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Comparison of the protective nature of antioxidants on stored erythrocytes

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ABSTRACT

Erythrocytes undergo oxidative damage during storage which overwhelms their antioxidant defense system. The employment of antioxidants as additives during storage could protect erythrocytes against storage induced oxidative stress. This study was designed to evaluate the effects of antioxidants (Vitamin C, L-carnitine and Curcumin) on osmotic stress in erythrocytes of stored blood.

Blood was obtained from male *Wistar* rats (4 months old) during the year 2015 and stored for up to 25 days at 4 ºC in CPDA-1 solution. Samples were divided into 10 groups (n=5) with and without antioxidants (Vitamin C, L-carnitine and Curcumin) at concentrations of 10mM, 30mM and 60mM. Erythrocytes were isolated from stored blood every fifth day and the effects of storage on osmotic fragility and membrane sulfhydryls were studied.

Osmotic fragility varied significantly in controls. Osmotic fragility increased in 0.3% and 0.4% BSS during storage in VC 10, VC 30, all LC groups, Cur 10 and Cur 60. Osmotic fragility levels were similar in Cur 30. Sulfhydryls were insignificant in VC groups, while it incremented in LC 30 against controls. Cur groups showed decreases in sulfhydryls.

L-carnitine at 30mM decreased osmotic stress at 0.4% and increased sulfhydryls. The addition of L-carnitine showed maximum storage of 20 days and is a good candidate for further studies on erythrocytes during blood storage.

KEY WORDS: Erythrocytes; Osmotic Fragility; Storage; Antioxidants.

INTRODUCTION

Erythrocytes are a major cellular component of blood, functioning as the transporter of oxygen and carbon dioxide throughout the body. In order to efficiently transport respiratory gases, the erythrocytes must be deformable but must also resist fragmentation [1]. The red cell membrane is highly deformable yet stable, and alteration of this property has been implicated in red cell destruction [2]. The erythrocyte membrane consists of a lipid bilayer, integral proteins, and a skeletal protein network of spectrin, actin, ankyrin, tropomyosin, and proteins 4.1 and 4.9 [1]. The membrane skeleton maintains the shape, the mechanical properties of the erythrocyte and is responsible for the rheological properties of normal erythrocytes such as deformability and mechanical resistance to shear stress [3]. Direct interactions between some proteins of the skeleton and lipid bilayer provide additional stability [4].

Erythrocytes are highly susceptible to oxidative stress (OS) situations and the presence of polyunsaturated membrane lipids and iron, a potent catalyst for free radical reactions makes the erythrocyte a good substrate for oxidative damages. Membrane oxidations by reactive oxygen species (ROS) modify the intrinsic membrane properties, by altering membrane fluidity, ion transport and loss of enzymatic activities of the cell [5], and thereby trigger membrane disruption [6]. The effects of OS on erythrocytes during storage is characterized by morphological, physiological, biochemical, metabolic and biomechanical changes, collectively known as the "storage lesion" [7-10]. The extensive oxidative damage during storage causes alterations in the membrane and variations in membrane stability and deformability, which in turn lead to shape change and loss of function of the erythrocytes. Oxidative modifications of the erythrocyte membranes contribute to increased fragility of red blood cells [11].

The osmotic fragility test is most frequently used to evaluate the response of erythrocytes to osmotic pressure [12] and determines their resistance to hypotonicity and hemolysis [13]. Osmotic fragility is affected by extrinsic factors such as pH, temperature, osmolality and blood storage [14-17] and intrinsic factors such as (i) presence of osmotically active constituents (ii) modifications of the lipids and proteins in the membrane [18]. The osmotic fragility provides a guide to assess the membrane integrity [19, 20] and can echo the changes in membrane properties in the erythrocytes [21]. The erythrocyte membrane contributes significantly to the effective functioning of the erythrocyte. There exists an inverse correlation between the sulfhydryl concentrations of the erythrocyte membrane and osmotic fragility [22]. The sulfhydryl groups maintain the redox balance of the membrane and provide mechanical strength. Oxidation of these sulfhydryl groups may form the basis for increased osmotic fragility [23].

