Original Article

The Effects of Glutathione Monoethyl Ester on Different Biochemical, Oxidant, and Antioxidant Levels During Storage in Leukoreduced Red Blood Cells

Behrooz Ghezelbash[1], Mohammadreza Deyhim[2], Azita Azarkeivan[2], Ali Akbar Pourfathollah[3], Nahid Eskandari[1]

[1]Department of Immunology, Faculty of Medicine, Isfahan University of Medical Science, Isfahan, Iran, [2]Department of Biochemistry, Faculty of Medicine, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran, [3]Department of Immunology, Faculty of Medical Science, Tarbiat Modares University, Tehran, Iran

Abstract

Background: It is essential to maintain the quality of the stored blood, because various factors affect the stored red blood cells (RBCs) over time, some red blood cell storage lesions (RCSL) develop during storage, and it could reduce the function of the RBCs. The present study aimed to evaluate the effects of glutathione monoethyl ester on different biochemical changes, oxidant, and antioxidant levels in the leukoreduced RBCs (LR-RBCs) during storage.

Materials and Methods: About 10 units of LR-RBC were collected, processed and stored according to the standard operating procedures (SOPs) of the Iranian Blood Transfusion Organization. Each unit divided into 2 equal parts; LR-RBC treated with glutathione monoethyl ester and a control group. Exposure of phosphatidylserine (PS), reactive oxygen species (ROS) and microvesicle derived from the RBCs (RBC-MVs), were measured by the flow cytometry method. ELISA was used to measure the level of glutathione, and 2, 3-diphosphoglycerate (2,3-DPG). Glucose-6-phosphate dehydrogenase (G6PD) enzyme activity was measured with a chemistry autoanalyzer.

Results: The levels of glutathione reduced the initial value in the treated group (80%), and the control group (60%), respectively. Exposure of surface PS, ROS and RBC-MVs increased significantly during storage time for consecutive weeks to the amount of GSH. The levels of 2,3-DPG decreased with increasing storage time.

Conclusions: Overall, The study suggest that glutathione monoethyl ester is effective to reduce the oxidative stress and the quality of RBCs can be improved.

Keywords: Blood transfusion, glutathione, reactive oxygen species, red blood cells

Address for correspondence: Dr. Nahid Eskandari, Department of Immunology, School of Medicine, Isfahan University of Medical Sciences, Isfahan, Iran.
E-mail: neskandari@med.mui.ac.ir
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Introduction

The quality of prepared red blood cells (RBCs) is influenced by different factors such as the method of product preparation, type of bag, preservative solution, filtration, and storage time. RBCs undergo changes during storage, which leads to a decrease in their quality. RBCs changes include a decrease in pH, adenosine triphosphate (ATP), and 2,3-DPG, an increase of bioreactive substances, an increase in oxidants (ROS and NOS), etc. Oxidative damage is the main factor affecting RBC storage lesion which is caused by free radicals.[1] Free radicals damage the RBC products by lipid, protein oxidation and oxidative loss with changes in the structure of the RBCs membrane leads to an increase in the accumulation of band-3 protein, lipid oxidation و and apoptotic changes in the last weeks of storage. Therefore, it leads to surface externalization of phosphatidylserine (PS).[2]

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Modern storage additive solutions (AS) preserve the human RBC for up to six weeks along with the hypothermic storage.[3-5] This period is short storage time in the laboratory conditions, based on the viability of cells after the blood injection within 24 hours, it should be greater than or equal to 75% ± 9, by the United States Food and Drug Administration (FDA).[1,6] We have previously shown that the functional and physical changes in the leukoreduced RBC (LR-RBC) or filtered-RBC are lower than in the unfiltered-RBC.[7]

