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Evaluation of Lipid Peroxidation, Antioxidant Status and Trace Elements in Red Blood Cell Concentrates during Storage

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ABSTRACT

Background: Red blood cell concentrates (RBCs) undergo biochemical and structural changes during storage, commonly referred to as the RBC storage lesion (RSL) which reduces the survival of RBCs and affect transfusion efficiency. Lipid peroxidation and oxidative damage are the most important side effect of RSL. We aimed to evaluate oxidative damage and some related parameters in RBCs during storage.

Methods: In this experimental study, eight RBCs bags were randomly selected from healthy blood donors and stored at 2-6 °C for 35 days. Oxidative stress markers, trace elements and RBCs metabolism parameters including total antioxidant capacity (TAC), malondialdehyde (MDA), zinc, copper, manganese, selenium, iron, magnesium, sodium, potassium, lactate, glucose, lactate dehydrogenase (LDH) enzyme activity, pH and also RBCs hematological indexes including hemoglobin, hematocrit, MCV, MCH, MCHC and free plasma hemoglobin were evaluated during of RBCs storage.

Results: The results showed a significant increase in hemoglobin, hematocrit, calcium, phosphorus, iron, magnesium, lactate, potassium, free plasma hemoglobin, TAC and LDH activity during RBCs storage according to one way analysis of variance ($P<0.05$), while a significant decrease was shown in pH, sodium and glucose concentration ($P<0.05$). No significant mean changes were seen in MDA, zinc, copper, manganese and selenium concentration during RBC storage.

Conclusion: It seems that RBCs at the end of the storage period have a lower quality than newly prepared ones. Therefore, we commend that RBC products rather be used before third week of storage due to post transfusion side effects in blood recipients.

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Introduction

Red blood cell concentrates (RBCs) are the major blood component transfused worldwide to rescue severe anemia with the aim of improving the quality of life of patients, increasing oxygen delivery and improving blood circulation.[1, 2]

RBCs are stored at 2-6 °C for a maximum of 35 days before transfusion; however, under this condition red blood cells are exposed to metabolic, biochemical and morphological changes which reduces RBCs quality and decreases the viability and function of the red blood cells which is called "red blood cell storage lesion" (RCSL).[3-7] These changes include a decrease in pH, reduced level of 2,3 diphosphoglyserate (2,3 DPG) and adenosine triphosphate (ATP), increased in lactate dehydrogenase enzyme activity (LDH) and lactate concentration. Due

to long storage of RBCs, the sodium-potassium ATPase channel would be affected which causes an increase in potassium (K^+) outside the cell as well as an increase in sodium (Na^+) inside the cell.[8] On the other hand, increase in vesiculation of the RBCs, RBC membrane loss and lysis, RBC membrane lipid peroxidation and oxidative stress occurs during RBC storage.[9-15]

Trace elements protect the function and survival of the red blood cells, and any change in their concentration may reduce cell function. For example, zinc reduces oxidative stress by participating in the synthesis of antioxidant enzymes and in physiological concentrations inhibits the production of reactive oxygen species (ROS) and reduces lipid peroxidation.[16, 17] Copper and selenium are other trace elements in red blood cells that increase the activity of superoxide dismutase activity as an anti-oxidant enzyme.[10, 18] During RBCs' storage and subsequent RBC lysis, iron is released from the cells and accumulation of the iron in different tissues causes the formation of free radicals which are highly toxic.[19, 20] Generally, during prolonged storage of RBCs, changes in the concentration of the trace elements may occur which can reduce RBC function. The objective of this study was to evaluate the effect of long-time storage of RBCs on the biochemical and hematological parameters of RBCs, named as RCSL.