The erythrocytes possess an effective antioxidant defense mechanism, which is impaired by prolonged storage [24]. The cytosolic antioxidant system includes enzymatic (superoxide dismutase, catalase, and glutathione peroxidase) and non-enzymatic antioxidants (ascorbic acid, vitamin E, and glutathione), while the membrane mainly comprises of vitamin E as the major, lipid-soluble chain-breaking antioxidant [24]. Studies on the effect of antioxidants such as Vitamin C, Vitamin E, L-carnitine,

β-carotene, α-lipoic acid and melatonin, on osmotic fragility have focused on supplementation [25-36]. Studies on storage and osmotic fragility have shown that osmotic fragility increases with prolonged storage [8, 37-40] and varies with different additive solutions [41, 42]. There are very few studies on the effects of antioxidants on osmotic fragility of erythrocytes of stored blood. This study was designed to evaluate the effects of antioxidants (Vitamin C, L-carnitine and Curcumin) on osmotic stress during storage. The concentrations of the antioxidants were based on the studies on supplementation of the antioxidants. This study aims to provide insight into the role of osmotic stress during storage in erythrocytes and the possible effects of antioxidants in reducing oxidative stress.

MATERIALS AND METHODS

Animal care and maintenance was in accordance with the ethical committee regulations (841/b/04/CPCSEA). The work was carried out in the year 2015.

Chemicals

Curcumin and 5, 5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were purchased from Sigma-Aldrich Chemicals [St. Louis, MO, USA]. L-ascorbic acid (vitamin C), L-carnitine, bovine serum albumin (BSA) were purchased from Himedia Laboratories [Mumbai, India]. All chemicals used were of reagent grade and organic solvents were of spectral grade.

Blood Sampling

Animals were lightly anaesthetized with ether and restrained in dorsal recumbancy as described earlier [10]. In brief, the syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. 4-5 ml of blood was carefully aspirated from the heart into plastic collecting tubes with citrate-phosphate-dextrose-adenine-1 (CPDA-1) solution (2.63 g trisodium citrate, 327 mg citric acid, 222 mg sodium dihydrogen phosphate, 3.18 g dextrose, 27.5 mg adenine in 100 ml of distilled water).

Experimental design

Blood was drawn from male *Wistar* rats (4 months old) and stored over a period of time (25 days) at 4 ºC in CPDA-1 solution. Blood samples were divided into 10 groups (n=5) (Table 1). Erythrocytes were isolated from the stored blood at regular intervals (every fifth day) and the biomarkers of OS were studied.

Erythrocyte separation

The erythrocytes were isolated by centrifugation for 20 min at 3,500 rpm at 4 ºC. The plasma and buffy coat were removed using a micropipette. The cell pellet was washed three times and suspended in an equal volume of isotonic phosphate buffer [43]. This constituted the erythrocyte suspension.

Osmotic fragility

Osmotic fragility was determined by the method of O'Dell *et al* [44]. 10 μl of washed erythrocytes were each added to 0.3%, 0.4% and 0.9% buffered salt solution (BSS). Tubes were allowed to stand for 30 minutes, centrifuged at 2,000 rpm for ten minutes to pellet the cells and the absorbance of the supernatant was measured at 540 nm. Hemolysis in each tube was expressed as a percentage taking 100% as the maximum value of absorbance for hemolysis in distilled water. Buffered salt (0.9%) solution was used as a control sample (osmotic control).

Erythrocyte Membrane Separation

Hemolysate was prepared from the washed erythrocyte suspension by diluting 1:14 times with hypotonic buffer (20 mosm, pH 7.4) in a tube. The contents were mixed by gentle swirling and then centrifuged at 20,000 rpm for 40 min at 4ºC. The supernatant was decanted carefully and the ghost (membrane) was resuspended by adding the same strength buffer to reconstitute the original volume. Ghosts were washed three times subsequent to hemolysis. The membrane pellet was resuspended in an isotonic buffer for the assessment of protein sulfhydryls [43].

Table 1. Experimental Design

Groups	Controls (n=5) (Blood stored in CPDA-1)	Experimentals (n=5 in each group) (Blood stored in CPDA-1+ Antioxidants)		
Antioxidant	-------	Vitamin C	L-carnitine	Curcumin
Concentration (in mM)	-------	10 (VC 10) 30 (VC 30) 60 (VC 60)	10 (LC 10) 30 (LC 30) 60 (LC 60)	10 (Cur 10) 30 (Cur 30) 60 (Cur 60)
Storage Period (Days)	25	25*	25#	25$