Long-term storage, along with its advantages, also has problems. Since glycolysis is the only way to produce adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide hydrogen (NADH) energy in the RBCs, lactic acid produced in glycolysis leads to a decrease in pH during six weeks of storage.[8] Another chemical change during storage is the decrease in the internal concentration of 2,3-diphosphoglycerate (2,3-DPG). Decreasing allosteric substance levels primes to the shift of the -O2 balance curve of hemoglobin to the left, and effectively increases the affinity of O2 to hemoglobin. The loss of 2,3-DPG during storage could reduce the ability of RBCs deliver O2.[9] At low temperatures, glucose metabolism via the Pentose phosphate pathway (PPP) pathway is also reduced, which glucose-6-phosphate dehydrogenase (G6PD), and NADPH reduces leading to the loss of the RBCs antioxidant activity during the storage.[10] The decrease in the antioxidant activity during the storage could cause the increase of ROS and the formation of hemolysis and vesicles.[11] Reduced glutathione (GSH), ascorbic acid (AA), uric acid (UA) and α-tocopherol (vitamin E) play a role in protecting the low level of oxidants inside and outside the RBCs surface.[11]

The 2B7 antibody could reacts with CD235a (Glycophorin-A), an ~10 kDa type I sialoglycoprotein which present in the cell membrane of the erythrocytes and erythroid precursors.[12,13] Glycophorin-A bears the antigenic determinants for the MN and Ss blood groups and has been suggested to provide a great mucin-like surface to the erythrocytes that acts to minimize accumulation in flow. Glycophorin-A is visible on the morphologically identifiable erythroid precursors subsequently the colony-forming unit erythroid (CFU-E) stage, and reaches its greatest expression at the late normoblast stage.[14] Anti-glycophorin[15] is valuable in combination with anti-transferrin receptor (CD71)[16] to classify different stages of erythroid differentiation,[17] since CD71 expression precedes the Glycophorin-A expression, but is absent through the maturation of normoblasts to the mature RBCs.

GSH is found primarily (>99%) in the human erythrocytes with an average concentration of about 1 mmol to 3 mmol,[18] and the ratio of reduced glutathione to oxidized glutathione (GSH/GSSG) is also high.[19,20] This glutathione redox ratio (GSH/GSSG) is due to the presence of glutathione reductase (GSHr) and NADPH.[21] Glutathione reductase acts an main role in shielding hemoglobin, red cell enzymes, and biological cell membranes against oxidative damage by increasing the level of reduced glutathone (GSSGR) in the process of aerobic glycolysis.[22] It seems that under oxidative stress, transfering GSH, by a family of transporters, called ATP-binding cassettes (ABC) such as multidrug resistance protein (MRP1), is carried out from inside the RBC to the plasma.[23] Purification of glutathione cysteine synthase (GCS) and glutathione syntheses (GSHs) from the human red blood cells proved that glutathione synthesis takes place inside the cell. In order to synthesize glutathione in RBCs, three amino acids cysteine, glutamate and glycine and two ATP molecules are needed.[24,25] Currently, the role of GSH in ROS inhibition is emphasized. It suggests that the form of glutathione monoethyl ester is a cell-permeable derivative of GSH due to its ester content. Therefore, the aim of the presen study is to evaluate the effect of GSH monoethyl ester additive on RBCs function during the storage period in LR-RBC.

Materials and Methods

RBCs preparation and sampling

This study was a simple random study, in which the packed LR-RBCs (n = 10) were collected from the healthy donor volunteers following the guidelines of the Iranian Blood Transfusion Organization (IBTO). Each unit was collected in a final amount of 450 ± 45 ml of the blood along with additive solution containing combinations of citrate, phosphate dextrose (CPD), and saline, adenine, glucose and manitol (SAGM) anticoagulant. It is of note that SAGM provides extended shelf life of RBC up to 42 days with increased efficient possibility. In addition, by adapting CPD the whole blood storage bag, residual blood from a renal replacement therapy (RRT) circuit can be saved in pediatric patients, decreasing in donor exposure later.[25,26]

Briefly, the collection, processing and storage of the packed LR-RBC were split into two satellite bags; the LR-RBC treated with glutathione monoethyl ester and a control group. The amount addition of glutathione monoethyl ester was determined, similar to the previous studies and trials related to the additives to treat RBC.[25,26] About 1 ml of glutathione monoethyl ester AS (5 mmol in litre) was added to the LR-RBC groups and the same volume of the normal saline (NS) was added to the control groups. Then, samples were mixed gently. Sampling for all experiments was done on different days of 3, 14, 21, 34 and 42 of storage. In addition, to determine the percentage of the microvesicles, derived from the RBCs (RBC-MVs), sampling was done on days of 3, 28 and 42.

