Original Paper

Inhibition of Glutathione Synthesis via Decreased Glucose Metabolism in Stored RBCs

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Abstract
Background/Aims: Although red blood cells (RBCs) transfusions can be lifesaving, they are not without risk. RBCs storage is associated with the abnormal metabolism of glutathione (GSH), which may increase the risk of the oxidative damage of RBCs after transfusion. The responsible mechanisms remain unknown. **Methods:** We determined the L-cysteine efflux and influx by evaluating the changes of free -SH concentrations in stored RBCs. The glutamate cysteine ligase (GCL) activities and protein content in stored RBCs was determined by fluorescence assay and western blotting. In addition, the glucose metabolism enzyme activity of RBCs was measured by spectrophotometric assay under *in vitro* incubation conditions. **Results:** We found that both L-cysteine transport and GCL activity significantly declined, thereby inducing the dysfunction of GSH synthesis during blood storage, which could be attenuated by ATP supplement and DTT treatment. In addition, the glycometabolic enzyme (G6PDH, HK, PK and LDH) activity significantly decreased after 6 weeks storage. Oxidant stress-induced dysfunction in glucose metabolism was the driving force for decreased GSH synthesis during storage. **Conclusion:** These experimental findings reflect an underlying molecular mechanism that oxidant stress induced glucose metabolism dysfunction contribute to decreased GSH synthesis in stored RBCs.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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Introduction

Red blood cells (RBCs) routine storage is associated with the progressive accumulation of a long series of biochemical alterations in stored erythrocytes, collectively referred to as the "storage lesion" [1]. Several studies have suggested that RBCs transfusions are associated with higher risks of morbidity and that the storage time of transfused blood is an independent risk factor for post-injury multiple organ failure [2, 3]. Glutathione (GSH) is an important redox active biomolecule critical in maintaining cellular and organism homeostasis [4, 5]. Previous studies suggested that abnormal metabolism of GSH was closely related with various pathological and physiological conditions [6, 7]. Whillier et al. showed that the glutathione synthesis rate significantly decreased in stored RBCs after 6 weeks [8]. However, the mechanism underlying dysfunction of GSH antioxidant system during storage remains to be explored.

Aging RBCs are characterized with increased accumulation of structural, metabolic, and functional modifications [9, 10]. Oxidant stress injury was recognized as an important factor eliciting multiple changes at the metabolic and physiological levels during storage [11]. Erythrocyte GSH played vital role in mitigating the detrimental effects of reactive oxygen species (ROS) encountered in circulation and produced by the continuous oxidation of hemoglobin within the cytosol of erythrocytes [12]. Reduced GSH reacts with superoxide reaction products and degrades hydrogen peroxide and lipid peroxides by GSH peroxidase. In addition, GSH covalently modifies toxic xenobiotics and endogenous electrophiles to form water-soluble conjugates that are exported from erythrocytes for excretion [13]. In diseases associated with increased ROS production induced GSH depletion, the restoration of normal erythrocyte GSH concentration was proved with positive therapeutic effects [14, 15].

Although there is no protein synthesizing system in erythrocytes, GSH could be synthesized by glutamic acid, cysteine, and glycine [5]. Despite the fact that three amino acids were required for GSH synthesis, L-cysteine availability was the rate-limiting step of GSH synthesis [16]. Recently study by Yildiz demonstrated that L-cysteine efflux play vital role in maintaining a harmonious redox status in the plasma [17]. The decreased influx rate of L-cysteine is considered an important factor contributing to the development of oxidative stress in human erythrocytes during aging [6, 18]. However, the changes of L-cysteine transport capacity during storage and its influence on the antioxidant capacity of erythrocytes remains unclear. A previous study by M Güven demonstrated that oxidative stress significantly decreased in glucose transport and utilization efficiency [19]. Herein, we assumed that oxidant stress leads to decreased levels of glucose metabolism and ATP production in RBCs during storage, which may further induce the inhibition of GSH synthesis in erythrocytes due to the dysfunction of ATP-dependent L-cysteine transport.

In RBCs GSH is synthesized by two successive ATP-dependent reactions catalyzed by glutamate cysteine ligase (GCL, EC 6.3.2.2)and glutathione synthetase (GS, EC 6.3.2.3) [20]. GCL catalyzes the first and rate-limiting step in GSH biosynthesis [20]. GCL is a heterodimeric holoenzyme complex comprising a catalytic subunit (GCLC, 73 kDa), which contributes all the enzymatic activity and contains all the substrate and cofactor binding sites of GCL, and a modifier subunit(GCLM, 31 kDa), which modulates GCLC activity and affinity for substrates and inhibitors [20]. However, changes in the expression and activity of GCL and its influence on the antioxidant capacity of erythrocytes during storage are not clear.

Based on these observations, we investigated the effects of L-cysteine uptake and GCL change on erythrocyte GSH synthesis during storage. In addition, energy metabolism was also investigated under *in vitro* incubation conditions to explore the underlying molecular mechanisms of changes in L-cysteine transport at different storage times.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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www.karger.com/cpb

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Materials and Methods

Collection and processing of blood

Whole blood from 20 eligible young blood donors was collected. They underwent routine blood donation for this purpose. Blood donation and the entire preparation and storage of RBC units were performed according to international guidelines in the local blood bank centre.