CPDA-1: citrate, phosphate, dextrose and adenine; VC 10- Vitamin C as an additive at the concentration of 10mM; VC 30- Vitamin C as an additive at the concentration of 30mM; VC 60- Vitamin C as an additive at the concentration of 60mM; LC 10- L-carnitine as an additive at the concentration of 10mM; LC 30- L-carnitine as an additive at the concentration of 30mM; LC 60- L-carnitine as an additive at the concentration of 60mM; Cur 10- Curcumin as an additive at the concentration of 10mM; Cur 30- Curcumin as an additive at the concentration of 30mM; Cur 60- Curcumin as an additive at the concentration of 60mM.
*- VC 60 samples could be stored till day 15
- LC 30 and LC 60 samples could be stored till day 20
$ - Cur 30 and Cur 60 samples could be stored till day 15

Protein Sulfhydryls (P-SH)

The concentration of P-SH groups in the proteins of the membrane was measured as described by Habeeb [45]. In brief, 0.08 mol l^{-1} sodium phosphate buffer containing 0.5 mg ml^{-1} of Na_2-EDTA, and 2% SDS were added to each assay tube. 0.1 ml of 5, 5'-DTNB was also added. Absorbance was measured at 412 nm. P-SH was calculated from the net absorbance and molar absorptivity, 13,600 $M^{-1}l^{-1}cm^{-1}$.

Protein determination

Protein was determined in the membrane by the method of Lowry *et al* [46] using bovine serum albumin as the standard.

Statistical Analyses

Results are represented as mean ± SE. Values between the groups were analysed by two-way ANOVA and was considered significant at $P < 0.05$. Bonferroni's *Post* Test was performed for all the assays using GraphPad Prism 5 software. The statistical analyses was carried out in two stages for osmotic fragility: (i) Analysis of the changes occurring in each group with storage [controls (without antioxidants) and experimentals (with antioxidants)] (ii) Analysis of the changes occurring in 0.3% and 0.4% buffered saline with storage.

RESULTS

Osmotic Fragility

Changes in osmotic fragility in controls were significant with storage. Fragility increased by 15 fold in 0.3% BSS and 13 fold in 0.4% BSS against 0.9% BSS (osmotic control) on day 0.

A. Vitamin C

[1] Storage: Changes in VC 10 and VC 30 were significant with storage whereas osmotic fragility in VC 60 remained insignificant with storage (Figure 1).

VC 10: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS decreased against osmotic control on day 0 (60%), while it increased on days 10 (2 fold), 15 (190%), 20 (170%) and 25 (70%). At 0.4% BSS, fragility decreased on day 0 by 60% and increased on day 20 by 120% against 0.9% BSS.

On day 10, fragility at 0.3% BSS was higher over 0.4% BSS (90%).

VC 30: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS decreased on day 0 (30%) and increased on days 10 (100%), 15 (450%), 20 (135%) and 25 (175%) against osmotic control. Fragility