Materials and Methods

Preparation and Storage of RBCs

The present study was conducted on the samples of eight healthy blood donors. All donors were male with an average age of 38±11 years. The study was approved by the local ethical committee, and the informed consent was obtained from the volunteers by Iranian Blood Transfusion Organization (IBTO). Eight RBCs bags was obtained from the whole blood by routine phlebotomy into 450-ml bags containing 63 ml of citrate phosphate dextrose adenine (CPDA1) solution as anticoagulant in standard Quadruple containers (Fresenius Kabi Medicare, Hamburg, Germany). All of these RBCs were kept in a blood bank refrigerator at 2-6 °C for 35 days according to blood bank protocol. Oxidative stress markers, trace elements concentration and RBCs metabolism parameters including total antioxidant capacity (TAC), malondialdehyde (MDA), zinc, copper, manganese, selenium, iron, magnesium, sodium, potassium, lactate, glucose, lactate dehydrogenase (LDH) enzyme activity, pH and also RBCs hematological indexes including hemoglobin, hematocrit, MCV, MCH, MCHC and free plasma hemoglobin were measured during storage period. All assessments were performed in different periods of storage including days 0, 2, 7, 14, 21, 28 and 35. It should be noted that day 0 was considered the day that RBCs were prepared.

Sample Preparation

After gentle mixing under a class II laminar airflow cabinet, 10 mL samples were removed aseptically from each unit on days 0, 2, 7, 14, 21, 28 and 35. Three mL of the samples were analyzed for hematological parameters. Briefly, 7 mL aliquots of the samples were centrifuged at 2500 RPM for 10 minutes and supernatant plasma was used for the analysis of biochemical parameters.

Markers of RBC Oxidative Stress

Total Antioxidant Capacity (TAC) Measurement

TAC concentration was assayed using antioxidant assay kit (Zell Bio, Germany) according to manufacturer's protocol. The concentration of TAC (Mm) was calculated by standard calibration curve.

Malondialdehyde (MDA) Measurement

MDA concentration was assayed by MDA assay kit (Zell Bio, Germany) according to manufacturer's protocol. This test is based on the reaction between MDA and thiobarbitoric acid (TBA) at boiling temperature. The concentration of MDA (µmol) was calculated by standard calibration curve.

RBC Metabolism Parameters

Glucose, Lactate and pH Measurement

Glucose and lactate were measured based on enzymatic colorimetric method using assay kit (Pars Azmoon, Iran) and with a chemistry analyzer (Hitachi-911, Japan) according to manufacturer's protocol. pH was measured by calibrated pH meter (Melter, USA) during of RBC storage.

Lactate Dehydrogenase (LDH) Activity Measurement

LDH assay kit (Pars Azmoon, Iran) and auto analyzer (Hitachi 911, Japan) were utilized to determine LDH activity of samples.

Measurement of Calcium, Phosphorous, Iron and Magnesium

These parameters were measured by colorimetric assay according to manufacturer's protocol (Pars Azmoon, Iran). In all assay, biochemistry auto analyzer (Hitachi 911, Japan) was used.

Measurement of Sodium and Potassium

Na^+ and K^+ concentration was measured by using Flame-photometer (Eppendorf-Efox 5053, Germany).

Measurement of Trace Elements Such as Zinc (Zn), Copper (Cu), Manganese (Mn) and Selenium (Se)

Measurement of trace elements was performed by using atomic absorption spectrophotometer. For this assay, kit from Merck (Germany) was used. The samples after preparation were injected into the atomic absorption spectrophotometer, based on the standard calibration curve; the concentration of trace elements was calculated based on ppb unit.

Hemolysis Index (HI) in RBC

For measurement of free hemoglobin in plasma samples, we used hemoglobin cyanometry method. In this method, the created Methemoglobin is a stable compound and has the highest absorption at 540 nm wavelength which is measured by the Spectrophotometer. HI is calculated according to the following formula.

Red Blood Cell Indices

Red blood cell indices such as RBC count, Hb concentration, Hct, MCV, MCH and MCHC were measured by automated hematology analyzer (Sysmex KX-21, Cobe, Japan).

