Original Research Article

Impact of lipid peroxidation and antioxidants on erythrocytes during blood storage

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Abstract

Background: Blood transfusion plays important role in the management of certain clinical conditions like acute blood loss, injury and anemia. The red blood cells (RBCs) for transfusion can be stored for 35 to 42 days at 2–6°C. It has been reported that some biochemical changes occur during the course of storage. During storage, progressive morphological and biochemical changes occur which are often related to the reduction of ATP, 2,3-diphosphoglycerate, and NADH in RBCs. These changes are referred to as the "storage lesions". Oxidative damage is the most important factor causing RBC storage lesion. Free radicals can damage RBC products by lipid and protein oxidation affecting cell quality. The present study is aimed to study the impact of lipid peroxidation and potential role of enzymatic antioxidants in stored blood. **Material and methods:** The present study was observational study carried out in healthy blood donors at KEM hospital, Mumbai. Thirty healthy donors, who were fulfilling the inclusion and exclusion criteria, were enrolled in the study. Estimation of hemoglobin, levels of lipid peroxidation and some enzymatic antioxidants like glutathione peroxidase, catalase and superoxide dismutase activity was carried out in a properly stored blood samples at 4^0C. Enzyme levels estimation was carried out at every 7 days interval. Blood grouping of all the samples was also done to check if there is any change in the levels of lipid peroxidation and antioxidant levels across the groups. **Results:** Malondialdehyde (MDA) is an indirect marker of lipid peroxidation that can modify proteins. Increased MDA levels in the study indicate that lipid peroxidation in red cells has occurred during the preservation period. Throughout storage period, the levels of glutathione peroxidase and catalase declined. Statistically significant negative correlation existed between lipid peroxidation and glutathione peroxidase. Whereas study established positive correlation between lipid peroxidation and superoxide dismutase (SOD). On day 8, day 15 and day 22, lipid peroxidation was found to be positively correlated with SOD. But on Day 30, there was negative correlation between lipid peroxidation and SOD. Blood grouping of all samples indicate no significant susceptibility to lipid peroxidation when the different blood groups were compared. Methemoglobin levels in stored blood were increased over a period of 30 days. **Conclusion:** Red cell storage lesions due to oxidative injury during storage are now the reported fact, confirmed by the findings of the present study. This also indicates that antioxidant enzymatic machinery of the system comes into play adequately to circumvent the damage done. To investigate further therapeutic role of antioxidants in preventing oxidative damage to red cells during storage, large sample studies will be required.

Key words: lipid peroxidation, antioxidant, catalase, glutathione peroxidase, superoxide dismutase, enzymes.

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INTRODUCTION

The history of blood transfusion originated with William Harvey's discovery of blood circulation in 1628.[1] The earliest known blood transfusions occurred in 1665 by Richard Lower[2] and the first human blood transfusion was performed by Dr. Philip Syng Physick in 1795. The first transfusion of human blood for the treatment of hemorrhage was performed by Dr. James Blundell in London in 1818. Technology making the transfusion of allogeneic blood products feasible includes Karl