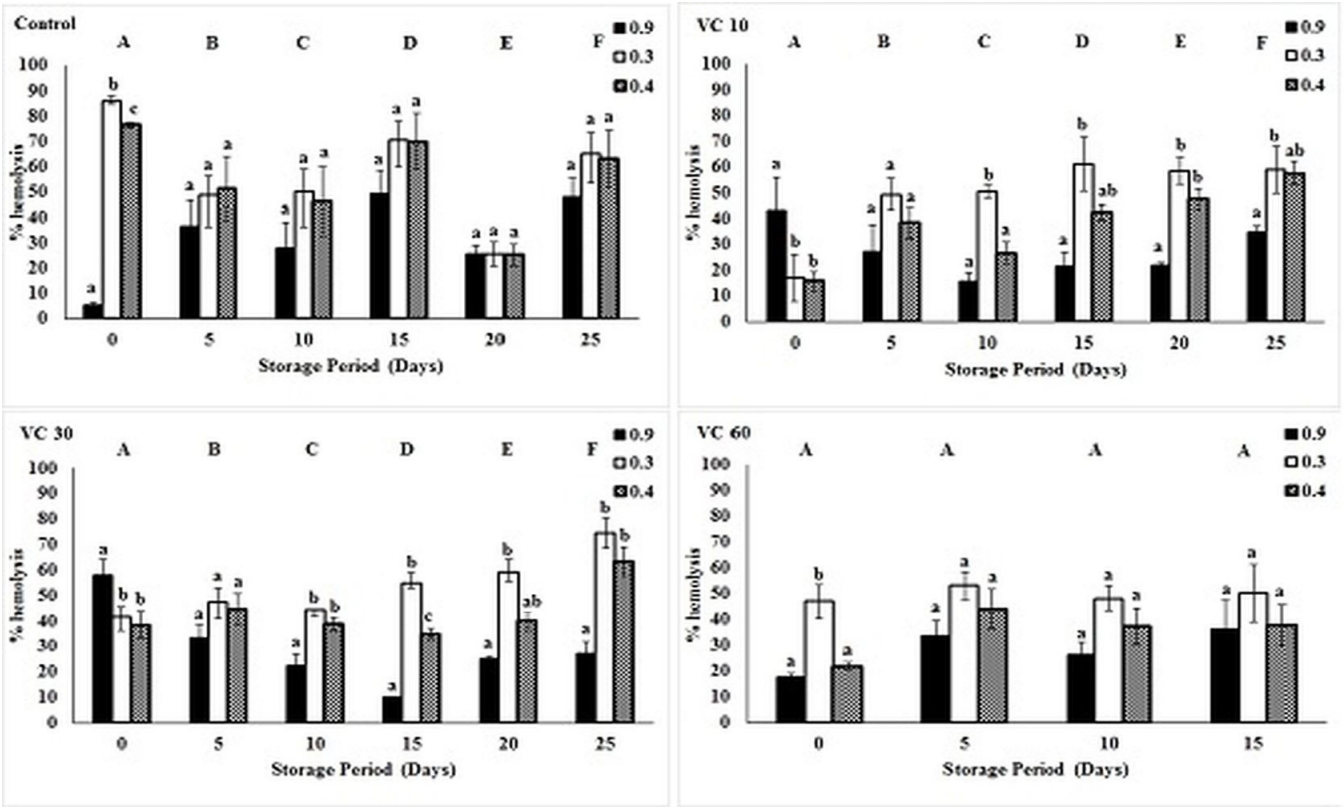

Figure 1. Osmotic Fragility in Erythrocytes of Stored Blood with Vitamin C as Additive
Values are expressed as Mean ± SE from 5 samples. VC 10 – Vitamin C (10mM); VC 30 – Vitamin C (30mM); VC 60 – Vitamin C (60mM); 0.3 - 0.3% buffered salt solution; 0.4 - 0.4% buffered salt solution; 0.9 - 0.9% buffered salt solution (osmotic control). Changes were analyzed by two-way ANOVA followed by Bonferroni's *Post* Test using Graphpad Prism software. *P*<0.05 was considered significant. Values between groups are represented in upper case, while values within a group are represented in lower case. Those not sharing the same letters are significant.

at 0.4% BSS reduced by 35% on day 0, while it elevated over 0.9% BSS on days 10 (75%), 15 (250%) and 25 (135%). Fragility at 0.3% BSS incremented over 0.4% BSS on day 15 (60%).

B. L-carnitine

[1] Storage: Changes in all concentrations of L-carnitine were significant with storage (Figure 2).

LC 10: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS increased over osmotic control on days 5 (175%), 10 (115%), 15 (300%), 20 (185%) and 25 (130%). Changes in fragility at 0.4% BSS were significantly higher than 0.9% BSS on days 5 (100%), 15 (140%) and 25 (80%). Fragility at 0.3% BSS increased on days 10 (100%), 15 and 20 (60%) when compared to fragility at 0.4% BSS (days 10-20).

LC 30: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS was higher than controls on all days (130%, 240%, 210%, 150% and 45% respectively). Fragility at 0.4% BSS increased over osmotic controls on days 0 (57%), 5 (125%), 10 (140%) and 15 (85%). At 0.3% BSS, fragility was higher than 0.4% BSS on all days (47%, 53%, 32%, 36% and 20% respectively).

LC 60: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. The fragility at 0.3% BSS elevated over osmotic controls on all days (110%, 270%, 150%, 80% and 35% respectively). Fragility at 0.4% BSS was significantly higher against 0.9% BSS on days 5 (175%), 10 (85%) and 15 (50%). The fragility at 0.3% BSS elevated over 0.4% BSS on days 0 (50%), 5 and 10 (35%).

[2] 0.3% BSS: Changes between the antioxidant concentrations were significant with storage.

L-carnitine at higher concentrations (LC 30 and LC 60) showed increases in osmotic fragility over the control group and LC 10 on days 5, 10, 15 and 20.

