleming², George J. Eckert³, Brenda B. Poindexter¹, Peter P. Nawroth² and Mervin C. Yoder¹

BACKGROUND: Preterm infants have a greater risk of necrotizing enterocolitis following transfusion. It is hypothesized that high glucose concentrations in red blood cell (RBC) preservatives lead to increased methylglyoxal (MG) metabolism, causing glycation-driven damage to transfused RBCs. Such changes to the RBCs could promote a proinflammatory state in transfusion recipients.

METHODS: Standard and washed RBCs in Adsol-3, two common neonatal preparations, were studied. Consecutive measurements were performed of glucose, MG, reduced glutathione, glyoxalase I activity (GLO-I), and D-lactate, the stable end product of MG detoxification by glyoxalase enzymes over the 42-d storage period.

RESULTS: RBC units consume glucose and produce D-lactate and MG during storage. In 28/30 units, the MG concentrations showed only small variations during storage. Two units had elevated MG levels (>10 pmol/mg Hb) during the first half of storage. Washing of the RBCs significantly reduced both MG and D-lactate.

CONCLUSION: This study shows two patterns of MG metabolism in packed RBCs for neonatal transfusion and raises the possibility that RBC units with higher MG levels may have increased glycation-driven damage in the transfused RBCs. Whether transfused MG could trigger an inflammatory response such as necrotizing enterocolitis in preterm neonates and whether washing could prevent this require further study.

Transfusion-related acute gut injury (TRAGI) is a condition described in preterm infants who develop necrotizing enterocolitis in the 48-h period following a packed red blood cell (RBC) transfusion (1). While blood product and transfusion recipient factors jointly contribute to the risk of transfusion-related acute lung injury in adult and pediatric populations (2,3), only a few studies have investigated proinflammatory factors in blood products with respect to TRAGI. Blau *et al.* (1) did not detect anti-HLA or antineutrophil antibodies in a single donor unit, whose recipient died following TRAGI. However, plasma inflammatory cytokines, such as interleukin-8 and tumor necrosis factor- α , were found to increase following routine transfusions of preterm infants

\leq 28 wk of gestation, and this led to endothelial cell activation as evidenced by soluble intracellular adhesion molecule-1 release (4).

Methylglyoxal and Advanced Glycation End Product Formation

Packed RBCs for transfusion are stored in preservatives that allow the cells to remain metabolically active, utilizing the preservative's glucose supply in glycolysis. Methylglyoxal (MG) is a natural by-product of glycolysis, formed from the spontaneous degradation of the triose phosphate intermediates (Figure 1). MG belongs to a class of small molecular weight compounds known as the dicarbonyls, which are potent glycating agents. During glycation, either intact glucose or dicarbonyls react with proteins at lysine and arginine residues via the Maillard reaction to form advanced glycation end products (AGEs) (5,6). To minimize the extent of glycation, MG is metabolized by the glyoxalase I and II enzymes (GLO-I and GLO-II) using reduced glutathione as a cofactor (7). The glyoxalase system forms D-lactate, which is distinct from the L-lactate stereoisomer formed by glycolysis under anaerobic conditions (8). However, in the setting of hyperglycemia, higher levels of the dicarbonyls are generated, and reduced glutathione may be depleted (9,10).

The physiologic importance of MG and AGE formation remain under investigation; however, glycation can interfere with the structure and function of proteins (11). Studies in patients with diabetes mellitus have shown a substantial link between MG and AGE accumulation and the development and progression of nephropathy, neuropathy, and retinopathy (12–14). AGEs also potentiate cellular dysfunction through interaction with specific cell surface receptors, such as the receptor for AGEs (RAGE) (15–18). AGE–RAGE interactions lead to a proinflammatory phenotype (17,18) through the expression of cytokines, growth factors, and adhesion molecules (tumor necrosis factor- α , interleukin-1, platelet-derived growth factor, insulin-like growth factor-1, interferon- γ intracellular adhesion molecule-1, or vascular cell adhesion molecule-1) (19–21). RAGE activation also increases RAGE expression, suggesting that repeated exposures to AGEs can prime a cell for a larger magnitude inflammatory response on subsequent exposures (22).