450±50 mL of whole blood were collected and suspended in 100 mL of preservation solution saline-adenine-glucose-mannitol (SAGM; 8.77 g/L NaCl, 0.169 g/L adenine, 9.00 g/L dextrose monohydrate [d-glucose], 5.25 g/L mannitol) and leukoreduced by filtration (final concentration, less than 10^6 white blood cells/RBC unit). After filtration, RBCs were stored for 7 weeks at 2 to 6°C. Units were sterilely sampled (15 mL per time point) at days 0, 7, 21, 42 by a sterile sampling device, after gentle mixing of the unit content by inversion for approximately 5 minutes. The study gained approval from the local ethics committee and informed consent was obtained from all subjects.

All additive solutions (ASs) were made up in SAGM. When these various solutions were added to the RBCs suspended in SAGM they increased the volume in the packsby 2.5% and gave final concentrations of 1) SAGM only (control); 2) 5 mmol/L N-acetylcysteine (NAC), alanine and glycine; 3) 0.2U/ml HX 1.5mM XO (hypoxanthine/xanthine oxidase) to increase the oxidative load and 4) 1.0 mM dithiothreitol (DTT) to increase the antioxidant capacity. The solutions were added under sterile conditions and the RBCs were stored at 4°C. A 5mL sample was aseptically collected at Weeks 1, 3, and 6 of storage from each of the bags and pH was measured at 22°C using a laboratory pH meter.

RBC rejuvenation

To detect the changes of glutathione synthesis in RBCs after transfusion, a 5-mL sample of RBCs collected from the RBC bag with different preservation time was washed and suspended in fresh plasma (collected from the same donor) to a 45% Hct as rejuvenated group. The sample was divided into two equal volumes: one aliquot was retained as the "unrejuvenated control." Both rejuvenated and unrejuvenated RBCs were incubated in a 37°C bath for 2hours with continuous stirring. In some experiments, cells were treated with dehydroepiandrosterone (DHEA; 100 mM) for 12 h.

Preparation of RBC suspensions and ghosts

Blood was centrifuged at 900 × g at 4°C for 10 min. Plasma and the buffy coats were removed. After removal of plasma, cells were filtered through cellulose to remove leucocytes and platelets, as described in [21]. Packed RBCs were washed thrice in isotonic Hepes buffer (145 mM NaCl, 20 mM HEPES (4-(2-hydroxyethyl) -1-piperazineethanesulfonic acid), pH 7.4, osmotic pressure 300mOsm) and was further used in the experiments at 20% hematocrit.

Oxidant Stress Parameters studies

Determination of SH-radicals in erythrocyte membranes. SH-radicals of erythrocyte membrane proteins were quantified according to Anderson [22].

Membranous peroxide (thiobarbituric acid reactive substances, TBARS) level detection. The extent of lipid peroxidation of RBC membranes was estimated by measuring TBARS levels according to the method of Stocks and Dormandy [23].

Detection of L-cysteine influx in erythrocyte

A total of 0.25 ml of washed erythrocytes was suspended in 1 ml of PBS-glucose containing 1.4 and 10 mM concentrations of L-cysteine and incubated for 1 hr at 37°C in a water bath. At the end of incubation erythrocytes were removed, centrifuged and the supernatants were discarded. The free -SH concentrations in erythrocytes were determined as described by Yildiz [17].

Detection of L-Cysteine efflux in erythrocyte

A total of 0.25 ml of washed erythrocytes was resuspended in 1 ml of PBS-glucose in the presence of different concentrations of L-cysteine. Erythrocytes were incubated at 37°C in a water bath for 1 hr to allow the uptake process. At the end of incubation erythrocytes were centrifuged and the supernatants were

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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www.karger.com/cpb

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discarded. The erythrocytes were then resuspended in 1 ml of fresh PBS-glucose and incubated at 37°C for indicated times to allow the efflux process. At the end of incubation erythrocytes were centrifuged and the supernatants were transferred to fresh tubes. The free -SH concentrations in the supernatant was then measured as described above.

Calculation of intracellular concentration for GSH, GSSG and TFG in RBCs

For each sample, the amount (mole) of GSH and GSSG per RBC was determined via the standard curve specific to each run. First, the GSH amount was divided by the number of RBCs associated with each sample giving a value of mole cell^{-1}. The mole cell^{-1} value was then divided by the mean cell volume resulting in an intracellular concentration of mole L^{-1} for GSH and GSSG. The median of the three independently processed samples was taken to reflect the intracellular GSH and GSSG concentration of an individual. From these molar concentrations the status of the GSSG/2GSH couple was calculated using the Nernst equation $E_{hc} = -255 - 30 \log ([GSH]^2 / [GSSG])$ in mV [4], assuming an intracellular pH of 7.25 for RBCs and a temperature *in vivo* of 37 °C [24]. Erythrocyte total free glutathione (TFG) was calculated as GSH + 2×GSSG.

Enzyme Activity Assays

G-6-PD, HK, LDH, PK activities. Erythrocyte glucose 6-phosphatedehydrogenase (G-6-PD) activity was measured by Zinkham's methods [25]. Hexokinase (HK, EC 2.7.1.1), lactate dehydrogenase (LDH, EC 1.1.1.27), pyruvate kinase (PK, EC2.7.1.40) activities were determined in hemolysate essentially by Beutler's methods [26].