[3] 0.4% BSS: Changes between concentrations of antioxidants were significant with storage.

LC 10 and LC 30 showed decreases in osmotic fragility against the control group up to day 20 of storage, while LC 60 showed elevations in OF from day 10 onwards

C. Curcumin

[1] Storage: Changes in all concentrations were significant with storage (Figure 3).

Cur 10: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS decreased by 45% (day 0) and increased by 130% (day 20) and 75% (day 25) against osmotic controls. Changes in fragility in 0.3% BSS increased by 60% on day 20 against 0.4% BSS while fragility at 0.4% BSS increased significantly against 0.9% BSS on day 25 (60%).

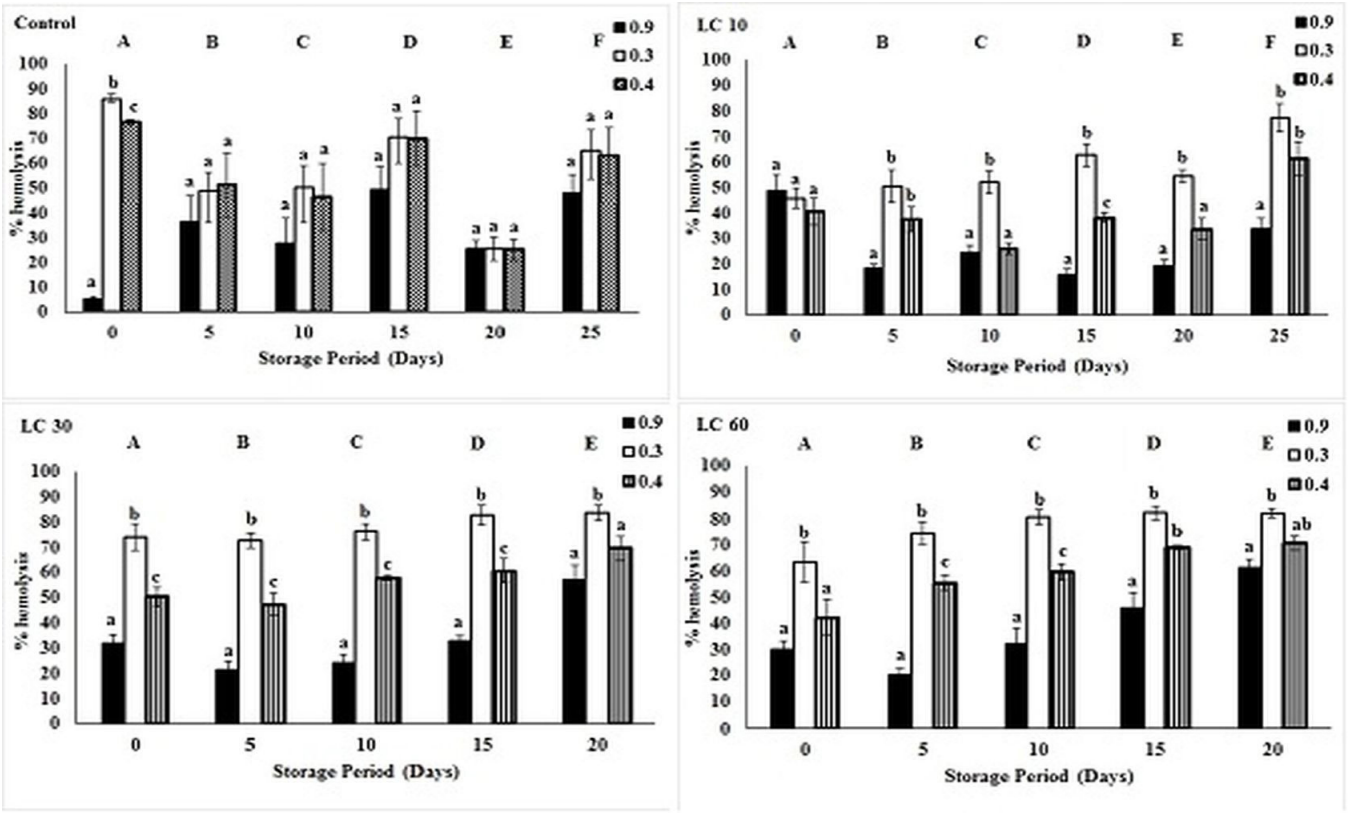

Figure 2. Osmotic Fragility in Erythrocytes of Stored Blood with L-carnitine as Additive
Values are expressed as Mean ± SE from 5 samples. LC 10 – L-carnitine (10mM); LC 30 – L-carnitine (30mM); LC 60 – L-carnitine (60mM); 0.3 - 0.3% buffered salt solution; 0.4 - 0.4% buffered salt solution; 0.9 - 0.9% buffered