Statistical Analysis

All data were analyzed using version 23 of SPSS software and presented as means±SD of duplicate determinants. One way ANOVA was used for the analytical data during storage. Paired *t* test was used to compare the data between different time intervals based on data distribution. Differences were considered statistically significant at P<0.05.

Results

RBC Metabolism Parameters

RBC metabolism parameters including glucose, lactate and pH were analyzed at defined interval times. ANOVA indicated that glucose concentration and pH significantly decreased during all determined days of storage (P<0.001, Table 1). Lactate concentration increased during this period. paired t test showed that there was a significant difference in mean glucose, lactate and pH between days 0-2, 2-7, 7-14, 14-21, 21-28 and 28-35 of storage (P<0.05).

Calcium, Phosphor, Sodium and Potassium Concentration

Calcium, phosphor and K^+ concentration significantly increased, while Na^+ concentration was significantly decreased during the whole storage period (P<0.001, Table 1). Paired t test showed a significant difference in mean concentration of calcium between days 2-7, 21-28 and 28-35 of storage. (P_{day2-7}=0.022, $P_{day21-28}$=0.002, $P_{day28-35}$=0.005) and there was a significant difference in mean concentration of phosphorous between days 2-7, 7-14 and 21-28 of storage. (P_{day2-7}=0.007, $P_{day7-14}$=0.001, $P_{day21-28}$=0.042). Also, there was a significant difference in mean concentration of Na^+ and K^+ between days 0-2, 2-7, 7-14, 14-21, 21-28 and 28-35 of storage (P<0.05).

LDH Enzyme Activity

LDH enzyme activity increased during all days of storage (P<0.001) (Table 1). Paired T-test showed that there was a significant difference in LDH activity between days of RBC storage ($P_{day0-2=}$ 0.002 P_{day2-7}=0.001, $P_{day7-14=}$ 0.001 $P_{day14-21=}$ 0.001, $P_{day21-28}$=0.001) (Figure 1).

Trace Elements Concentrations

Trace elements including zinc (ZN), iron (Fe), selenium (Se), manganese (Mn) and copper (Cu) were measured at defined interval time periods. No significant changes were observed in trace elements concentrations during RBC storage except for iron. Iron concentration significantly increased during all days of storage (P<0.001) (Table 1). Paired t-test showed a significant difference in mean concentration of iron between days 2-7, 21-28 and 28-35 of storage (P: 0.001) (Figure 2).

Markers of Oxidative Stress

Oxidative stress markers including, malondialdehyde

Table 1: Hematological and Biochemical Parameters of Red Blood Cell Concentrates (RBCCs) During Storage