GCL Activity Assay. GCL activity was determined by the fluorescence assay described by Chen et al. [27]. The values are expressed in millimoles per min per milligram protein.

Western blotting

The protein contents of the GCLc and GCLm subunits of GCL were determined by western blotting. Briefly, the supernatants containing equal amounts of protein (20 μg for GCLc and 50 μg for GCLm as determined by the linear responses of respective antibody) were loaded onto 12% SDS-polyacrylamide gels and separated by electrophoresis using Mini-Vertical Gel Electrophoresis Units (Bio-Rad, Hercules, CA, USA). The proteins that were resolved on the gels were transferred to polyvinylidenedifluoride (PVDF) membranes using a Trans-Blot Semi-Dry Transfer Cell (Trans-Blot; Bio-Rad) at 10 V for 30 min.

The protein-bound PVDF membranes were incubated overnight at 4°C with the polyclonal GCLc antibody (1:3, 000; ab80841,Abcam, Cambridge, UK) or monoclonal GCLm antibody (1:3, 000; ab124827,Abcam). The blots that were probed with the GCLc or GCLm antibody were incubated with the second antibody, goat anti-rabbit immunoglobulin G (1:3, 000; Beijing Kangwei Technology Group Co., Ltd., Beijing, China) conjugated with horseradish peroxidase, at room temperature for 1 h. The immunoblots were imaged using a JS-680D automatic gel imaging analyzer (Shanghai Peiqing Science and Technology Co., Ltd., Shanghai, China).

Measurements of ATP levels

ATP concentrations were determined enzymatically using a commercially available kit and controls (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). The amount of ATP in the sample was calculated as μmol/dl; this was further normalized using the total hemoglobin concentration (μmol/g Hgb) [28].

Determination of NADPH

Nicotinamide adenine dinucleotide phosphate (NADPH)value was measured as previously described [29].

Statistical analysis

All data from three or more than three groups of repeated experiments, and are expressed in the form of mean ± standard deviation (mean ± SD). Intergroup differences of between young and aged group were assessed by independent sample t-test. The intra group comparisons between treated groups and their respective control values were performed using one-way ANOVA. Analyses were performed with SPSS PC version 19.0.$P<0.05$ means the difference was significant.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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Results

Variation in quality parameters in stored RBCs

Table 1 describes the differences of *in vitro* quality parameters of RBCs with different storage time. The oxidative damage parameters TBARS levels increased 59.4% after 21 days of RBC storage (P<0.01). Additionally, the free thiol groups declined 14.2% after 21 days of RBC storage (P<0.05). The 2, 3-DPG levels declined 869% after the 7 days of RBC storage (P<0.001). After 42 days of RBC storage, the pH levels declined from 6.97 ± 0.05 to 6.15± 0.03. In addition, no significant variations were observed for MCH, MCV and MCHC.

GSH, GSH/GSSG ratio and TFG levels decreased in stored RBCs

To explore the impact of storage on RBC glutathione parameters, the levels of GSH, GSSG, GSH/GSSG and TFG were analyzed, and the results are presented in Fig. 1. After 21 days of RBCs storage, the GSH level significantly decreased (day 0, 1.12± 0.09 mmol/l vs. day 21, 0.71± 0.11 mmol/l; P < 0.05). There were no significant alterations in the GSSG level during storage. Significant decrement in GSH/GSSG ratio was found after 21 days storage (Fig. 1C). In addition, the TFG level of RBCs significantly decreased after 42 days of RBC storage (day 0, 1.74 ± 0.16 mmol/l vs. day 42, 1.14 ± 0.16 mmol/l; P < 0.05).

Table 1. Main red blood cell parameters followed during storage. MCH, mean corpuscular hemoglobin; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; TBARS, thiobarbituric acid reactive substances; 2,3-DPG, 2,3-diphosphoglyceric. Values are means ± SD. C, *P< 0.05 and **P< 0.01