salt solution (osmotic control). Changes were analyzed by two-way ANOVA followed by Bonferroni's *Post* Test using Graphpad Prism software. $P<0.05$ was considered significant. Values between groups are represented in upper case, while values within a group are represented in lower case. Those not sharing the same letters are significant.

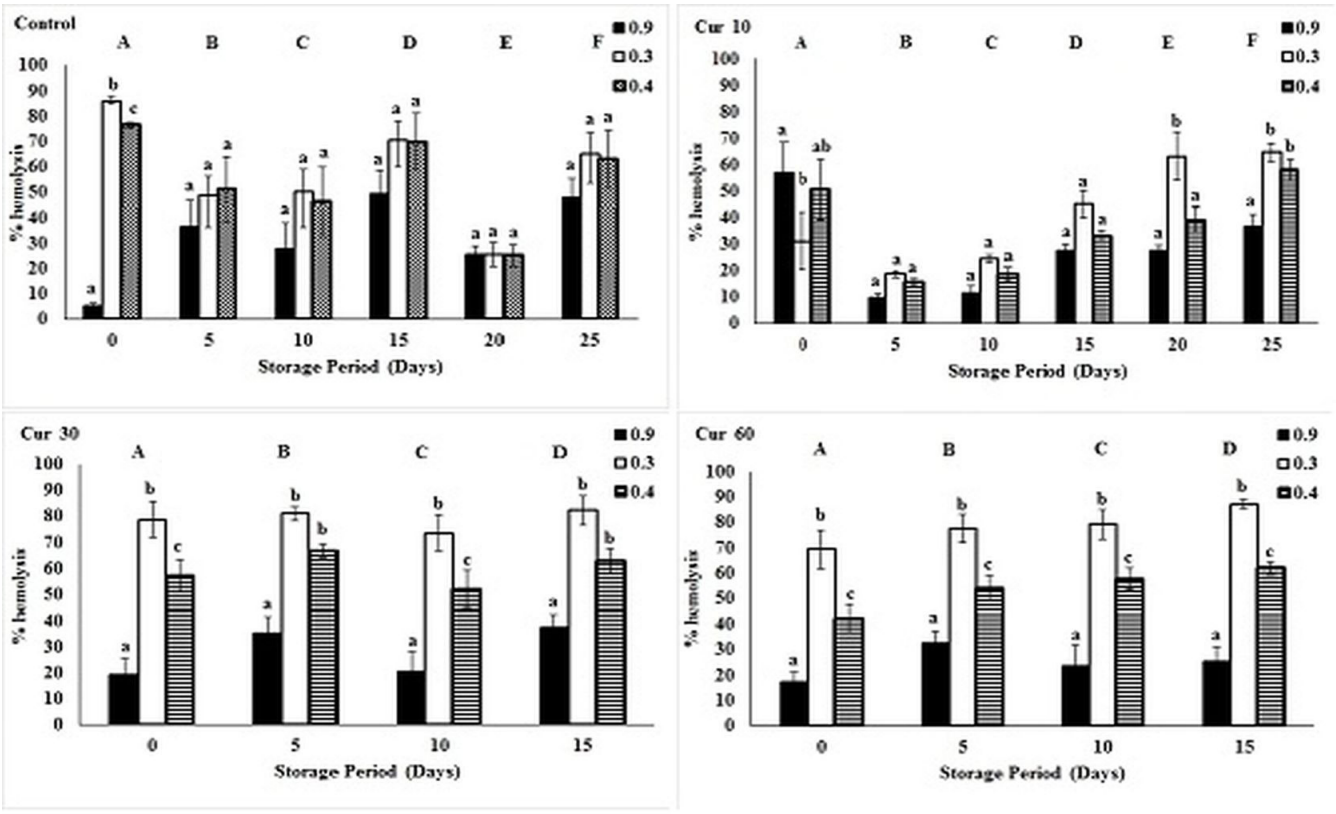

Figure 3. Osmotic Fragility in Erythrocytes of Stored Blood with Curcumin as Additive
Values are expressed as Mean ± SE from 5 samples. Cur 10 – Curcumin (10mM); Cur 30 – Curcumin (30mM); Cur 60 – Curcumin (60mM); 0.3 - 0.3% buffered salt solution; 0.4 - 0.4% buffered salt solution; 0.9 - 0.9% buffered salt solution (osmotic control). Changes were analyzed by two-way ANOVA followed by Bonferroni's *Post* Test using Graphpad Prism software. $P<0.05$ was considered significant. Values between groups are represented in upper case, while values within a group are represented in lower case. Those not sharing the same letters are significant.