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Landsteiner's landmark identification of the human blood groups A, B, and O in 1901.[3] Decastello and Sturli added the fourth group, AB, in 1902. Reuben Ottenberg used blood typing and cross-matching for the first time shortly thereafter; he also coined the terms universal donor and universal recipient in 1912. Subsequently, the development of long-term anticoagulants, such as acid-citrate-dextrose, allowed preservation of blood for later use. In 1939-1940, the Rhesus (Rh) blood group system was discovered, leading to the development of minor antigen compatibility testing.[4] In 1971, hepatitis B surface antigen testing heralded the advent of screening to minimize infection transmission complicating allogeneic transfusion. To maintain viability and activity of red cells, blood collected from the donor has to be stored under some physiological conditions until it is transfused to the recipient. The First World War acted as a catalyst for the rapid development of blood banks and transfusion techniques. The first blood bank was established in Leningrad in 1932. For storage of blood, the basic physical and chemical conditions are generally optimized that include temperature, pH, the source of energy for red cells and use of anticoagulant. RBCs are perhaps the most recognizable component of whole blood. The ability to store RBCs and other components for extended periods of time has dramatically expanded the availability and use of transfusion as a life-saving therapy. However, as soon as whole blood is collected from a donor, red blood cells begin to degrade. RBCs experience progressive biochemical and biomechanical changes during storage, collectively called the "storage lesion", that result in compromised physiological functions. Free radicals are highly reactive molecules generated by biochemical redox reactions that occur as a part of a normal cell metabolism and in the course of free radical mediated diseases such as cancer, diabetes mellitus, cardiovascular and renal diseases.[5] Free radicals may cause lipid peroxidation (the level of lipid peroxidation expressed as malondialdehyde) and damage macromolecules and cellular structure of the organism, endothelium and erythrocytes. Plasma Malondialdehyde (MDA) is the breakdown product of the major chain reactions leading to definite oxidation of polyunsaturated fatty acids such as linoleic and linolenic acid and thus serves as a reliable marker of lipid peroxidation. [6,7] Free radicals are eliminated from the body by their interaction with enzymic and non enzymic antioxidants such as uric acid, albumin, vitamin E, C, A, glutathione, glutathione peroxidase, superoxide dismutase and catalase.[5] There are few reports describing the use of stored blood without any alteration in biochemical factors. In a study, Acid-Citrate-Dextrose was used as preservative in stored blood in 1947. [8,9] In other study, Citrate-phosphate-Dextrose and Citrate-phosphate-Dextrose-Adenin were used as blood preservative factor in 1957 and 1960 respectively. In further study, Glucose was added to the final constituent and the CPDA-1 was formed and the useful blood storing time increased to 35 days which with adding of Mannitol to CPDA-1, this time increased to 42 days.[10] Antioxidants are molecules which are exogenous or endogenous. Storage lesions can cause either metabolic or structural changes in the red cell. These antioxidant molecules neutralize the oxidative damage caused by oxidants by their own intra- and extracellular defense mechanisms. The extracellular defense mechanisms include various molecules, such as albumin, bilirubin, transferrin, ceruloplasmin, uric acid, ascorbate, and α-tocopherol. Intracellular free radical-scavenging enzymes provide the primary antioxidant defence mechanism. These enzymes are superoxide dismutase (SOD), glutathione-S-transferase and glutathione peroxidase (GSH-Px), glutathione reductase, catalase, and cytochrome oxidase [11,12]. The present work is an attempt to study the oxidative defects in terms of lipid peroxidation and some antioxidant enzymes which may develop during preservation.

MATERIALS AND METHODS

The present study was carried out in the department of Biochemistry of KEM Hospital after obtaining the sanction of the hospital Ethics committee. For the present study, blood samples were collected from healthy voluntary blood donors. In this study, healthy voluntary blood donors and not the patients with any clinical disorders are involved hence no additional clinical investigations were conducted in these volunteers.

30 healthy donors who were willing to donate their blood were selected based on their fulfilling inclusion and exclusion criteria. Nature, objectives and benefits of the study were explained to each donor. Informed consent was obtained prior to entry into the study.

Major Inclusion Criteria	Major Exclusion Criteria
1. Age between 18 to 50 years	1. Person with any clinical history of diseases and disorders like cough, cold, fever, jaundice, malaria, tuberculosis or any other infections, diabetes, hypertension, asthma and heart disease.
2. Weight more than 50kg	2. Person undergoing any type of medical treatment
3. Blood pressure not higher than 180 mm Hg systolic and 100 mm Hg diastolic	3. Unwilling to sign the consent form
4. Hemoglobin more than 12.5g/dl	4. Pregnancy
5. Interval between donations must be minimum of 10 weeks	
6. No recent illness	

The blood bags were preserved at $4^0C \pm 2^0C$ in citrate phosphate dextrose adenine (CPDA) preservative in cold room of blood bank for thirty days. Aliquots were drawn from the bag on the 1stday, 8th day, 15th day, 22nd day and 30th day of storage. All the under mentioned parameters were studied in each of 30 bags. Each bag was studied five times within a period of 30 days at an interval of 7 days.

Study Plan

Volunteer's first underwent tests confirming negative results for HbsAg, HIV and VDRL. After this confirmation following laboratory investigations were carried out;

a. Hemoglobin estimation
b. Blood grouping
c. Oxidative stress: Study of lipid peroxidation measured by the amount of Malondialdehyde (MDA) produced.
d. Enzymatic antioxidant activity in stored blood
    1. Glutathione peroxidase activity
    2. Catalase activity
    3. Superoxide dismutase activity
e. Methemoglobin levels

All the investigations were carried out with red cell membrane that was isolated by the method of hypnotic lysis of the red cells as suggested by Dodge.

Estimation	Method
Isolation of Red cell membrane	Dodge 's Method[13]
Membrane protein	Lowry's Method [14]
Lipid Peroxidation	Uchiyama and Mihara Method[15]
Glutathione peroxidase	Hafeman's Method[16]
Catalase	Aebi's Method[17]
Superoxide dismutase	Marklund's Method [18]
Methemoglobin	Evelyn and Malloy Method [19]

The present work was undertaken with a view to study oxidative stress and enzymatic oxidants during blood preservation at 4^0C. All the statistical calculations namely mean, standard deviation, students paired and unpaired 't' test and correlation of coefficient were done using SPSS 10.01.

