

Effects of prior antioxidant supplementation on quality of stored erythrocytes: vitamin C + vitamin E and vitamin C + N-acetylcysteine

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Abstract

Background: The antioxidant status of donors may impact the quality of stored blood and its components. Therefore, oral antioxidant supplementation prior to blood donation could be a promising approach to improve the efficacy of stored erythrocytes. **Materials and methods:** Wistar rats (3 months old, n = 5/group) were categorized into controls, antioxidant group 1 (vitamin C and vitamin E, VC + VE group), antioxidant group 2 (vitamin C and N-acetylcysteine, VC + NAC group). Experimental rats were supplemented for 30 days. Erythrocytes were stored in additive solution (AS-7) for 42 days and analyzed at two-week intervals. **Results:** Hemoglobin increased on days 28 and 42 in both the antioxidant groups. Superoxide dismutase and catalase activities elevated during initial days and, glutathione peroxidase activity towards the end of storage in the antioxidant groups. Protein carbonyls and malondialdehyde levels decreased in the antioxidant groups. Glutathione levels increased on day 42 in VC + VE. Hemolysis was lower in the VC + VE group until day 28 and was similar in the VC + NAC group. **Conclusion:** VC + VE and VC + NAC supplements protected hemoglobin, blood proteins and lipids from oxidation and elevated antioxidant defenses. Therefore, prior supplementation augments the antioxidant status and quality of stored blood.

Keywords: erythrocytes · vitamin C · vitamin E · N-acetylcysteine · supplementation

Citation

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Abbreviations

- AS-7 – additive solution-7
- OS – oxidative Stress
- PS – phosphatidyl Serine
- VC – vitamin C
- VE – vitamin E
- NAC – N-acetylcysteine
- MetHb – Methemoglobin
- GSH – reduced glutathione
- GSSG – oxidized glutathione
- ROS – reactive oxygen species
- NADH – nicotinamide adenine dinucleotide
- NADPH – nicotinamide adenine dinucleotide phosphate
- H₂O₂ – hydrogen peroxide

Introduction

Erythrocytes experience physiological and biochemical modifications under blood bank conditions referred to as “storage lesions”. Oxidative stress (OS) leads to a decline in antioxidant defenses, oxidation of proteins and lipids, and phosphatidyl serine (PS) externalization [1-3].

Attenuation of oxidative stress could be a promising strategy to diminish storage lesions. Therefore, blood banking research continues to focus on enriching the antioxidant defenses to combat oxidative stress. It has been hypothesized, that enhancing the nutritional status of a donor prior to blood donation could improve the viability and efficacy of stored erythrocytes [4]. Therefore, the primary objective of this study was to explore, whether the antioxidant status of donors influences the quality of stored blood.

There are limited studies on the supplementation of donors with antioxidants. Supplementation of vitamin C and vitamin E resulted in a decrease in plasma malondialdehyde and hemolysis [5]. Nonetheless, the impact of these antioxidant supplements on the OS profile, antioxidant defenses and metabolism of stored erythrocytes has not been explored. The supplementation of β -carotene, vitamin E, vitamin C and selenium decreased the release of hemoglobin and potassium and protected antioxidant enzymes [6].

Animal models offer a platform to mitigate the effects of donor variations, such as age, ethnicity, sex, genetics, and habits (such as smoking), which directly impact the characteristics of stored erythrocytes [7-9].

Therefore, this study aimed to investigate the influence of vitamin C + vitamin E and vitamin C + N-acetylcysteine supplements on the efficacy of banked erythrocytes.

Materials and methods

Ethical committee approval (IAEC/NCP/117/2022) was obtained from the Nargund College of Pharmacy (Bengaluru, India). Animal care and maintenance were in accordance with the ethical committee regulations.

Experimental design

Wistar rats [male, 3 months old, 160-170 g body weight (b.w)] were categorized into three groups (n = 5/group).

A) controls – distilled water (without any antioxidants);

B) antioxidant group 1 (VC + VE group) – vitamin C (400 mg/kg b.w) and vitamin E (50 mg/kg b.w);

C) antioxidant group 2 (VC + NAC group) – vitamin C (400 mg/kg b.w) and N-acetylcysteine (100 mg/kg b.w).

The rats were acclimatized in the animal house for one week and then orally supplemented for 30 days. Erythrocytes were isolated from whole blood and stored for 42 days in AS-7 (additive solution-7) [10]. Hemoglobin, hemolysis, and phosphatidyl serine externalization were measured in erythrocytes. Oxidative stress and antioxidant markers were analyzed in hemolysate at two-week intervals.

Erythrocyte separation

Erythrocytes were isolated by centrifugation at 3500 rpm at 4 °C for 20 minutes (min). The erythrocyte pellet was washed and resuspended in isotonic phosphate buffer to a final hematocrit of 50%.