Cur 30: Osmotic fragility remained similar in 0.3% BSS and 0.4% BSS during storage. Fragility at 0.3% BSS increased against osmotic control during 15 days of storage (310%, 130%, 260% and 120% respectively). Similarly, elevations of 200%, 88%, 150% and 70% were observed in 0.4% BSS against 0.9% BSS on all days (till 15). Fragility at 0.3% BSS was significant higher against 0.4% BSS on days 0 and 10 (40%).

Cur 60: Osmotic fragility increased in 0.3% BSS and 0.4% BSS during storage. During storage of 15 days, fragility at 0.3% BSS (300%, 140%, 230% and 240%) and 0.4% BSS (65% - day 5 and 140% - days 0, 10 and 15) increased over osmotic control. Osmotic fragility at 0.3% BSS elevated over 0.4% BSS throughout storage (65% - day 0; 40% - days 5, 10 and 15).

[2] 0.3% and 0.4% BSS: Significant changes were observed in all concentrations with storage.

Cur 10 showed decreases in osmotic fragility with storage than control group, while Cur 30 and Cur 60 showed elevations over controls and Cur 10.

Protein Sulfhydryls (Membrane) (Table 2):

A. Vitamin C: Changes in SH were insignificant with storage.

B. L-Carnitine: Changes in SH membrane were significant with storage. In controls, SH decreased by 68% (days 5 and 15), 25% (day 10) and 46% (day 20). SH decreased by 53%, 70% and 45% on days 5, 10 and 15 respectively, while it increased by 75% on day 20 in LC 10. LC 30 showed an increase of 4 fold on day 20, while LC 60 showed increases of 42% (day 15) and 130% (day 20).

SH incremented by 2 fold on day 20 in LC 30 against controls.

C. Curcumin: Variations in SH membrane were significant with storage in all groups. In controls, SH decreased by 68% (days 5 and 15) and 25% (day 10). Cur 10 showed decreases of 86% (day 5) and 97% (days 10 and 15) while Cur 60 showed an increase of 30% (day 10) followed by a decrease of 25% on day 15.

On day 0, Cur 30 and Cur 60 showed significant reductions in SH levels by 85% against controls and Cur 10.

DISCUSSION

Storage of blood and its components is characterized by the formation of storage lesions. The inherent antioxidant system of the erythrocytes cannot protect them against the oxidative insult efficiently and hence this study was designed to determine the effects of antioxidants as additives during storage.

Table 2. Protein Sulfhydryls in Erythrocytes of Stored Blood

Days	Control	LC 10	LC 30	LC 60
0[A]	691.17 ± 135.58	528.08 ± 263.17	233.16 ± 92.27	497.27 ± 274.08
5[B]	224.85 ± 78.93	250.76 ± 127.36	207.24 ± 85.49	473.07 ± 155.55
10[C]	514.66 ± 216.09	154.90 ± 36.78	275.19 ± 176.91	580.69 ± 393.34
15[D]	212.13 ± 48.36	290.61 ± 86.39	207.51 ± 38.05	709.03 ± 334.40
20[E]	370.66 ± 77.84	924.86 ± 435.18	1278.92 ± 129.46[*]	1160.56 ± 308.62
25[F]	405.49 ± 76.07	1275.71 ± 652.40	------	-----
	Control	**Cur 10**	**Cur 30**	**Cur 60**
0[A]	691.17 ± 135.58	1118.84 ± 488.78	101.21 ± 19.40[#]	122.54 ± 43.21[#]
5[B]	224.85 ± 78.93	156.01 ± 40.49	81.45 ± 27.91	110.08 ± 13.64
10[C]	514.66 ± 216.09	26.82 ± 9.19	114.02 ± 47.95	159.48 ± 59.86
15[D]	212.13 ± 48.36	37.35 ± 4.27	111.99 ± 45.22	91.14 ± 21.98
20[E]	370.66 ± 77.84	46.58 ± 15.83	-----	-----
25[F]	405.49 ± 76.07	57.91 ± 9.36	-----	-----