¹Department of Pediatrics, Indiana University School of Medicine, Indianapolis, Indiana; ²Department of Internal Medicine and Clinical Chemistry, University of Heidelberg, Heidelberg, Germany; ³Department of Biostatistics, Indiana University School of Medicine, Indianapolis, Indiana. Correspondence: Autumn S. Kiefer (askiefer@hsc.wvu.edu)

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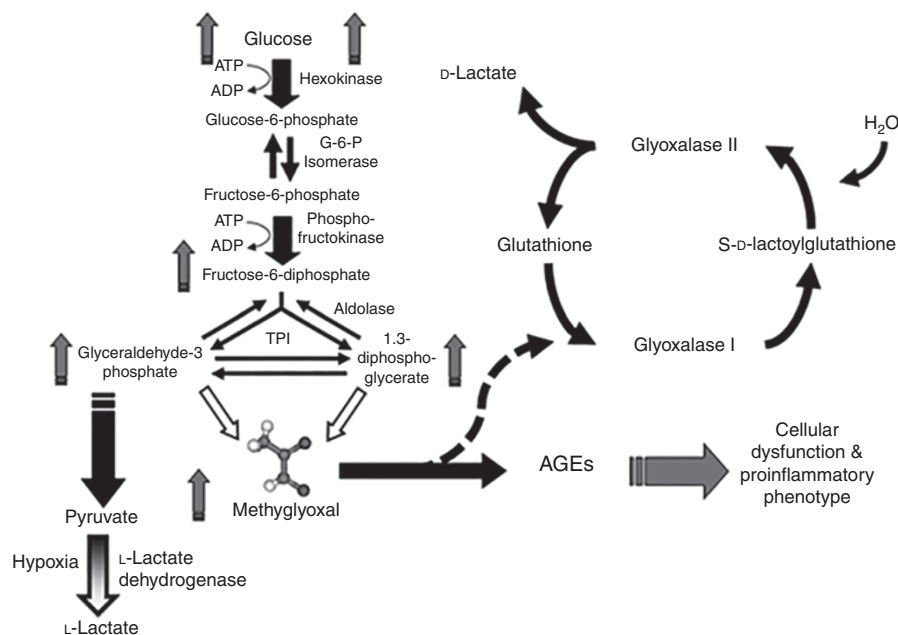


Figure 1. Intracellular methylglyoxal (MG) formation and removal. The breakdown of glucose to pyruvate via the Embden–Meyerhof pathway of glycolysis is shown on the left. The early steps of glycolysis and the formation of triose phosphate intermediates (TPI) are detailed, as the spontaneous dephosphorylation of TPI leads to the formation of MG and other dicarbonyls. MG either forms AGEs by binding to free amino groups or it is removed by the activity of the glyoxalase enzymes with conversion to D-lactate (dashed arrow on right). The gray arrows show the effects of increased glucose supplies, with increased MG and AGE formation, leading to a proinflammatory state. The shadowed arrow shows the distinct pathway by which L-lactate may form. ADP, adenosine diphosphate; AGE, advanced glycation end product; ATP, adenosine triphosphate.