Unit definitions

1. Glutathione peroxidase (GSH P_X): 1 enzyme unit is defined as a decrease in log (GSH) of 0.001/minute after the decrease in log (GSH)/ minute of non-enzymatic reaction was subtracted.
2. Catalase (CAT): 1 Unit of enzyme catalase is that activity which decomposes 50% hydrogen peroxide in 100 seconds at 25^0C.
3. Superoxide dismutase (SOD): 1 unit of SOD is the amount of required to cause 50% inhibition of pyragallol antioxidation per minute.

RESULTS

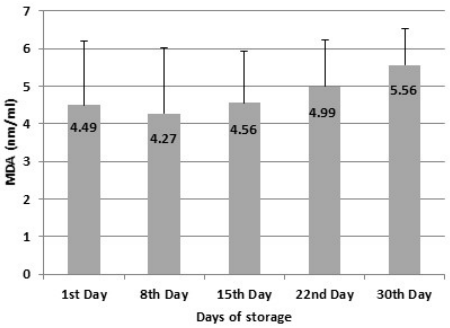
Summary of the biochemical parameters studied at 7 days interval in the subjects is presented below.

Table 1: Extent of Lipid peroxidation occurred during storage

Days of storage	MDA (nm/ml) Mean±SD
1st Day	4.49 ±1.72
8th Day	4.27 ±1.76 NS
15th Day	4.56 ±1.36 NS
22nd Day	4.99 ±1.23*
30th Day	5.56 ±0.96***

Day1vs Day 8- $p > 0.05$ NS; Day1vs Day 15- $p > 0.05$ NS; Day1vs Day 22- $p < 0.05$*, Day1vs Day 30- $p < 0.001$***

Extent of lipid peroxidation measured on all 30 days by the amount of Malondialdehyde (MDA) is given table and depicted in a graph below.



Graph 1: Extent of Lipid peroxidation occurred during storage

Table 2: Values of Lipid peroxidation and antioxidants during storage over a period of 30 days

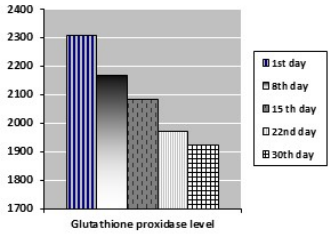
Days	MDA (nm/ml)	Glutathione Peroxidase (IU/protein content)	Catalase (IU/protein content)	Superoxide dismutase (IU/protein content)
1st Day	4.49 ±1.72	2308.46 ± 0.795	0.167 ±0.075	42.33 ±14.06
8th Day	4.27 ±1.76	2169.90 ±0.783***	0.154 ±0.074**	43.67 ±12.73*
15th Day	4.56 ±1.36	2085.39 ±0.650***	0.146 ±0.056***	48.00 ±13.75***
22nd Day	4.99 ±1.23	1970.65 ±0.630***	0.143 ±0.057***	55.33 ±12.52***
30th Day	5.56 ±0.96	1924.00 ±0.508***	0.130 ±0.051*	61.67 ±8.34***

GP: Day1vs Day 8- $p < 0.001$***, Day1vs Day 15- $p < 0.001$***. Day1vs Day 22- $p < 0.001$***, Day1vs Day 30- $p < 0.001$***

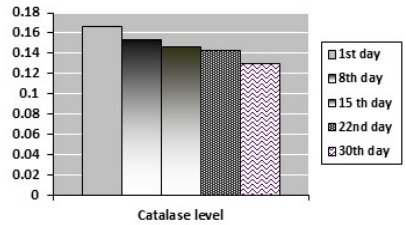
Catalase: Day1vs Day 8- $p < 0.01$**, Day1vs Day 15- $p < 0.001$***. Day1vs Day 22- $p < 0.001$***, Day1vs Day 30- $p < 0.05$*

SOD: Day 1vs Day 8- $p > 0.05$* ; Day 1vs Day 15- $p < 0.001$*** ; Day 1vs Day 22- $p < 0.001$***, Day 1vs Day 30- $p < 0.001$***

From the data obtained in table 2, it is seen that the glutathione peroxidase level decreased from day 1 of storage upto day 30th. The decrease is statistically significant. Also indicates that catalase levels decreased from day 1 to day 30th. The decrease in levels of catalase on day 8th, day 15th, day 22nd and day 30th is statistically significant. Also observed are increasing levels of superoxide dismutase in 30-day measurement. Values of glutathione peroxidase and catalase levels in volunteers over a period of 30 days are shown in graphs below;