Values are expressed as Mean ± SE from 5 samples. VC 10 – Vitamin C (10mM); VC 30 – Vitamin C (30mM); VC 6 – Vitamin C (60mM); LC 10 – L-carnitine (10mM); LC 30 – L-carnitine (30mM); LC 60 – L-carnitine (60mM); Cur 10 – Curcumin (10mM); Cur 30 – Curcumin (30mM); Cur 60 – Curcumin (60mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's *Post* Test using Graphpad Prism software. $P<0.05$ was considered significant. Values between groups are represented in upper case, while values within a group are represented in lower case. Those not sharing the same letters are significant.
* - LC 30 was significant against controls on day 20.
# - Cur 30 and Cur 60 were significant against control and Cur 10 on day 0.

The addition of vitamin C to whole blood as an additive and the assessment of osmotic fragility and membrane sulfhydryls in the erythrocytes revealed that vitamin C could not successfully protect the erythrocytes from oxidative stress. In our study, the addition of vitamin C at concentrations of 10mM and 30mM increased osmotic fragility. Vitamin C has been shown to be a potent antioxidant which assists vitamin E in maintaining the properties of the erythrocyte membrane [47, 48]. Vitamin C regenerates vitamin E by directly reducing tocopheroxyl radicals generated when vitamin E scavenges reactive free radicals [47, 48]. The results in our study can be explained by the unavailability of the reduced vitamin E. The overall effects of vitamin C on the erythrocytes in our current and previous study [49] showed that vitamin C could not decrease the oxidative damage and was contradictory to previous studies which focused both on supplementation [28, 50-54] and storage [55-57].

The effects of L-carnitine on erythrocytes has been studied [58-61]. L-carnitine as an additive in blood storage and the subsequent analysis of the osmotic fragility and sulfhydryls was indicative that the antioxidant increased osmotic fragility and maintained sulfhydryls. The antioxidant enzymes were modulated, reactive species were scavenged and hemoglobin and sulfhydryls (in lysate) were maintained by L-carnitine. However, the utilization of L-carnitine as an additive did not show complete protection from oxidative damage [61]. L-carnitine at 30mM concentration was successful in protecting the erythrocytes from osmotic stress of 0.4% and in elevating membrane sulfhydryls. It has been shown that during situations of oxidative stress, in erythrocytes, the plasma membrane is usually the primary site of damage. The membrane is rich in polyunsaturated fatty acids [62] and proteins, which upon peroxidation and oxidation respectively, cause hemolysis [48].

Curcumin is a potent antioxidant and its effects have been explored in plasma and erythrocytes of whole blood storage [63, 64]. The results of osmotic fragility indicate that curcumin as an additive could not successfully protect all membrane proteins and lipids from osmotic stress, as observed in our results of osmotic fragility. The sulfhydryl levels in membrane decreased in curcumin groups whereas sulfhydryls in lysate and plasma were successfully maintained by curcumin [63, 64]. However, the products of lipid peroxidation and protein oxidation were not completely protected from OS as observed in our results of malondialdehyde and hemolysis [64].

The addition of antioxidants to the storage solutions elucidated that the different concentrations influenced the storage period. Vitamin C samples at 60mM concentrations could not be stored beyond day 15, while L-carnitine samples at concentrations of 30mM and 60mM had a maximum storage period of 20 days. Curcumin groups at concentrations 30mM and 60mM lasted only till day 15. The erythrocytes (stored in whole blood) with L-carnitine as an additive had a maximum shelf life of 20 days. L-carnitine at 30mM decreased the effects of osmotic stress at 0.4% and increased sulfhydryls.

L-carnitine is a good candidate for further studies on erythrocytes during blood storage as proved by this study and our earlier studies [61]. The variations between the storage of rat and human erythrocytes have shown that the storage lesion formed during 1 week of rat storage is

equivalent to that of storage of human erythrocytes for 4 weeks [65]. Hence, this study forms a basis for tracking the changes in erythrocytes during storage and assists in the correlation of the storage lesion of animal and human models. Further examination of antioxidants as additives in blood storage would provide the basis for better management of blood storage.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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