Measurement of GSH

To determine the amount of GSH, the packed LR-RBC was evaluated into two groups before and after the addition of glutathione monoethyl ester additive using a glutathione kit (ZellBio Company, Cat. No ZX-44100-96, Berlin, Germany). For this purpose, according to the kit's instruction, a cell suspension with a dilution of 1 million cells/ml was prepared in the phosphate-buffered saline (PBS) at pH 7.2-7.4.

Isolation o f the red blood cells-microvesicles (RBC-MVs)

The RBCs were isolated by the gradient density to prepare a supernatant containing MVs. Then, 20 ml of RBC were transferred to the Falcon tubes and centrifuged at 1850 g for 10 minutes. Then, the supernatant plasma was removed and re-centrifuged it at 4000 g for 10 minutes. Then, the supernatant was transferred to the Falcon tubes and centrifuged at 20,000 g for one and a half hours. Then, the PBS was added to the sample deposit, mixed it, and centrifuged ? g for ? minutes. Finally, the supernatant was removed and PBS was added to its precipitate. In this way, a suspension containing RBC-MVs was prepared.

Flow cytometry method

The count and phenotype of the RBC-MVs were evaluated by the flow cytometry device (Partec PAS, Germany). Mouse IgG2b k (BD Company, Cat. 14-4732-82, Waltham, USA) PE isotype control was used as a negative control. In this study, the cellular origin of microvesicles and their concentration were determined on days 3, 28, and 40. Then, 50 µl of the samples were mixed with 5 µl of anti-human CD235a and incubated at 4°C for 30 minutes in the dark room. About 5 µl of 1.500 of dilution with double distilled water was added to each sample. Then, the concentration of EMVs was calculated. Based on the MVs size (less than 1 µm), the origin of erythrocyte (positive expression of Gly. A) were defined. Data were analyzed using the Flomax software.

Measurement of phosphatidylserine (PS)

Annexins are a group of proteins that bind to phospholipids (preferably PS) and their binding depends on calcium. After the initiation of apoptosis, PS loses its asymmetric distribution and moves to the outer part of the cell membrane. To determine the externalization of PS on the RBCs surface, Annexin V kit, (eBioscience Company, Cat No. 88-8005-74, Warsaw, Poland) was used. Cells were washed in PBS according to the kit instructions. Then, the cell suspension was prepared (with a dilution of 1-5 × 10^6) in binding buffer. Approximately 5 µl of Annexin V conjugated fluorochrome was added to 100 µl of the cell suspension and incubated for 5 minutes, and then washed with 1% binding buffer. Cells were stained with 5 µl of the Propidium Iodide (PI) and results were read with a flow cytometer device. Fresh samples were used as negative control and samples exposed to the UV light, were used as a positive control. Based on the previous studies, the UV light was used to cause apoptosis and the surface appearance of PS.[27] For this purpose, 100 µl of the tested samples, were placed under the hood with the UV light for 2 hours and, then analyzed according to the instructions of the kit.

Measurement of the ROS

The ROS kit (eBioscience Company, Cat. and lot numbers?, the names of city and state?, USA) was used to check the ROS on the surface of the RBCs during storage. The cell suspension with a concentration of 105 to 108 was prepared by PBS. Then, 100 µl of the prepared reagent was added to 1 ml of cell suspension and incubated for 1 hour in a CO2 incubator. The ROS on the surface of the RBCs was determined by the flow cytometry method. To stimulate and create the ROS and to prepare the ROS-positive control, different concentrations of H2O2 were used. Samples were exposed to H2O2 in a 37°C incubator for 2 hours and then were evaluated.[28] Samples were exposed to H2O2 in a 37°C incubator for 2 hours and then were evaluated.