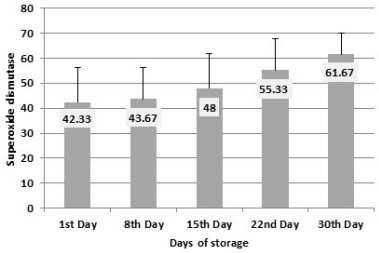


Graph 2



Graph 3

Graph 2: Values of Glutathione peroxidase (IU/ protein content) over a period of 30 days; **Graph 3:** Values of Catalase (IU/ protein content) over a period of 30 days



Graph 4: Values of Superoxide Dismutase (IU/ protein content) over a period of 30 days

Table 3: MDA levels over a period of 30 days Mean±SD in different blood group patients

Days	A (+ve) (n=8)	B (+ve) (n=8)	O (+ve) (n=8)	AB (+ve) (n=8)	B (-ve) (n=8)
1st Day	5.13±1.56	4.12±1.96	3.87±1.95	5.27±0.61	3.39
8th Day	4.78±1.10 NS	3.96±1.69 NS	3.69±2.93 NS	5.09±0.54 NS	3.39 NS
15th Day	4.78±1.105 NS	4.25±1.30 NS	4.58±2.21 NS	5.09±0.54 NS	3.39 NS
22nd Day	5.13±0.95 NS	4.81±1.18 NS	5.00±2.10 NS	5.27±0.53 NS	4.64 NS
30th Day	5.76±0.70 NS	5.49±0.79 NS	5.71±1.71 NS	5.36±0.50 NS	4.64 NS

Values expressed are mean±SD. NS : Not significant (P>0.05)

From the data presented in the table 3, it can be observed that MDA level in stored blood decreased upto 15 days in blood groups of A+ve, B+ve, O+ve and AB+ve, but then there is sudden increase in its level. The decrease as well as increase is statistically non-significant. For B-ve, MDA level was constant upto 15 days after which it increased.

Table 4: Value of Glutathione peroxidase over a period of 30 days in different blood group patients

Days	A (+ve) (n=8)	B (+ve) (n=8)	O (+ve) (n=8)	AB (+ve) (n=8)	B (-ve) (n=8)
1st Day	2.109±0.848	2.285±0.777	2.742±0.654	2.261±0.105	1.739
8th Day	2.015±0.828 NS	2.131±0.736 NS	2.583±0.727 NS	2.102±0.106 NS	1.615 NS
15th Day	1.897±0.650 NS	2.109±0.848 NS	2.404±0.651 NS	2.194±0.864 NS	1.615 NS
22nd Day	1.825±0.693 NS	2.109±0.848 NS	2.255±0.601 NS	1.982±0.766 NS	1.551 NS
30th Day	1.800±0.526 NS	2.109±0.848 NS	2.196±0.463 NS	1.886±0.648 NS	1.551 NS

Values expressed are mean±SD. NS : Not significant (P>0.05)

It is evident from the above table that there is statistically non-significant decrease in glutathione peroxidase over a period of 30 days in blood groups of A+ve, B+ve, O+ve, AB+ve and B-ve.

Table 5: Value of Catalase over a period of 30 days in different blood group patients

Days	A (+ve) (n=8)	B (+ve) (n=8)	O (+ve) (n=8)	AB (+ve) (n=8)	B (-ve) (n=8)
1st Day	0.155±0.046	0.159±0.066	0.208±0.133	0.145±0.035	0.186
8th Day	0.144±0.048 NS	0.145±0.055 NS	0.190±0.140 NS	0.142±0.033 NS	0.177 NS
15th Day	0.143±0.047 NS	0.144±0.058 NS	0.156±0.088 NS	0.138±0.027 NS	0.174 NS
22nd Day	0.136±0.039 NS	0.141±0.063 NS	0.157±0.088 NS	0.134±0.027 NS	0.163 NS
30th Day	0.137±0.037 NS	0.137±0.063 NS	0.104±0.059 NS	0.129±0.026 NS	0.165 NS

Values expressed are mean±SD. NS: Not significant (P>0.05)

Above table 5 indicates that catalase levels decreased in blood groups of A+ve, B+ve, O+ve, AB+ve and B-ve over a period of 30 days which is statistically non-significant decrease.