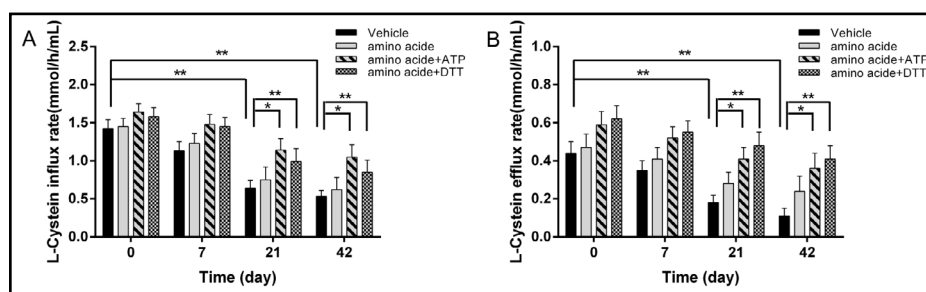| Value	2d	7d	21d	42d
MCH, pg	31.25 ± 1.79	31.23 ± 1.81	31.29± 1.61	31.41± 1.92
MCV, fl	91.79 ± 4.45	93.12 ± 4.31	97.91 ± 5.46	99.6 ± 6.11
MCHC, g/dl	34.03 ± 0.99	33.54 ± 0.94	31.91 ± 0.98	31.63 ± 1.15
TBARS, nmol/ml	4.36 ± 0.46	4.69 ± 0.51	6.95± 0.66**	9.35 ± 0.95**
SH-radical,μmol/g protein	76.2 ± 4.9	72.3 ± 5.3	65.4± 5.9*	59.5± 6.1**
Na+, meq/l	142.04 ± 1.51	131.25 ± 2.12	118.42± 2.64**	107.51± 3.32
K+, meq/l	1.35 ± 0.51	13.9 ± 0.69**	24.2± 9.69**	30.1 ± 1.54**
PH	6.97 ± 0.05	6.75 ± 0.06	6.43± 0.04	6.15± 0.03
2,3-DPG, mmol/l	3.81 ± 0.04	0.5 ± 0.03**	0.05± 0.01**	0.02 ± 0.01**
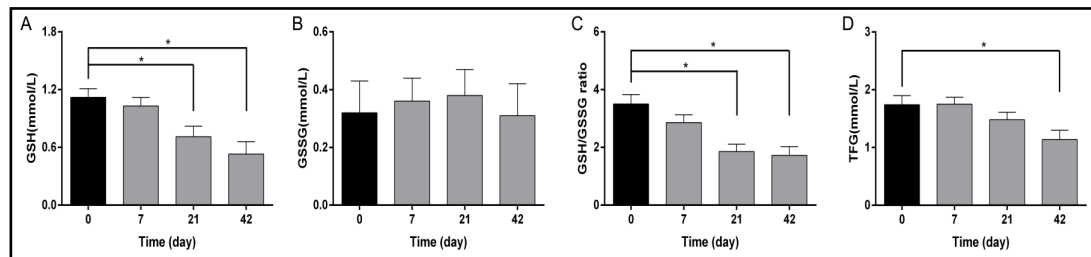
Fig. 1. Effect of storage on GSH, GSSG, GSH/GSSG ratio and TFG levels of RBCs. The levels of GSH (intracellular reduced glutathione) (A), GSSG (intracellular oxidised glutathione) (B), GSH/GSSG ratio (C) and TFG (total of GSH) levels (D). Data are expressed as means ± SEM with the significance level set at *P<0.05 and**P<0.01.

Fig. 2. Effect of storage on L-cysteine transport of RBCs. (A) L-cysteine influx rate in different groups after in vitro treatment of HX 0.2U/ml XO 1.5mM or 1.0 mM DTT, (B) L-cysteine efflux rate in different groups after in vitro treatment of HX 0.2U/ml XO 1.5mM or 1.0 mM DTT. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01.

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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www.karger.com/cpb

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ATP supplement and DTT treatment attenuate decreased L-cysteine influx rate induced by storage which was positively correlate with TFG levels

L-cysteine is involved in the synthesis of glutathione, the transport of L-cysteine was determined in stored RBCs with different storage period. As shown in Fig. 2, the L-cystein influx rate (day 0, 1.42±0.12 mmol/h/mL vs. day 21, 0.64±0.10 mmol/h/mL; P < 0.01) and efflux rate (day 0, 0.44±0.06mmol/h/mL vs. day 21, 0.18±0.04 mmol/h/mL; P < 0.01) decreased significantly after 21 days of RBC storage.

To evaluate the influence of storage induced oxidation and energy metabolism change on L-cysteine transport of RBCs, erythrocyte suspensions were incubated for 30 min at 37°C in the presence and absence of 5 mmol/L N-acetylcysteine (NAC), alanineand glycine, 0.5mM ATP and 1.0 m Mdithiothreitol (DTT), and then were analyzed for L-cysteine transport in different storage period. As shown in Fig. 2, in vitro treatment of amino acids+ATP and amino acids+DTT treatment significantly attenuate decreased L-cysteine influx rate induced by long term storage.

Furthermore, Pearson correlation analysis suggested significant positive correlation (r=0.7983, 0.8177, 0.7809 and 0.7195 in 1, 7, 21 and 42days) between the L-cysteine influx rate and the TFG levels in different storage time (Fig. 3).

ATP supplement and DTT treatment attenuate decreased GCL activities induced by storage which was positively correlated with TFG levels

GCL is the rate-limiting enzyme of GSH synthesis. As compared day 0 control group, the average GCL activity of RBCs significantly decreased from 0.64±0.09 nmol/min/mg protein to 0.46±0.10nmol/min/mg protein after 42 days storage (P<0.01). In addition, *in vitro* treatment of amino acids+ATP and amino acids+DTT treatment significantly attenuate decreased GCL activity induced by long term storage (Fig. 4A). Pearson correlation analysis suggested that the GCL activity levels positively correlated with TFG level (r=0.84, 0.75, 0.79 and 0.77 in 1, 7, 21 and 42days, P<0.05) (Fig. 4B, C, D).
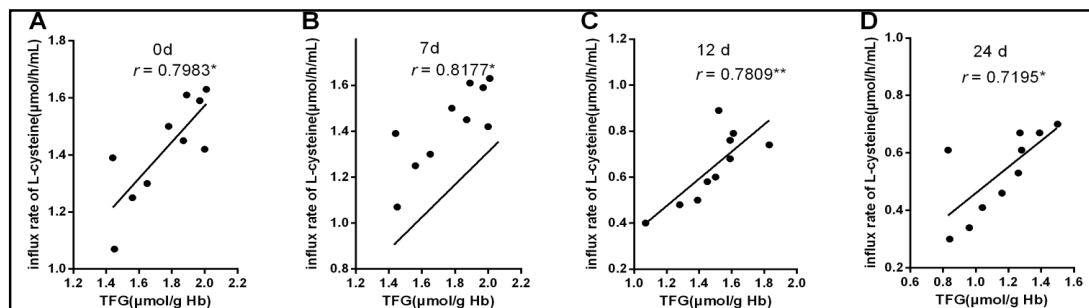