Determination of 2,3-DPG

According to the instructions of the 2,3-DPG kit (ZellBio Company, Berlin, Germany), cell suspension with a dilution of about 1 million cells/ml was prepared using PBS. Cells were lysed by repeated freezing and thawing. Then, the samples were centrifuged at 4000 g for 10 minutes. A supernatant was used for the experiment. A standard curve was prepared with the different dilutions, specified in the kit.

G6PD enzyme activity

Firstly, the hemoglobin concentration (gr/dl) was measured with a cell counter (Sysmex KX-152 21, Japan). Then, 200 µl of the homogenized RBCs was washed three times with 0.9% NS. 9 ml of lysing reagent was added to the washed RBCs and then incubated for 15 minutes at 2-8°C, then centrifuged. The supernatant was examined for the G6PD enzyme activity by using the kit 155 (Biolabo Company, France).

Statistical analysis

All results represented as mean ± standard deviation of the mean (SD), were analyzed by the one-way analysis of variance (ANOVA) and paired t-tests, followed to compare the groups with the non-normal distribution. The Pearson comparison of variables and their effect on each other was used. All statistical calculations were performed using the GraphPad Prism version 6.0 (GraphPad Software, Inc., San Diego, CA, USA) and SPSS Inc., (V. 22, IBM, Chicago, IL., USA). The acceptable level of significance was established at $P < 0.05$ unless otherwise indicated.

Results

Glutathione levels in the LR-RBCs storage

The basic levels of glutathione were examined on different days before and after the addition of the glutathione monoethyl ester additive in the LR-RBCs storage [Table 1]. Given that the glutathione monoethyl ester penetrate the cell due to its ester content, it was used instead of reduced glutathione. The results of this study showed that the levels of GSH had a significant difference from each other in 5 different periods (ANOVA, $P < 0.000$). The amount of glutathione decreased significantly in the LR-RBCs storage ($P = 0.000$).

The RBCs-MVs quantification

The results showed that the number of RBC-MVs increased during the LR-RBC storage period. In addition, the numerical average of the RBC-MVs on days 28 and 42 had significant differences, compared to the third day ($P < 0.05$). The results of this study also showed that the number of the RBCs-MVs in the treated-LR-RBCs was lower than the untreated samples

Table 1: The amount of glutathione in the LR-RBCs during storage in different periods

Test	Product	Day 3	Day 14	Day 21	Day 35	Day 42	ANOVA *P*
Amount of glutathione (mmol/ LRBCs Mean±SD	LR-RBCs	0.77±0.021	0.69±0.013	0.63±0.096	0.53±0.103	0.47±0.077	0.000
	RBCs + GSH	0.77±0.022	0160.86±0.	0.78±0.081	0.68±0.076	0.63±0.068	0.000
	Paired Test *P*	a	0.001	0.001	0.000	0.000	

LR-RBCs; Leukoreduced RBCs, GSH; Glutathione

with the glutathione monoethyl ester, which could be effective in the formation of the RBCs-MVs [Figure 1].

PS exposure in the LR-RBCs storage

With the start of apoptosis, PS loses its asymmetric distribution and moves to the outer part of the cell membrane, therefore, to determine the externalization of PS on the RBCs surface, the Annexin V kit was used. The results showed that apoptosis was low in the cells treated with the glutathione derivative, compared to the control group [Figure 2].

Levels of the ROS on the LR-RBCs

As the RBCs expose to the ROS, their quality decrease. Therefore, to reduce the effect of these ROS, the LR-RBCs were exposed to the glutathione monoethyl ester. Therefore, the average percentage of the ROS in the treated group on days 35 and 42 of the study, was 2.01% and 2.85%, respectively. Likewise, the average percentage of the ROS in the control group, on days 35 and 42 was 2.45% and 3.71%, respectively. The levels of the ROS were different significantly in the treated and control groups on days 35 and 42 [Figure 3]. Moreover, there was a confident relationship between the externalization of the surface PS and the creation of free radicals (r = 0.65 and *P* = 0.000) [Figure 4].