Few studies have evaluated the effects of high glucose storage conditions on transfused RBCs. Lysenko et al. (23) found that AGE levels increased in packed RBCs over a 42-d storage period. Mangalmurti et al. (24) showed that stored RBCs had significantly greater levels of a lysine-derived AGE, carboxymethyllysine, and stored RBCs caused significantly more *in vitro* RAGE activation than fresh RBCs. It can therefore be hypothesized that this AGE accumulation occurs due to MG generation by the stored RBCs during glycolysis. Under physiologic conditions, MG rapidly forms AGEs or is removed by the glyoxalase enzymes. However, we hypothesize that the low temperature and pH conditions used for blood storage will slow MG binding to free amino groups and lead to a larger pool of unbound MG. Thus, the previously described elevations in AGE levels may not represent the full extent of glycation damage within stored RBCs. This hypothesis is supported by the observation that another intermediate in glycation reactions, labile hemoglobin A1c (HbA1c), is a minor variant of HbA1c *in vivo* but can represent a larger proportion of Hb in stored blood (25). Determining the amount of unbound MG in the transfused blood is a key first step to understanding the potential impact of glycation-damaged RBCs on transfusion recipients and ultimately determining if these changes are linked to the development of posttransfusion inflammatory responses like TRAGI in preterm infants.

RESULTS

Donor Unit Profiles

Of the 30 packed RBC units, 7 were blood type AB positive and 23 were A positive. All units tested negative for red cell

antibodies, hepatitis B and C viruses, human immunodeficiency virus, human T-lymphotropic virus, West Nile Virus, and syphilis antibody. None of the packed RBC units had bacterial infection on day 20 or day 42. One unit did have a contaminated culture at day 20, but subsequent cultures on days 25 and 42 were negative for any bacterial growth without intervention.

Glucose Metabolism

Glucose concentrations were determined in packed RBC samples, which were hemolyzed for analysis. The mean initial glucose concentration was 907 ± 10 mg/dl, and this decreased to 578 ± 14 mg/dl by the end of the storage life (Figure 2). With washing, the mean glucose concentration decreased significantly to 27 ± 1 mg/dl ($P < 0.0001$). Washing did not reduce the glucose concentration as much if it was performed at the end of the storage period ($P < 0.0001$), with a mean decline in glucose on day 0 of 886 ± 51 mg/dl, day 20 of 628 ± 100 mg/dl, and day 42 of 550 ± 32 mg/dl.

Glucose-Mediated Changes in Packed RBCs

Donor whole blood samples had a mean labile HbA1c of $2.1 \pm 0.05\%$ and mean stable HbA1c of $5.6 \pm 0.17\%$. In the packed RBC units from these donors, the stable form slowly increased over the 42 d of storage ($5.8 \pm 0.17\%$ on day 42; $P < 0.0001$). Stable HbA1c was higher for AB+ than for A+ units regardless of storage age ($P = 0.0499$). Labile HbA1c had more abrupt changes with the greatest percent present on day 0 of storage ($4.7 \pm 0.05\%$; $P < 0.0001$), declining to $2.9 \pm 0.07\%$ on

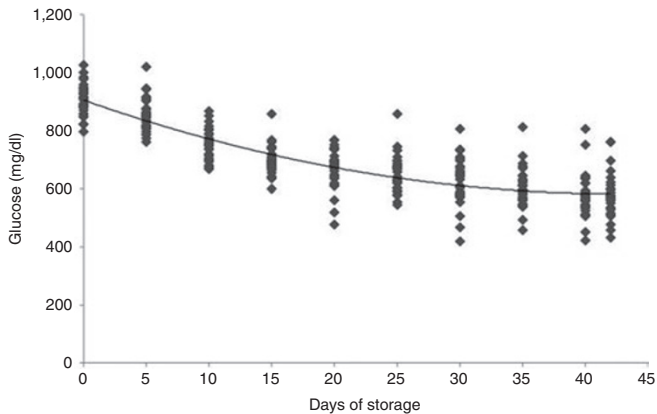


Figure 2. Glucose levels show greatest decline in first half of packed RBC storage. Glucose concentrations over the 42-d storage life of 30 packed RBC units stored in Adsol-3 are shown. Packed RBCs were exposed to very high initial glucose concentrations, near 900 mg/dl. The packed RBCs had an initial period of rapid glucose consumption, followed by a plateau of glucose levels near 600 mg/dl. A trend line ($R^2 = 0.73$) demonstrates the nonlinear pattern of glucose consumption. Individual measurements from the 30 RBC units are represented by the diamond markers. RBC, red blood cell.

day 42, which was still significantly increased compared to the baseline donor measures ($P < 0.0001$). Washing of RBCs significantly reduced the amount of labile HbA1c ($1.8 \pm 0.08\%$; $P = 0.0001$) but had no effect on the stable form ($P = 0.83$).