Fig. 3. Pearson correlation analysis between L-cysteine influx rate and TFG level in stored RBCs. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01 for the exercise group compared with the corresponding control group.
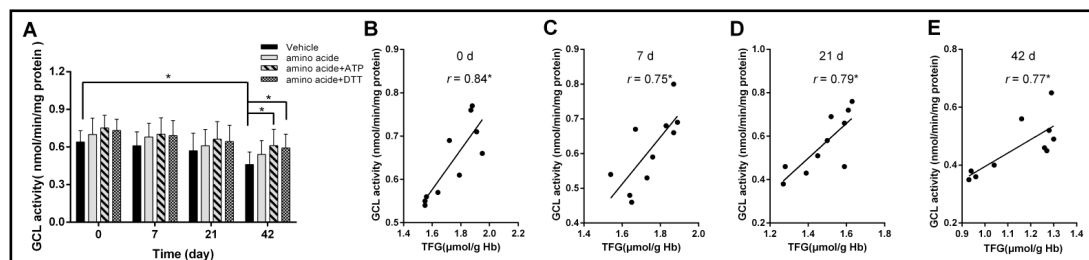

Fig. 4. GCL activity and correlation analysis in stored RBCs. (A) GCL activity, (B) Pearson correlation analysis between GCL activity and TFG value. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01 for the exercise group compared with the corresponding control group.

Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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The content of GCLC and GCLM significantly decreased in stored RBCs

To explore the mechanism of decreased GCL activity during RBCs storage, the protein content of the two subunits of GCL was determined. Fig. 5 shows representative immunoblots (A) and densitometric analysis (C) of GCLC from stored RBCs. The relative GCLC protein expression significantly decreased in 42 days storage group compared to the values observed in day 0 control(P<0.05).

As shows in Fig. 5B, D, the relative GCLM protein expression level significantly decreased in 42days storage group compared to the values observed in day 0 control (P<0.05).

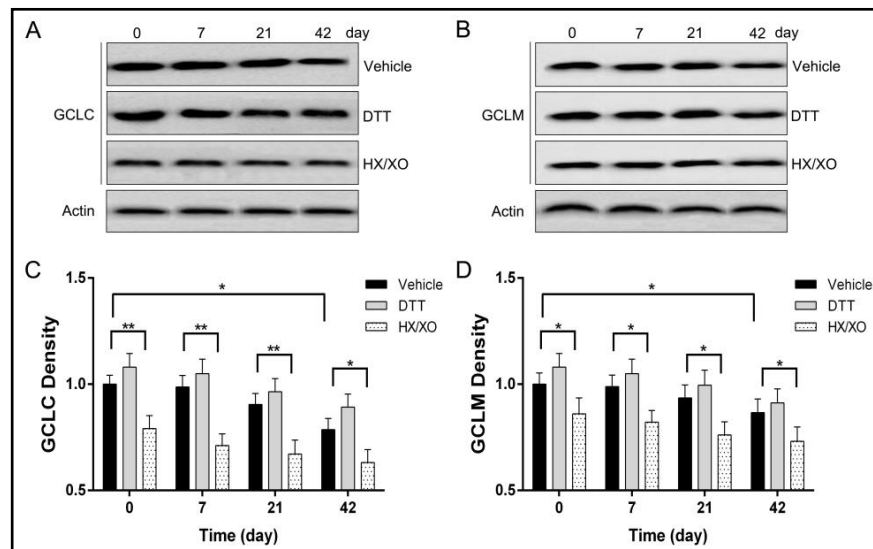*Glucose metabolism parameters decreased in stored RBCs*

To evaluate the influence of storage induced oxidation on glucose metabolism of RBCs, erythrocyte suspensions were incubated for 30 min at 37°C in the presence and absence of HX 0.2U/ml XO 1.5mM (hypoxanthine/xanthine oxidase) and 1.0 mM dithiothreitol (DTT).