Table 6: Comparative Values of MDA and Methemoglobin

Days	MDA (nm/ml)	Methemoglobin (%)
1st Day	4.49 ±1.72	1.49±0.94
8th Day	4.27 ±1.76	1.55±0.99***
15th Day	4.56 ±1.36	1.60±0.100***
22nd Day	4.99 ±1.23	1.72±0.94***
30th Day	5.56 ±0.96	2.02±0.038***

Day 1vs Day 8- $p < 0.01$* ; Day 1vs Day 15- $p < 0.001$*** ; Day 1vs Day 22- $p < 0.001$***, Day 1vs Day 30- $p < 0.001$***

Based on the data presented in Table 6, it can be observed that Methemoglobin levels in stored blood were increased over a period of 30 days. The increase on day 8th, day 22nd and day 30th is statistically significant.

Table 7: Correlation coefficients between SOD and Methemoglobin

Day	Superoxide dismutase (IU/mg PRC)	Methemoglobin (%)
	0.324	0.224
	(P= 0.081)	(P= 0.233)
15	0.097	0.117
	(P= 0.611)	(P= 0.537)
22	0.020	0.154

	(P= 0. 919)	(P= 0.415)
30	- 0.013	- 0.146
	(P= 0.947)	(P= 0.442)
	0.204	0.291
	(P= 0.279)	(P= 0.119)
ALL	0.0282	0.142
	(P= 0.057)	(P= 0.440)

*Correlation Significant at the 0.05 level (2-tailed)

Table 7 above summarizes correlation coefficient between lipid peroxidation and antioxidant – SOD over a period of 30 days.

DISCUSSION

In comparison to various factors, oxidative stress remains to be a major factor affecting RBCs. Even though risk of oxidative damage among RBCs is high but their antioxidant system is also more sensitive and powerful than other cells [20]. A balance between RBCs' antioxidant enzymes and free radicals[21, 22] is observed in normal physiological conditions but when erythrocytes are against oxidative stress, such as being in blood storage for a long period, RBCs' antioxidant enzymes cannot protect erythrocytes against oxidative damage by free radicals. RBC storage lesion can occur due to oxidative damage, with a negative effect on RBC quality during storage. Lipid peroxidation, the oxidative deterioration of polyunsaturated fatty acids, is a common mechanism of cell injury and death. Although, earlier studies failed to detect MDA, a marker of lipid peroxidation in stored red cells, more recent investigations using sensitive techniques have found increased levels of this marker during red cell storage.[23,24] Knight *et al.*[25] observed a progressive and significant increase in mean MDA levels over 28 days of storage. The mean MDA on day 0 was 2.25 mmol/mg of protein and increased to 3.76 mmol/mg of protein on day 28 ($p<0.005$). Our study results are in agreement with earlier published data.[26,27] Our study results indicate that there is an overall rise in the lipid peroxidation level throughout the storage period which suggest that the red cells yield to oxidative damage during preservation. Gaetani *et al.*[28] published study results claiming catalase and glutathione peroxidase are equally active in detoxification of hydrogen peroxide in human erythrocytes. Present study showed an increase in SOD levels over the period of preservation. Comparative analysis of MDA and SOD levels indicate gradual increase of both over the storage period of 30 days. SOD also showed positive correlation with lipid peroxidation, correlation coefficient being 0.22 (p=0,057). Webster *et al.*[29] found that SOD activity appeared to reduce with storage beyond 10 days. In this study, data indicates that throughout storage period, the levels of glutathione peroxidase and catalase declined but increase observed in SOD levels. Statistically significant negative correlation existed between lipid peroxidation and glutathione peroxidase. This mix observation in enzymes levels itself explains that the antioxidants produced are unable to completely protect the red cells from oxidative damage. Blood grouping of all the samples was done to check if there is any change in the levels of lipid peroxidation and antioxidant levels amongst the groups. The study shows that there was no significant susceptibility to lipid peroxidation when the different blood groups were compared, although some changes were marginally evident in O+ ve group.

CONCLUSION

In the present study, slight increase in MDA level over a period of storage indicates lipid peroxidation of the red cell membrane during preservation. Antioxidant enzymes like glutathione peroxidase, catalase and SOD are active throughout the preservation period which acts as safeguards. There was decrease in glutathione peroxidase and catalase levels and statistically significant increase in SOD levels during storage period indicating that correlation existed between lipid peroxidation and the enzymes. Red cell storage lesions due to oxidative injury during storage are now a well established fact, confirmed by the findings of the present study. Further studies are required to investigate the therapeutic role of antioxidants, either given to the donor before donation or added to the blood bag after red cell separation, in preventing oxidative damage to red cells during storage.

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