Parameter	Day 0 (x±SD) (n=8)	Day2 ($\bar{x}$±SD) (n=8)	Day7 ($\bar{x}$±SD) (n=8)	Day14 ($\bar{x}$±SD) (n=8)	Day21 ($\bar{x}$±SD) (n=8)	Day28 ($\bar{x}$±SD) (n=8)	Day35 ($\bar{x}$±SD) (n=8)	Anova P value
RBC (1 *10 ^{6}/ml)	7.7±1.4	8.4±0.9	8.3±1.2	8.6±0.4	8.6±0.4	8.2±1.1	8.8±0.5	0.307
Hb (g/dl)	21.9± 2.9	23.9±2.1	25.0±1.5	25.1±1.5	25.0±1.5	25.0±1.6	25.1±1.6	0.014
HCT (%)	66.8±7.1	70.7±5.6	71.3±8.7	75.9±3.7	77.4±3.7	74.3±9.1	79.2±4.7	0.003
MCV (fl)	86.40±6.07	84.7±6.63	86±6.94	88.3±7.05	90.6±7.5	90.3±7.2	90.1±7.18	0.177
MCH Pg))	28.30 ±0.43	28.63±2.52	30.98±7.36	29.25±2.84	28.62±2.64	31.11±7.22	28.63±2.46	0.701
MCHC (g/dl)	32.7±1.1	33.8±1.1	35.8±6.8	33.0±1.4	31.9±0.6	34.3±6.6	31.7±0.6	0.099
Glu mg/dl)	357.8±20.3	324.5±18.3	272.8±17.7	201.8±17.9	149.7±20.1	112±27.62	96.5±38.8	0.001
Ca (mg/dl)	4.44±0.12	4.48±0.13	4.64±0.21	4.81±0.33	4.78±0.30	4.70±0.28	4.79±0.26	0.001
P (mg/dl)	11.05±0.45	11.12±0.91	12.47±1.86	15.27± 1.68	16.16±0.82	14.73±1.67	13.68±2.87	0.001
Zn (µg/l)	531.42±76.6	532.41±82.1	524.36±78.8	533.62±84.0	524.63±79.2	536.10±78.8	524.56±75.2	1.000
Fe (µg/dl)	267.5±28.1	279.5±30.5	304.3±28.2	350.8±42.7	387.1±58.1	434.3±75.7	484.7±95.4	0.001
Mg (mg/dl)	1.84±0.083	1.810.12	1.78±0.15	1.91±0.22	1.87±0.30	2.67±0.63	2.81±0.89	0.001
Lac (mg/dl)	76.78±12.5	109.70±13.6	162.27±16.8	211±16.4	247.75±18.3	311.25+_19.0	339.28±22.2	0.001
LDH (IU/L)	310.7±72.8	572.5±219.9	1335.8±461.2	2359.6±747.1	3217.3±959.6	3846.6±1073	4495.3±1636.7	0.001
Na (mEq/L)	152.2±6.8	147.3±7.1	138.3±8.4	128.5±7.3	114.3±6.8	108.1±6.8	97.2±6.1	0.001
K(mEq/L)	1.63±0.21	1.72±0.36	2.54±0.45	3.6±0.48	4.21±0.38	4.53±0.28	4.97±0.41	0.001
Se (µg/l)	125.71±44.7	124.67±47.0	123.98±46.1	123.42±44.8	123.06±45.2	120.21±44.3	123.22±44.2	1.000
Mn (µg/l)	5.48±2.65	5.50±2.76	5.37±2.33	5.51±2.90	5.31±2.15	5.43±2.78	5.37±2.28	1.000
Cu (µg/l)	99.72±15.4	100.66±16.8	100.92±14.9	102.32±17.3	101.50±15.3	101.26±13.7	101.71±17.5	1.000
MDA (nmol/l)	41.34±18.76	42.77±11.97	44.84±14.16	44.43±15.25	47.54±15.29	50.77±14.98	49.13±11.09	0.327
TAC (mmol/l)	0.593±0.149	0.582±0.185	0.541±0.154	0.536±0.087	0.643±0.132	0.723±0.0927	0.932±0.112	0.001
PH	6.89±0.13	6.76±0.073	6.65±0.075	6.58±0.088	6.50±0.071	6.48±0.066	6.43±0.067	0.001
HI (%)	0.241±0.17	0.259±0.14	0.325±0.20	0.451±0.18	0.488±0.26	0.755±0.44	0.880±0.41	0.001
Plasma Hb (mg/dl)	212±146.4	274.2±166.7	359.38±231.7	482.50±207.1	582±15.7	783.13±369.5	1145.38±590.2	0.001

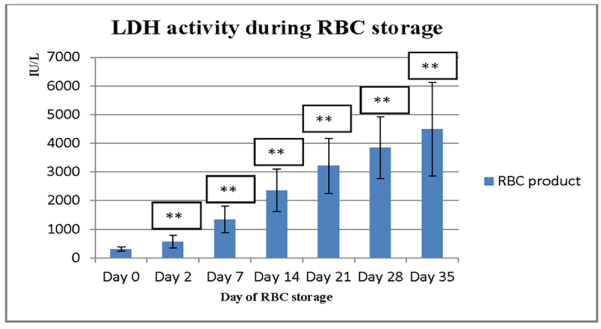

Figure 1: Lactate dehydrogenase enzyme (LDH) activity at different days of RBCCs storage. Data was shown as mean±SD. *P<0.05 and **P<0.01.