As the substrate of glucose metabolism, the levels of glucose displayed significant decrease along with time, which declined by 55.9% after 42 days storage. Meanwhile, significantly increase of glucose consumption was observed after *in vitro* incubation with 1.0 mM DTT in 7, 21 and 42 groups (Fig. 6A). Lactate, which is a frequently measured parameter in metabolic analyses of RBC, constantly accumulated as storage progressed, significantly increased in RBCs stored for more than 21 days as compared to day 0 control levels. In parallel, significant increase of lactate was observed after *in vitro* incubation with 1.0 mM DTT in all tested groups (Fig. 6B). ATP appeared to increase up to day 7 and was then rapidly consumed soon after the first week, so as to reach initial values and below. In addition, significant increase of ATP was observed after *in vitro* incubation with 1.0 mM DTT in 0, 21 and 42 groups (Fig. 6C). We observed a moderate, albeit constant accumulation of pentose phosphate pathway metabolite: NADPH levels continued to rise and increased by 164% after 42 days storage (Fig. 6D). As shows in figure 6EFGH, the glycometabolic enzyme (G6PDH, HK, PK and LDH) activity significantly decreased after 42 days storage.

TFG synthesis increased in rejuvenated RBCs

To evaluate the changes of TFG anabolism of stored RBCs after transfusion. Stored RBCs were suspended in fresh plasma (collected from the same donor) to a 45%Hct as rejuvenated group.

Fig. 5. Effect of storage on GCLC and GCLM protein levels. Western blot of GCLC (A) and GCLM (B). Densitometric analyses of immunoblots of RBCGCLC (C) and GCLM (D) in different groups after in vitro treatment of HX 0.2U/ml XO 1.5mM or 1.0 mM DTT. The data are the relative proportion (%) of each protein to the total membrane proteins normalized to the controls. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01 for the exercise group compared with the corresponding control group.

Cellular Physiology and Biochemistry

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As show in Fig. 7, the L-cystein influx rate (SAGM, 0.53±0.08mmol/h/mL vs. Rejuvenated, 1.21±0.16mmol/h/mL; P<0.01), GCL activity (SAGM, 0.46±0.06 nmol/min/mg protein vs. Rejuvenated, 0.52±0.05nmol/min/mg protein; P<0.05) and TFG synthesis rate (SAGM, 0.65±0.24 mmol/L RBCs/min vs. Rejuvenated, 0.99±0.24 mmol/L RBCs/min; P<0.01) significantly increased after rejuvenation in RBCs in 42 days storage group. Reduction treatment with DTT did induce significantly increase in GCL activity (Rejuvenated, 0.52±0.05nmol/min/mg protein vs. Rejuvenated+DTT, 0.61±0.07 nmol/min/mg protein; P<0.05) and TFG synthesis rate (Rejuvenated, 0.99±0.24 mmol/L RBCs/min vs. Rejuvenated + DTT, 1.27 ± 0.26 mmol/L RBCs/min; P<0.05).

To confirm the importance of glucose metabolism on glutathione synthesis of RBCs, we inhibited G6PDH enzyme activity by treatment with DHEA (100mM), a recognized noncompetitive inhibitor of the enzyme. As show in Fig. 7, the level of L-cystein influx rate, GCL activity and TFG synthesis rate significantly decreased in DHEA-treated compared with untreated cells in all tested groups.
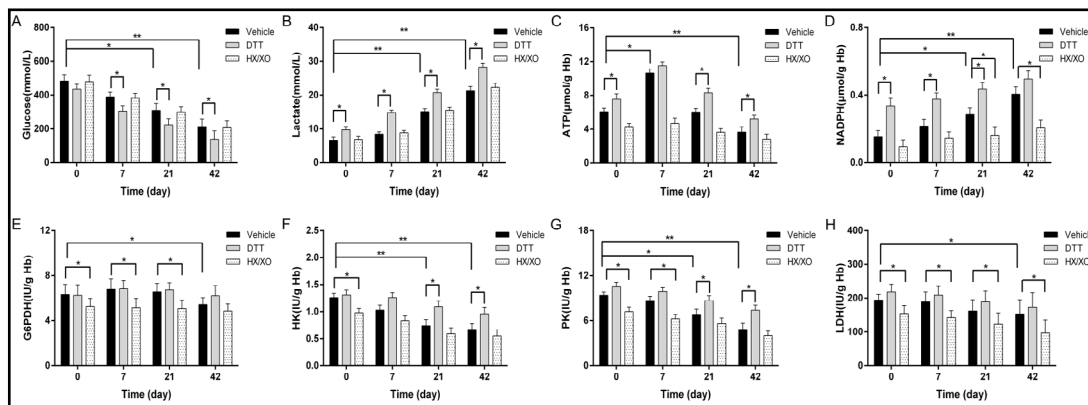

Fig. 6. Effect of storage on glucose metabolism parameters of RBCs. (A) Glucose levels, (B) Lactate levels, (C) ATP levels, (D) NADPH levels, (E) G6PDH levels, (F) HK levels, (G) PK levels, (H) LDH levels in different groups after in vitro treatment of HX 0.2U/ml XO 1.5mM or 1.0 mM DTT. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01.ATP, adenosine triphosphate; NADPH, nicotinamide adenine dinucleotide phosphate; G6PDH, glucose-6-phosphate dehydrogenase; HK, Hexokinase; PK, phosphofructokinase; LDH, lactate dehydrogenase.
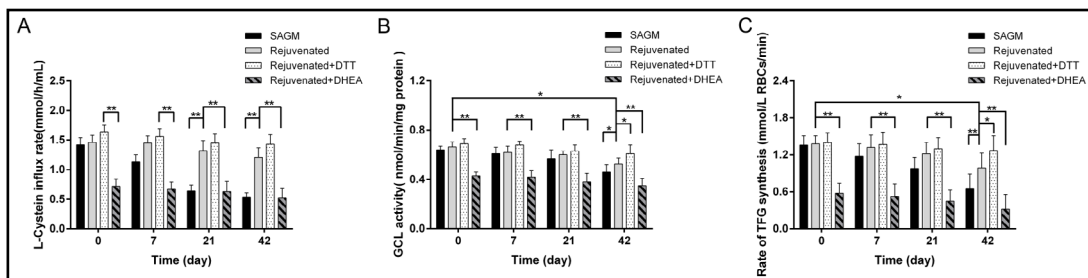