MG Metabolism

GLO-I activity was stable throughout the storage period with a mean of 0.13 ± 0.003 mmol/min/mg Hb, which was not affected by washing. The concentration of reduced glutathione significantly decreased during storage, from a mean of 348 ± 13 $\mu\text{mol/l}$ on day 0 to 261 ± 14 $\mu\text{mol/l}$ on day 30 ($P = 0.0003$) and remained low until day 42. Washed samples showed a significant drop in reduced glutathione from day 0 to day 20 or 42 ($P < 0.005$). D-Lactate concentrations significantly increased during storage from 111 ± 13 $\mu\text{mol/l}$ on day 0 to $1,345 \pm 37$ $\mu\text{mol/l}$ by day 42 ($P < 0.0001$), as seen in Figure 3. Washing significantly reduced the D-lactate concentration ($P < 0.0001$). The longer packed RBCs were stored, the greater the reduction in D-lactate concentrations with washing (day 0 mean reduction of 57 ± 16 $\mu\text{mol/l}$ vs. day 42 reduction of 711 ± 44 $\mu\text{mol/l}$; $P < 0.0001$). Blood type did not affect the degree that D-lactate was reduced.

Donor whole blood had a MG concentration of 6.79 ± 0.67 pmol/mg Hb, with a plasma concentration of 129.3 ± 4.4 nmol/l consistent with published values for healthy control individuals. Preparation of packed RBCs from the whole blood reduced the MG concentration (3.72 ± 0.13 pmol/mg Hb; $P < 0.0001$). There was a significant correlation between the initial donor MG concentration and the MG concentration of the packed RBCs ($r = 0.59$; $P < 0.001$). Washing of packed RBCs further reduced MG concentration (2.74 ± 0.30 pmol/mg Hb; $P < 0.0001$).

Small, but statistically significant, changes in MG concentrations occurred during storage, with levels initially declining from day 0 (4.10 ± 0.30 pmol/mg Hb) to day 20 (3.57 ± 0.44 pmol/mg Hb) ($P = 0.0059$). MG concentrations then increased

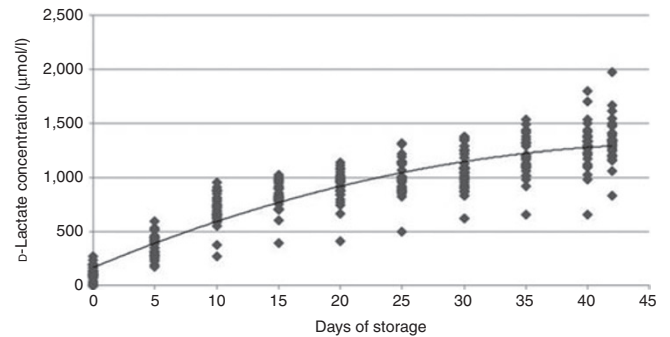


Figure 3. D-Lactate concentrations significantly increase until the limits of packed RBC storage. D-Lactate increases in packed, leukoreduced RBCs stored in Adsol-3 until day 40 of storage, and the trend line ($R^2 = 0.83$) demonstrates the nonlinear nature of D-lactate accumulation. In a sterile collection of human RBCs to which no medications are added, the only known source of D-lactate is glyoxalase enzyme activity (35,36). Humans do not produce D-lactate dehydrogenase, and RBCs lack the mitochondria which metabolize D-lactate through other enzyme pathways *in vivo*. Thus, we propose that D-lactate is a reasonable surrogate for the total MG broken down by glyoxalase enzymes in the closed packed RBC system. Individual measurements from the 30 RBC units are represented by the diamond markers. MG, methylglyoxal; RBC, red blood cell.