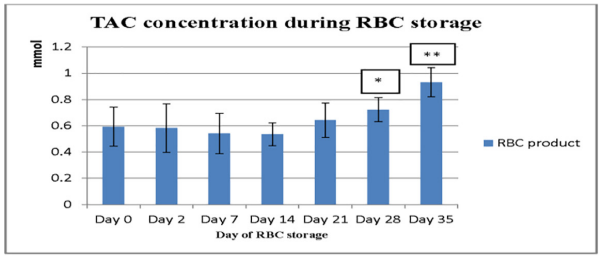

Figure 3: Total anti-oxidant (TAC) concentration at different days of RBCCs storage. Data was shown as mean±SD. *P<0.05 and **P<0.01.

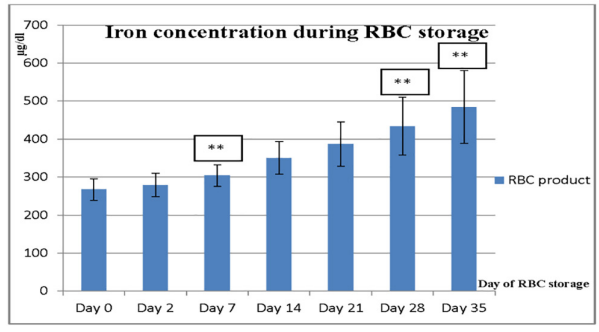

Figure 2: Iron concentration at different days of RBCCs storage. Data was shown as mean±SD. *P<0.05 and **P<0.01.

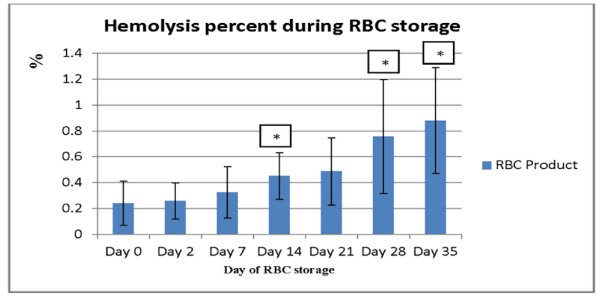

Figure 4: Hemolysis percentage at different days of RBCCs storage. Data was shown as mean±SD. *P<0.05 and **P<0.01.

(MDA) and total anti-oxidant capacity (TAC) was measured in different time periods of RBCs storage. There were no significant changes in MDA concentration during all days of storage, while significant increase in TAC concentration was showed (P<0.001, Table 1). Paired *t* test showed significant difference in mean concentration of TAC between days of 14-21 and 28-35 of RBC storage. ($P_{day14-21= 0.023}$, $P_{day28-35}$=0.001, Figure 3).

Hemolysis Index (HI)

Hemolysis index was increased slowly during the storage period (P<0.001) (Table 1). On the other hand, paired T-test showed a significant difference in mean percentage of HI between days 7-14, 21-28 and 28-35 of storage. ($P_{day7-14}$=0.021, $P_{Day\ 21-28}$=0.011, $P_{Day\ 28-35}$=0.028) (Figure 4).

RBC Indices

There were no changes in mean value of RBC indices except hematocrit (Hct). The mean value of Hct significantly increased during the storage period (P=0.003) (Table 1). Paired T-test showed a significant difference in mean Hct between days 14 and 21 of RBC storage. ($P_{day14-21}$=0.004).

Discussion

RBC products undergo a series of biochemical, hematological, structural and morphological changes during storage which are considered as RBC Storage Lesion (RCSL) and can affect the quality of this product. As we know, oxidative damage is one of the major causes of RCSL.[4, 5, 16]

There are limited data on the antioxidant capacity of RBC and biochemical changes such as trace element status and red blood cell metabolism during storage of RBCs. For this reason, in the present study, we tried to conduct more comprehensive research in this regard.