Fig. 7. Effect of storage on L-cystein influx, GCL activity and TFG synthesis in rejuvenated RBCs. (A) L-cysteine influx rate, (B) GCL activity, (C) TFG synthesis rate in different groups. Data are expressed as means ± SEM with the significance level set at *P<0.05 and **P<0.01.

Cellular Physiology and Biochemistry

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DOI: 10.1159/000495864
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Discussion

Oxidative damage has long been implicated in the blood storage process. GSH served as intracellular antioxidant which plays important roles in the maintenance of the redox state and cellular protection from damage by free radicals. Time-dependent oxidative changes in stored RBCs could decrease the RBCs GSH concentration [30, 31]. Previous studies by Whillier showed that the glutathione synthesis rate significantly decreased in stored RBCs [8]. In the present study, we confirmed that the decline of L-cysteine transport capacity and GCL activity attributed to the inhibition effect of GSH synthesis in stored RBCs. The mechanism that energy metabolism abnormality induced decline in L- cysteine transport activity has been revealed.

During refrigerated storage, banked RBCs underwent alterations, termed as the "storage lesion" [32]. These changes potentially affect the *in vivo* survival and function of RBCs after transfusion. There is evidence that the efficacy of banked RBCs products may be compromised due to decreased GSH dependent antioxidant potential over the time of storage [33]. When RBCs were continuously exposed to oxidative stress while cellular systems were unable to counteract the ROS-mediated insults, the amount of free GSH decreased, leading to irreversible cellular degeneration and death [34]. In this study, we observed a significant reduction of GSH, GSH/GSSH ratio and TFG level in rat RBCs after 6 weeks storage, consistent with the significant increase of TBARS levels. Increased oxidative damage in blood storage have been reported in previous study [35]. In our study, the TFG level of RBCs also significantly decreased. There might be two different explanations for this observation: (i) the increased oxidative stress-induced consumption of GSH and (ii) the dysfunction of GSH synthesis during storage.

RBCs possess the ability to synthesize GSH by glutamic acid, cysteine, and glycine. As the main factor that regulating the synthesis rate of GSH, the L-cysteine uptake changes were studied under numerous physiological and pathological conditions [17, 36]. In the present study, we observed significant decrease in L-cysteine transport capacity, which was significantly positively correlated with erythrocyte TFG synthesize during storage. Furthermore, we observed significant reduction of ATP concentration of RBCs after 6 weeks storage. In addition, *in vitro* incubation with ATP significantly improved L-cysteine influx rate in all tested groups. Given the significant decline of the GSH concentrations in RBCs, the ATP-dependent transport dysfunction might be responsible for decreased L-cysteine uptake capacity in stored RBCs.

GSH is synthesized *de novo* by two successive ATP-dependent reactions catalyzed by glutamate cysteine ligase (GCL) in RBCs. GCL catalyzes the first and rate-limiting step, in which glutamate is ligated with cysteine to form γ-glutamylcysteine (γ-GC), which is rapidly linked to glycine to form GSH via the action of GS. In the present study, the enzymatic activity of GCL significantly decreased after storage. In addition, *in vitro* reductive treatment with DTT or incubation with ATP significantly increased the levels of GCL activity. These data suggest that the GCL activity was regulated by the redox state of RBCs during storage. Increased oxidative stress induced the dysfunction of GCL during storage. *In vitro* reduction treatment with DTT induced the increase of TFG synthesis and GCLC and GCLM density. These results indicate that the reversible oxidative damage of GCLC and GCLM subunit may decrease GCL activity.

Oxidative damage to erythrocytes has been widely studied to explain destructive events in numerous hematological disorders [37, 38]. In the present study, we observed a significant decrease in glucose metabolism enzyme activity of rat RBCs after 6 weeks storage. There are two principal pathways for glucosemetabolism: (i) the Embden-Meyerhof pathway (EMP), which generates adenosine triphosphate (ATP), NADH and 2, 3-diphosphoglycerate (2, 3-DPG) and (ii) the hexose monophosphate pathway (HMP), which serves as the sole source of NADPH in erythrocytes. Relative flux through the EMP and the HMP is modulated by O_2-linked transitions in Hb conformation due to competitive binding for the cytoplasmic domain of Band 3 between deoxyHb and key EMP enzymes (PFK, Aldo, G3PD,PK, and LDH).