2 2,3-DPG levels and the G6PD enzyme activity in the LR-RBC

Our results showed that the levels of 2,3-DPG decreased with increasing storage time. No significant difference was observed between the treated and control groups. It suggests that its activity has decreased with the passage of storage time ($P < 0.05$). The average activity of the G6PD enzyme decreased during the storage days, and this finding indicates that its activity has decreased with the passage of the storage time ($P < 0.05$) [Table 2]. In addition, there was a negative correlation between the G6PD enzyme activity and the ROS (r = -0.737 and *P* = 0.000) [Figure 5].

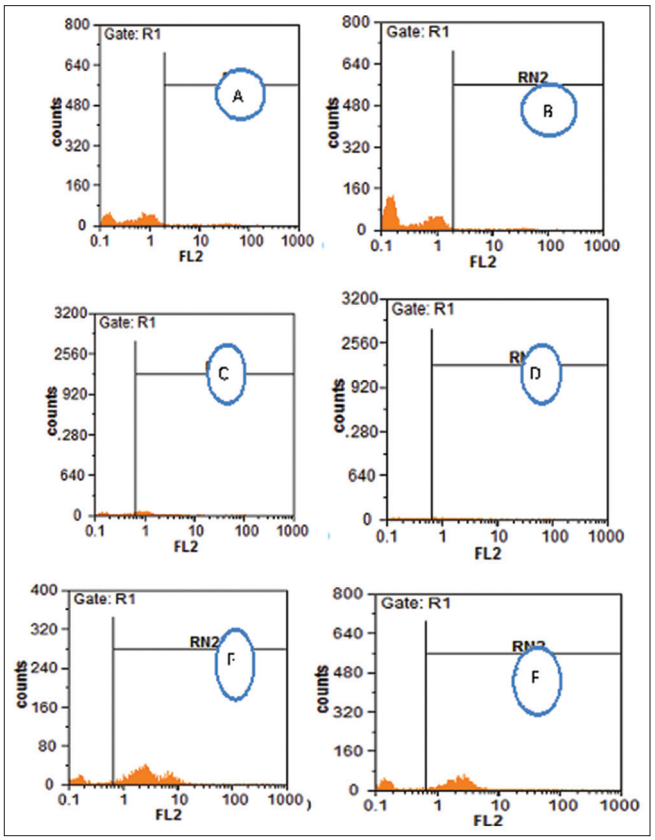

Figure 1: Identification and analysis of microvesicles = MVs,: Left side (LR-RBCs), right side (LR- RBCs + GSH). The percentage of LR-RBCs on the day 3; A) control group and B) LR-RBCs + GSH group, the percentage of RBCs-MVs on day 28; C) control group and D) LR-RBCs GSH goup, the percentage of RBCs-MVs on day 42; E) control group and F) LR-RBCs + GSH group. The population selected for 437 analysis was labeled with CD235 Phycoerythrin (PE) antibody, which was analyzed in FL2

Discussion

We aimed to examine the effect of GSH monoethyl ester additive on the RBCs function during the storage period in the LR-RBC. The effect of storage damage in the packed RBCs products and the LR-RBCs and its clinical aspects are not fully known, and various studies are still being shown around the world. Glutathione is considered the most important intracellular regenerator RBCs and as an antioxidant, plays an important role in neutralizing intracellular and cell surface oxidants.[29] In this study, the glutathione additive or a derivative of glutathione called glutathione monoethyl ester was used as an antioxidant additive to reduce oxidation and cell damage of LR-RBC products during storage. Glutathione monoethyl ester could penetrate the cell due to its ester content, so it was used instead of reduced glutathione.