from day 30 (3.49 ± 0.34 pmol/mg Hb) to day 42 (3.97 ± 0.39 pmol/mg Hb) ($P = 0.0127$). Of note, two packed RBC units had increasing concentrations of MG in the first 20 d of storage (Figure 4). The effect of storage time on MG concentrations did not change when these two outlier units were excluded from the analysis, though the mean MG concentrations were reduced (MG concentrations in pmol/mg Hb on day 0 (3.83 ± 0.24), on day 20 (3.02 ± 0.22), and on day 42 (3.65 ± 0.33)). Washing significantly reduced MG levels by a mean of $25.9 \pm 3.3\%$ ($P = 0.0009$); neither wash day ($P > 0.60$) nor blood type ($P > 0.23$) significantly affected the changes. When the two outlier units were excluded, washing still resulted in a significant reduction in MG concentration ($25.3 \pm 3.5\%$; $P = 0.0035$).

Additional Analyses With Exclusion of Two Outlier Units

The moderate correlation between the donors' initial MG levels and packed RBC unit MG levels remained (0.53 ; $P = 0.004$) after exclusion of the outliers. D-Lactate accumulation was also evaluated without the two outlier units. There was still an increase in D-lactate levels over the storage time with a mean concentration on day 0 of 114 ± 13 $\mu\text{mol/l}$ to a mean of $1,345 \pm 40$ $\mu\text{mol/l}$ on day 42 ($P < 0.0001$). There was an interaction seen between blood type and D-lactate accumulation over the storage time when the two outliers (one blood type A+, one AB+) were excluded from the analysis, with AB+ blood having an overall increase in D-lactate but with nonsignificant increases over some 5-d intervals. Blood type still did not affect the reduction in D-lactate with washing of the packed RBCs ($P = 0.27$).

DISCUSSION

RBCs for transfusion do generate MG during storage in high glucose preservatives. In the majority of RBC units studied, the MG level showed only small variations over the storage time and did not exceed the donor's initial MG level. This is likely

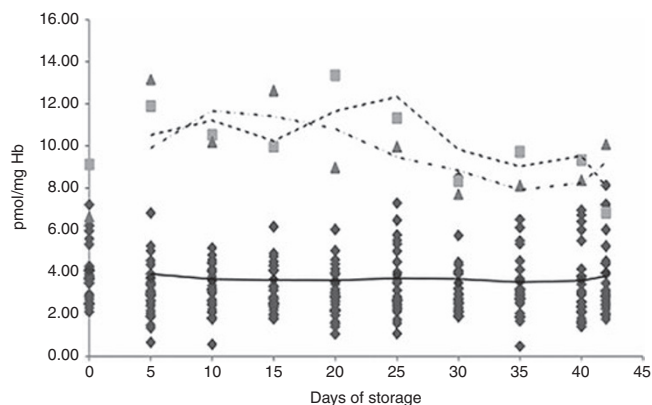


Figure 4. Two patterns of methylglyoxal (MG) accumulation in stored, packed red blood cells (RBCs). For 28 of the packed RBC units in this study (diamond markers), the peak MG concentration occurred either at day 0 or in the final days of storage (day 40–42). Two RBC units (triangle and square markers) had early peaks in MG concentration, >10 pmol/mg Hb. A moving average is shown to demonstrate the relatively stable concentration of MG in most units (solid line), with the MG concentrations in the two outlying units shown by dashed moving average trend lines.

explained by the decline in glucose consumption during the latter parts of storage, effective detoxification by GLO-I, and ongoing binding of MG to form AGEs, although the formation of the latter was not measured in this study. The rising D-lactate levels within the packed RBC units suggest that a dynamic system of ongoing production and removal of MG exists within the RBC unit, despite the relatively stable total unbound MG levels. Because there are limited sources of D-lactate in physiologic systems and bacterial contamination was shown not to be a factor in our experimental model, D-lactate accumulation is a surrogate marker of MG turnover in the packed RBC unit. The small increases in MG concentration, which occurred in the majority of units after 30 d of storage, are likely related to the decline in reduced glutathione beginning at 20–30 d of storage, depending on the RBC preparation. Though the activity of GLO-I was unchanged during our study, reduced glutathione is a necessary cofactor for GLO-I, and it has been shown *in vitro* that depletion can decrease the *in situ* activity and thereby increase the concentration of MG.