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Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;51:2172-2184

DOI: 10.1159/000495864
Published online: 7 December 2018

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Fig. 8. Hypothesized pathway of L-cysteine transport and GCL activity dysfunction induced inhibition of GSH synthesis. With the increase of blood preservation time, the metabolic substances depleted and the content of free radical increased in RBCs. Oxidative stress result
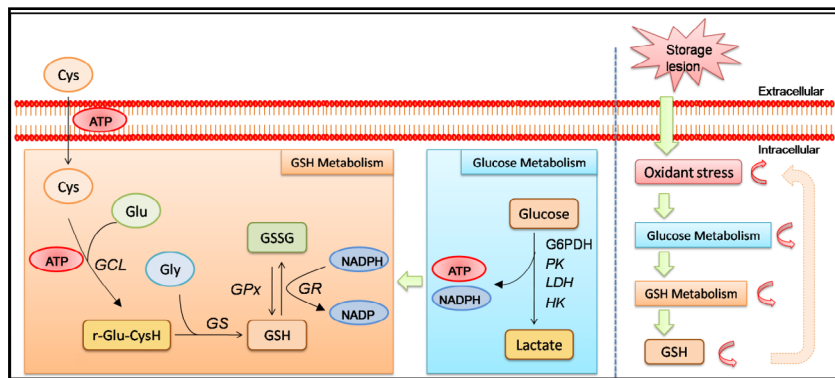
in decreased energy metabolism efficiency and decreased production of intracellular ATP and NADPH in stored RBCs. These effects in turn induce the decline of L-cysteine transport capacity and GCL activity, leading to the decreased efficiency of GSH synthesis. The lower GSH content in erythrocytes finally increases the risk of oxidative damage to the cells.

Previous observations suggested that blood storage results in elevated band-3 tyrosine phosphorylation and alters band-3 membrane organization [39]. The oxidative damage of Band-3 may lead to the decline of related enzymes activity. In this study, *in vitro* incubation with HX/XO significantly decreased levels of glucose metabolism enzyme activity in control group. In addition, we observed that inhibited G6PDH activity with DHEA induce the dysfunction of GSH synthesis. Therefore, these data suggested that the change of glucose metabolism enzyme activity of RBCs was influenced by intracellular redox state during storage. Decreased glucose metabolic efficiency could decrease ATP production, leading to inhibition of ATP-dependent L-cysteine uptake and GCL activity in RBCs. This finding is consistent with studies of L-cysteine influx rate and GCL activity change under *in vitro* oxidation or reduction treatment conditions. During storage, GSH *de novo* synthesis is ATP-dependent and is therefore impaired when the stocks of intracellular ATP are depleted. The loss of metabolites and antioxidants defenses correlates with the accumulation of oxidized biomolecules and the apparition of irreversible lesions (Fig. 8).

Due to the difference of extracellular environment between preservation solution and body, particularly the lack of amino acids required for GSH synthesis. Changes of GSH synthesis in RBCs after transfusion remain unclear. We further investigated the effects of GSH synthesis in RBCs after rejuvenated by suspended in fresh plasma. These data suggested that resuspension in rejuvenation solution significantly increased TFG levels in stored RBCs. These results indicate that storing RBCs in a solution containing the amino acids necessary for GSH synthesis might improve TFG synthesis after transfusion. In addition, the reduction of GSH synthesis rate in RBCs preserved for 42 days was more significant after *in vitro* reductive treatment with DTT. These results indicate that long-term blood preservation induced oxidative stress leads to a decrease in GSH synthesis efficiency in RBCs, which is partially irreversible during normal blood transfusion. The decline of GSH synthesis efficiency in banked blood may be the underlying mechanism of reperfusion injury.

Circulating RBCs are an heterogeneous populations of cells with different properties related to cell age [40]. They also respond to storage in an age-related manner. In addition, enzymatic activities decline in RBCs during ageing, so it could also be that the altered activity of GCL is only present in a subpopulation of RBCs. Previous studies by Piccinini provided evidence that although the total cell GSH was decreased with aging, there is no significant difference in its concentration in the RBCs preparations of different ages when correlated with cell water content [41].Therefore, the effect of age difference of RBCs on GSH synthesis during blood storage regimens should be evaluated further.

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Conclusion

In summary, we presented evidence that L-cysteine transport dysfunction and decreased GCL activity induced the inhibition of GSH synthesis during blood storage. In addition, oxidant stress-induced glucose metabolism dysfunction was the driving force for decreased L-cysteine uptake and GCL activity during storage. These experimental findings reflect an underlying molecular mechanism by which decreased antioxidant potential via GSH leads to oxidative damage in stored RBCs.

Abbreviations

RBC (red blood cell); GSH (glutathione); TFG (total free glutathione); GCL (glutamate cysteine ligase); ROS (reactive oxygen species); ATP (adenosine triphosphate); DHEA (dehydroepiandrosterone); SAGM (salineadenineglucosemannitol); NAC (N-acetylcysteine); DTT (dithiothreitol); HX/XO (hypoxanthine/xanthine oxidase); TBARS (thiobarbituric acid reactive substances); HK (hexokinase; LDH, lactate dehydrogenase); PK (pyruvate kinase); G-6-PD (glucose 6-phosphatedehydrogenase); NADPH (nicotinamide adenine dinucleotide phosphate).

Acknowledgements

Disclosure Statement

No conflicts of interest, financial or otherwise, are declared by the authors.

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Cellular Physiology and Biochemistry

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DOI: 10.1159/000495864

Published online: 7 December 2018

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