In this study, the amount of glutathione was evaluated before and after adding of glutathione monoethyl ester additive. The results of this study showed that the amount of glutathione in both groups (treated and untreated) was significantly reduced. The average glutathione on the 42nd day of storage showed a significant difference, compared to the third day, but reduction in the amount of GSH in the treated bags was lower than the control bags. This finding is consistent with other studies. Dumaswala *et al*.[30] proved that the level of

Table 2: The levels of 2,3-DPG and G6PD in the treated and control groups during the storage period

Test	Product	Day 3	Day 14	Day 21	Day 35	Day 42	ANOVA *P*
(2,3-DPG) µmol/ml Mean±SD	LR-RBCs	35.8±5.33	21.3±3.02	7.09±2.47	3.15±1.07	1.48±0.50	0.000
	RBCs + GSH	35.3±8.23	22.06±3.4	7.06±2.22	3.50±1.34	1.60±0.90	0.000
	Paired Samples Test *P*	a	0.54	0.93	0.30	0.73	
(G6PD) (IU/g Hb) Mean±SD	LR-RBCs	12.16±0.29	11.87±0.36	11.67±0.27	10.96±0.71	10.48±0.74	0.000
	RBCs + GSH	12.22±0.30	11.96±0.38	11.78±0.38	11.27±0.56	11.07±0.47	0.000
	Paired Samples Test *P*	a	0.37	0.16	0.000	0.000	

LR-RBCs; Leukoreduced RBCs, GSH; Glutathione

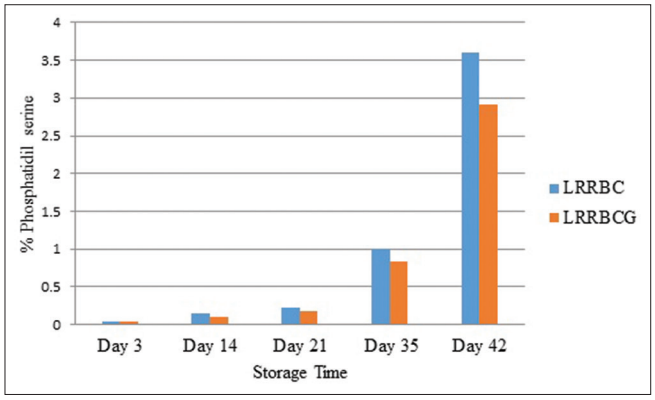

Figure 2: The amount of PS and comparison of the effect of the glutathione monoethyl ester additive on its externalization on the surface of the RBCs during storage. In the analysis of average PS in 5 different time periods, there are significant differences with each other ($P < 0.000$). With comparing the effect of glutathione monoethyl ester on the amount of PS, on day 35 ($P = 0.027$) and 42 ($P = 0.035$), there was a significant difference between the two groups ($P < 0.05$)

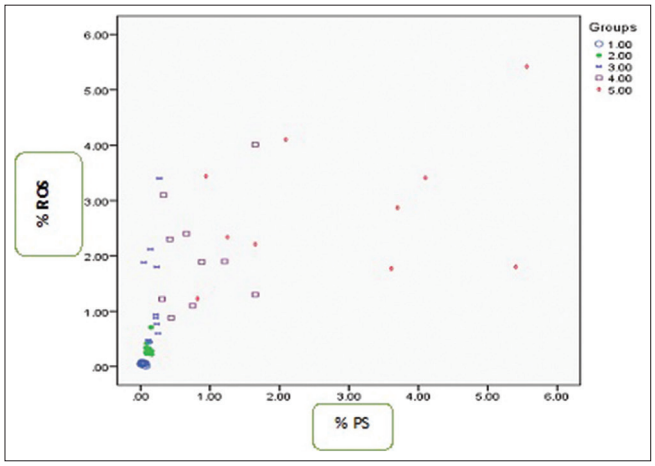

Figure 4: The relationship between the incidence of PS and ROS in the LR-RBCs. There is a positive correlation between the externalization of surface PS and the creation of ROS (r = 0.65 and $P = 0.000$)