Washing of RBCs has been previously proposed as a method of reducing inflammatory mediators (26), and in this study, it has been shown to be an effective means of reducing MG. Supplementation of either antioxidants or scavengers of MG, such as N-acetyl-cysteine (27,28), may provide an additional means of protection. However, it remains unclear whether the range of concentrations of MG observed in most packed RBC units prior to washing (3.5–4 pmol/mg Hb) would be sufficient to trigger a proinflammatory response in preterm infants receiving RBC transfusions. Yet, two of the 30 units in this study showed a differing pattern of MG accumulation, with concentrations greater than 10 pmol/mg Hb in the first 20 d of storage. Higher MG concentrations may result from a donor cells' tendency to generate more MG under similar glucose conditions, which has been shown in diabetic RBCs (29). The correlation between the donor's initial MG levels and the

packed RBC units' levels support our hypothesis that donor factors may be responsible for the elevated MG in these two units.

By demonstrating patterns of MG metabolism in packed RBC units, this study expands our knowledge of potential glycation damage in transfused cells. MG formation will have a direct effect on the RBCs themselves through modification of RBC proteins, affecting their function as well as potential to become a proinflammatory signal. It has been shown that MG modifies hemoglobin at physiologic concentration, and these modifications can increase the likelihood of methemoglobin formation, which impairs oxygen delivery to the tissues (30,31). Furthermore, it has been shown that exposure of RBCs to MG impairs energy production and antioxidative defenses, both of which contribute to enhanced phosphatidylserine exposure on the surface of RBCs, leading to anemia and deranged microcirculation (32). Thus, MG-modified RBCs may contribute to the development of TRAGI through mechanisms other than inflammation.

However, none of the packed RBC units in our study reached the MG concentrations (≥ 20 pmol/mg Hb) described in the RBCs of diabetic patients with late complications (29). While this might mean that proinflammatory effects from glycation changes would be minor in transfusion recipients, the response threshold is unknown in preterm infants, and unbound MG may be a lesser trigger compared to fully formed AGEs.

The possibility that a small percentage of packed RBCs produce higher concentrations of MG in response to the high glucose conditions in storage preservatives and then carry a stronger proinflammatory signal to transfusion recipients, which could be reduced by washing, warrants further study. Both *in vivo* and *in vitro* approaches are required to define not only the MG-mediated changes that arise in the RBCs themselves but also the physiological consequences in transfusion recipients.

METHODS

Packed RBC Unit Processing

Thirty packed RBC units were collected over a 4-mo period in 2012 from the Indiana Blood Center (IBC) after study approval from the Institutional Review Board at Indiana University. As all whole blood donors at IBC consent for a portion or the entirety of their donation to be used for research, the Institutional Review Board granted a waiver of consent for this specific study. Units were available for research if they were unlikely to be used clinically, based on a large number of donors (A+ blood type) or few eligible recipients (AB+ blood type). Research units were collected following IBC protocol with donor screening for healthy adults. Whole blood was collected in citrate phosphate double dextrose anticoagulant and then underwent prestorage leukoreduction via filtration. Plasma was separated by centrifugation, and the packed RBCs were resuspended in 100 ml of Adsol-3 and refrigerated at 4 °C for 42 d. To allow sampling from the units on the day of collection, research units were released from IBC before infectious testing was complete. Samples from each unit of packed RBCs were taken at 5-d intervals from day 0 to day 40 and on date of expiration (day 42).