According to our results, the concentration of glucose by day 35 of RBC storage was significantly decreased. Glucose is the only source of energy for RBC through the glycolysis pathway which was consumed and reduced during RBC storage. On the other hand, glucose is sensitive to temperature changes and during the RBC storage, glucose and ATP levels decreased so the viability of cell membrane is disrupted.[2] The study of Simone and colleagues showed similar results to our study.[21]

According to our results, significant increase in lactate concentration was observed during RBC storage followed by a decrease in pH. Lactate is the last anaerobic product of glucose metabolism in RBC and following decrease in glucose, production of lactate will be increased and pH becomes acidic.[15, 21]

The measurement of LDH enzyme activity was another parameter in this study. LDH activity significantly increased during the storage of RBCs. It is the result of the cell lysis, so that the intracellular enzymes leak into the plasma and cause increase in the activity of this enzyme in the plasma.[4]

According to our results, plasma potassium concentration was significantly increased during RBC storage. On the other hand, plasma Na^+ concentration was significantly decreased during RBC storage. Due to reduction of temperature and energy production in RBCs, the function of sodium-potassium pump impairs and sodium accumulates in RBCs, whereas release of K^+ from the cells causes the plasma Na^+ concentration to be decreased and increased plasma K^+ concentration will be followed. Potassium level of stored blood increases daily by approximately 1 mEq/L.

Increased plasma potassium during RBC storage is one of the signs of the RBC lysis.[16, 22-25]

Lipid peroxidation was other parameter which was evaluated by the measurement of malondialdehyde (MDA) as an oxidative stress marker. According to our results, increase in MDA concentration was observed during the storage period, but this change was not statistically significant (P>0.05). In a similar study, increase in MDA levels during the storage was also observed which was not significant on different days of measurement.[26] Increased levels of MDA during storage indicate oxidative stress, lipid peroxidation and membrane damage due to RBC hemolysis.[27]

RBC hemolysis index (HI) is one of the most important factors in evaluating the quality of RBC product during the storage that we evaluated in our study. The amount of hemolysis (free Hb in plasma) was significantly increased (P<0.05) during RBC storage. During storage, increasing hemolysis and formation of microvesicles occurs and hemoglobin is effluxed from the cell.[28] Increased in HI indicates irreversible changes in the morphology of RBCs and with the loss of part of the membrane in the form of microvesicles causes a decrease in blood quality.[29] Taking into account the results of the other studies indicate an increase in hemolysis in the RBCs during the storage.[15, 27] In agreement with Hashemi et al.,[30] hemolysis may have happened as a result of irreversible changes in cell membrane leading to membrane loss by microvesiculation, so hemolysis is considered as an important marker for evaluating the quality of stored RBCs.[30]

In our study, a significant direct correlation between increased MDA level and increased free plasma hemoglobin concentration (r=0.516, P=0.001) was observed (Figure 5). On the other hand, there was a significant correlation between increased LDH activity and increased free plasma hemoglobin concentration during RBC storage (r=0.716, P=0.001) (Figure 6). These results express the fact that oxidation and deterioration of membrane lipid and loos of RBC membrane during prolonged RBC storage may contribute to increased RBC lysis. As a result of this lysis, not only RBC's hemoglobin is released into the plasma, but as mentioned before intracellular enzymes, such as LDH and cations (K^+) are released. The correlation observed between these parameters, all indicate RBC lysis during RBC storage. Our results are in accordance with those of previously published studies.[4]

According to our results, a significant increase in the concentration of calcium during storage was observed in our samples. During RBC storage, reactive oxygen species (ROS) are formed which impairs cell membrane and function and reduces membrane anionic junction and Ca-ATPase pump performance. Leakage of Ca out of the cells results in increased concentration of Ca in the plasma.[8] In addition, increased osmotic fragility and Calcium levels have been reported.[31]