GSH and glutathione peroxidase (GSH-PX) are involved in the oxidative changes of the lipids, proteins and the loss of membrane integrity. Similar to this study, Dumaswala *et al*.[26] have artificially added the antioxidant GSH, and antioxidant enzymes such as catalase to the RBCs Bags, and they have indicated that oxidative damage to the RBCs membrane was prevented. They have also demonstrated that GSH has the

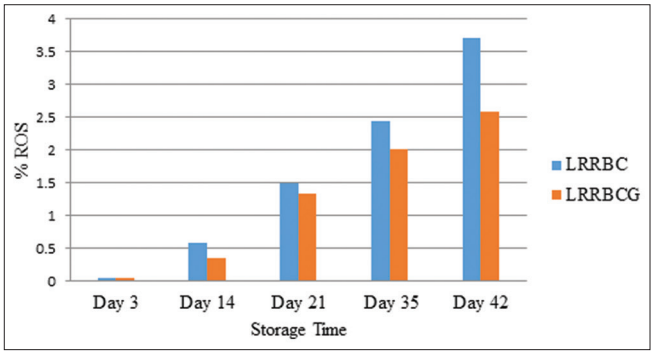

Figure 3: The changes in the ROS amount level and comparison of the effect of glutathione additive on its amount during storage of the LR-RBCs. The mean percentage of the ROS in 5 different time periods has significant differences with each other ($P < 0.000$), compared the effect of glutathione monoethyl ester on the ROS level, except for the third day, in the rest of the study days, the difference between the two groups was significant ($P < 0.05$)

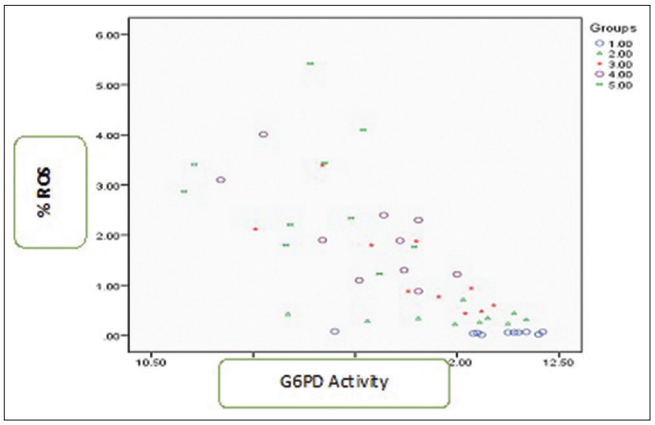

Figure 5: Examining the relationship between the G6PD enzyme activity and the ROS. There is a negative correlation between the G6PD enzyme activity and the ROS, (r = -0.737 and $P = 0.000$)

protective role for the RBCs stored at 1-6°C and on days 0, 42, and 84. In another study, they have shown a decrease in the GSH during the RBCs storage.[31] The results of this study showed that, the amount of the GSH decreased (more decrease occurred in the last two weeks of storage) in the LR-RBCs. Of course, in the group, which was used the GSH additive, the reduction rate was less than in the control group. In another study, the RBCs stored with the GSH precursor amino acids, did not show any decrease in total GSH concentration from

weeks 1 to 6. The percentages were different in the glutathione concentration in the treated RBCs, compared to the control RBCs, which was 22.3% in the third week and 36.5% in the sixth week.[25] The results of this study also showed that the percentage difference in the GSH concentration in the treated RBCs groups, compared to the control groups was 12.5% in the third week and 25.5% in the sixth week. It suggests that by adding GSH and its precursor amino acids, the GSH level of the bags can be maintained at an optimal level.

In this study, the RBCs-MVs were evaluated in three different periods. The results showed that the number of the RBCs-MVs increased significantly during the storage period in both groups, which in the treated bags were lower than the control bags. Similarly, another study has shown the formation of MVs is related to the oxidation of the RBCs spectrin protein during the storage by examining the cholinesterase enzyme as an erythroid marker in the membrane of vesicles.[32] the RBC-MVs were identified with the anti-CD235 or anti-Gly. A specific antibody, because this marker was to mark specific proteins in the membrane of the RBCs, which are also present on the surface of MVs derived from them, and this antibody is reactive with both parts of the RBC membrane. The results of this study also showed that the number and percentage of the RBCs-MVs gradually increased during the RBCs storage. In general, about 64% and 77% of the total number of the MVs had the CD235 marker on their surface on day 42 in the treated group and the control group, respectively.