Samples were obtained according to blood banking procedure using a sterile docking system to divert packed RBC samples into a multiple-bag pediatric aliquot system, commonly referred to as "quad packs." As units were accessed many times, sterility cultures were obtained on days 20 and 42 of storage to determine

the presence of any bacterial contamination. Each sample was irradiated according to the standard blood bank protocol for neonatal transfusions. Samples were frozen at -80°C within 4 h of collection.

Donor Blood Collection

During the standard blood donation procedure at IBC, the first 5 ml of the donor's whole blood is collected in a separate diversion pouch to reduce the risk of contamination of the main unit with bacteria from the donor's skin and to provide a sample for infectious disease testing. Though the packed RBCs from the study donors were not available for therapeutic transfusions, the donors' plasma was still available for clinical use, necessitating the standard infectious screening. Approximately 2.5 ml of whole blood remains after bloodborne pathogen testing is complete, and this remaining blood is typically discarded. For the 30 random donors in our study, the remaining whole blood was scavenged and frozen at -80°C within 4 h of collection for MG analysis.

Washing of Packed RBCs

Units were selected at random for washing on day 0, 20, or 42 of storage. A 150 ml aliquot was removed in a sterile manner from the selected unit and then irradiated to mimic the routine handling of blood for neonatal transfusion. Following irradiation, the aliquot was washed with 1 l of normal saline using a COBE 2991 Cell Processor (Gambro BCT, Lakewood, CO). Cells were washed over three cycles, and a 5 ml washed sample was then collected and frozen at -80°C within 4 h.

Measurement of MG Metabolism

Glucose concentrations were measured using an Aviva Accu-Chek Glucose Monitor and validated by comparison of 100 samples to the clinical standard, the hexokinase method performed at the University of Heidelberg's hospital laboratory (data not shown). HbA1c and labile HbA1c were measured by ion-exchange chromatography at Indiana University's hospital laboratory. Activity of GLO-I was determined by the spectrometric method (29), which monitors the initial rate of change in absorbance at 240 nm resulting from the formation of S-D-lactoylglutathione. GLO-I, rather than GLO-II, activity was measured because of its direct interaction with MG. Reduced glutathione concentrations were determined by high-performance liquid chromatography assay according to the manufacturer's instructions (Immunodiagnostik AG, Bensheim, Germany). D-Lactate was measured by enzymatic end point fluorescence assay as previously described (33). The concentration of MG was determined by derivatization with 1,2-diamino-4,5-dimethoxybenzene and high-performance liquid chromatography analysis of the quinoxaline adduct by fluorescence detection, as previously described (34). GLO-I activity and the amount of MG were normalized to hemoglobin concentration, as determined by the Drabkin assay. All samples were analyzed within 6 mo of collection.

Statistical Analysis

A mixed model repeated-measures analysis was used to evaluate the changes in MG, D-lactate, and HbA1c levels over time. The model included effects for days of storage, blood type, and their interaction, with an unstructured variance/covariance matrix for the repeated measurements within a sample over time. A natural logarithm transformation of the MG levels was used for the analyses. A similar repeated-measures ANOVA was used to examine changes in reduced glutathione during storage; however, this simplified model did not include blood type. Paired *t*-tests were used to evaluate changes between blood preparations, and ANOVA was used to evaluate the effects of blood type and wash day (0, 20, or 42) on these changes. Similar analyses were used to examine changes in glucose concentration and HbA1c due to washing. A Pearson correlation coefficient was calculated to evaluate the association between MG levels in the whole blood donor sample and the RBC unit. After identifying two RBC units with a distinct pattern of elevated MG concentrations, key analyses were repeated without these two units to evaluate the effect of these outliers. Data are expressed as means \pm SEM unless otherwise noted. Sample size of 30 units was selected for an 80% power, after a

small pilot sample (unpublished data), which suggested that a 25% difference existed between blood from packed RBCs and a previously described population of diabetic adults (29). A *P* value < 0.05 was considered significant.

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