The evaluation of iron status was another parameter in our study. The results showed significantly increased iron concentration during RBC storage. Due to oxidative stress, hemoglobin and iron are released and excess iron causes formation of free radicals which in turn results in membrane lipid and protein oxidative damage during long term storage.[27, 32-34] Excess iron causes serious complications such as cytotoxicity, systemic inflammation, lung injury and accumulation of iron in the liver and kidney of the blood recipient.[35]

Total anti-oxidant Capacity (TAC) was another parameter measured in this study. The results showed that there was no significant change in TAC concentration until day 14 of RBC storage, but from day 21 to 35 of storage, a significant increase in TAC concentration was observed. It seems that increasing the TAC from day 14 is based on a compensatory mechanism to respond to the increase in oxidative stress during the RBC storage. According to the study by Bardyn et al., TAC increased after one week of storage due to environmental changes and increased RBC metabolism.[36] Also, there are other studies which have reported the changes in anti-oxidant capacity during RBC storage. It has been shown that changes in RBC antioxidant enzymes start from two weeks after RBC storage. As a result, storage of RBC for more than 14 days causes a significant decrease of RBC quality.[37-39]

We did not find any statistically significant change in the mean concentration of trace elements including zinc, selenium, copper and manganese during the RBC storage in different time intervals. No similar study was found to compare the results of this study with the others in this matter.

According to the results of this study, it seems that the first 14 days after storage can be considered a safe interval in transfusion practice for minimizing oxidative stress of the RBCs subjected in the storage medium.

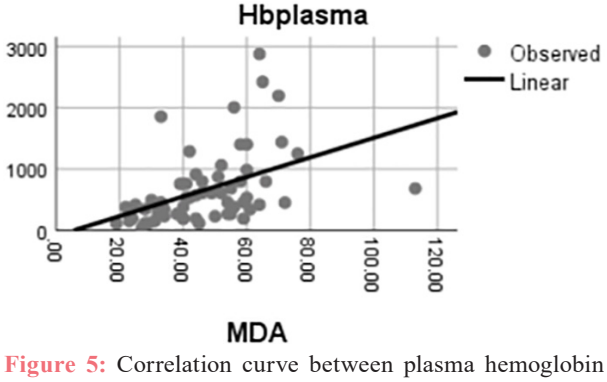

Figure 5: Correlation curve between plasma hemoglobin concentration and Malondialdehyde (MDA)

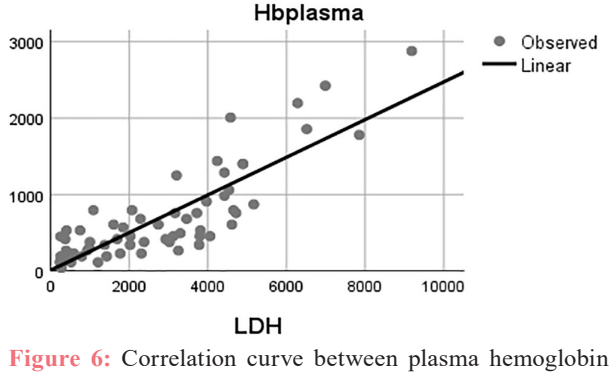

Figure 6: Correlation curve between plasma hemoglobin concentration and Lactate dehydrogenase enzyme activity (LDH)

Conclusion

RBCs in the last days of storage have a lower quality in comparison to the time of preparation. Therefore, it is recommended to transfuse RBCs before the third week to minimize the damage caused by the RCSL and the subsequent side effects that may occur in the recipients. These results need to be confirmed with a larger number of samples and on a larger scale.

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Financial Disclosure

The Authors have no financial relationships relevant to this article to disclose.

None of the authors have any conflicts of interest to declare.

Ethical Approval

The study was approved by the ethical committee and the informed consent was obtained from the blood donor participants by Iranian Blood Transfusion Organization (IBTO).

Conflict of Interest: None declared.

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