In the present study, the amount of the MVs was almost doubled on day 42, compared to day 21, which is similar to the study of Grisendi *et al*. (2015).[33] Likewise, another study has observed a 2-fold and 15-fold increase in the RBCs-MVs on days 14 and 50, compared to day 4.[34] Collectively, these results suggest that changes in the MVs could be clinically significant and more research is needed.

The externalization of PS on the surface of the RBCs not only indicates the level of cell damage during the storage, but is also a sensitive marker for the rapid detection and removal of the RBCs by the reticuloendothelial system and phagocytes. The results of this study showed that the level of PS in the treated RBCs was lower than the control group. This study also compared the effect of glutathione monoethyl ester on the externalization of PS, on days 35 and 42, and found that there was a significant difference between the two groups, which agrees with another study.[35,36] In contrast, Geldwerth *et al*.[37] have reported 45% surface of PS at the end of two months the externalization of PS on the surface of the RBCs. The results of this study also showed that, there was a positive correlation between the externalization of PS and the generation of ROS ($r = 0.65$). Therefore, GSH, by an inhibiting flip-flop, prevents the externalization of PS in the stored RBCs and could also improve the quality of the products.

In this study, to check the indicators of oxidative stress, the amount of ROS was analyzed. There was an inverse relationship between the GSH level and the increase of oxidants. The results of this study also showed significant changes in the ROS levels after day 21 in the treated and untreated groups in parallel. In this regard, other studies have indicated that the amount of oxidative stress increases during the blood storage, and one of the markers, which has been increased, is the amount of carbonylation membrane proteins of the RBCs.[38,39] In another study, the investigators have found significant differences in the amount of ROS, stored in the RBCs in three groups treated with ascorbic acid (Vitamin C), N-Acetylcysteine (NAC) and NAC + vitamin C.[40] These results indicate that the above additive solutions are effective in reduction of oxidants, and are consistent with the results of this study.

The exact mechanism of the reduction of GSH level in the RBCs is unknown, but it may be caused by the reduction of G6PD. It seems that this product is necessary for the protection of the GSH and oxidative stress.[29] The results of this study showed that glutathione monoethyl ester was effective in improving the G6PD activity, and the activity level of this enzyme was higher in the treated group than the control group, which is in agreement with the previous reports.[41,42] In contrast with the results of this study, other studies have reported that the level of G6PD enzyme activity does not change during the storage days[43] Storage of the red blood cells at 1-6 0 C does not maintain the 2,3-DPG level.[44] The results of this study indicated that the glutathione monoethyl ester additive did not affect the level of 2,3-DPG in the bags during the storage, which is in agreement with the previous studies.[40]

Overall, the results of this study suggest that the storage damage compromises the RBCs quality and may cause the side effects after the blood transfusion. There is a significant difference in all considered factors, except 2,3-DPG in the treated group with the glutathione monoethyl ester, compared to the control group. It seems that the glutathione monoethyl ester additive could reduce the storage damage of the LR-RBCs, oxidative stress, and therefore, the RBCs quality will be improved.

Ethics approval and consent to participate

The present study was carried out in accordance with the ethical guidelines of the 1975, declaration of Helsinki, as reflected in a prior approval by the Institution's Human Research Committee. This study approved by the Ethical Committees of the High Institute of the Research and Education of the Transfusion Medicine, Tehran, Iran (Thesis NO: 34).

Author contribution

BG, MD, AA, AP, and NE interpreted, and provided major contributions in writing the manuscript up. All authors read and approved the final manuscript.

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Conflict of interest

There are no conflicts of interest.

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