Hemoglobin (Hb)

Hemoglobin content of the erythrocytes was measured by the cyanomethemoglobin method using Hemocor-D Kit and represented in terms of g/dl [11].

Superoxide Dismutase [SOD, EC 1.15.1.1]

Epinephrine was added to the hemolysate diluted in carbonate buffer and measured at 480 nm. SOD activity was expressed as Units/mg protein [12].

Catalase [CAT, EC 1.11.1.6]

Hemolysate was treated with absolute alcohol, 6.6 mM hydrogen peroxide (H₂O₂) and the absorbance was measured at 240 nm. Catalase activity was expressed as Units/mg protein [13].

Glutathione Peroxidase [GPX, EC.1.11.1.9]

Glutathione reductase (0.24 units), and 10 mM glutathione (GSH) were added to hemolysate and preincubated for 10 min at 37 °C, followed by the addition of nicotinamide adenine dinucleotide phosphate (NADPH) in 0.1% sodium bicarbonate. The overall reaction was started by adding pre-warmed H₂O₂ and the decrease in absorption was monitored at 340 nm for three min. The enzyme activity was expressed as Units/mg protein [14].

Protein Carbonyls (PrC)

An equal volume of 1% trichloroacetic acid (TCA) and the samples were suspended in 10 mM 2,4-dinitrophenyl hydrazine (DNPH). Samples were kept in the dark for 1 hour. An equal volume of 20% TCA was added and left in ice for 10 min, centrifuged at 3000 g, and the pellet was washed with ethanol-ethyl acetate mixture (1:1). The final pellet was dissolved in guanidine HCl, and absorbance was measured at 370 nm [15].

Protein Sulfhydryls (P-SH)

Hemolysate was treated with sodium phosphate buffer containing 0.5 mg/ml of Na₂-EDTA and 2% sodium lauryl sulfate (SDS). 5,5'-dithiobis-2-nitrobenzoic acid (DTNB) was added to the above mixture, vortexed, and absorbance was measured at 412 nm [16].

Malondialdehyde (MDA)

Hemolysate samples were treated with 8.1% SDS, and incubated at room temperature for 10 min. 20% acetic acid was added followed by 0.6% thiobarbituric acid, and the samples were boiled until pink colour developed. Butanol-pyridine was added, and centrifuged for 5 min, and the absorbance was measured at 532 nm [17].

Thiobarbituric acid reactive substances (TBARS)

Hemolysate was treated with 0.9% NaCl and incubated at 37 °C for 20 min. 0.8 M HCl containing 12.5% TCA and 1% thiobarbituric acid was boiled for 20 min and cooled. The absorbance was measured at 532 nm [18].

Total Antioxidant Capacity (TAC) Cupric ion-reducing antioxidant capacity – BCS assay [CUPRAC-BCS]

Hemolysate was treated with 0.25mM bathocuproinedi-sulfonic acid disodium salt (BCS) and the initial absorbance was measured at 490 nm. 0.5 mM Copper sulfate was added and incubated for 3 min, followed by the addition of Na₂EDTA and read at 490 nm [19].

Glutathione (GSH)

Hemolysate was treated with 4% sulfosalicylic acid, and centrifuged at 2500 g for 15 min. The supernatant was treated with 10 mM DTNB and the absorbance was read at 412 nm [20].

Uric acid

Uric acid levels were determined by the Uricase/POD method kit from Aspen Laboratories [21].

Metabolic markers

Glucose

Glucose levels were assessed using the glucose oxidase and peroxidase (GOD-POD) method [22].

Lactate dehydrogenase (LDH)

Hemolysate was treated with the LDH reagent [a mixture of reagent 1 (80 mM Tris, 1.6 mM pyruvate, and 200 mM NaCl) and reagent 2 (0.2 mM NADH) at a ratio of 4:1], and incubated at 37 °C. The absorbance was measured at 340 nm [23].

Superoxides

Hemolysate was treated with cytochrome C (160 μM), incubated at 37 °C and the absorbance was measured at 550 nm [24].

Nitrites

Hemolysate was added to the coupling reagent, incubated for 10 min and the absorbance was measured at 520 nm [25].

Hemolysis

A 5% suspension of packed erythrocytes were mixed with an equal volume of 8 mM H₂O₂ and incubated at 37 °C. Hemolysis was determined by measuring the amount of Hb released into the supernatant at 540 nm [26].

Phosphatidyl Serine (PS) externalization by flow cytometry

The PS exposure on the erythrocyte membrane was analyzed by flow cytometry (CytoFLEX, Beckman Coulter, Inc. USA) according to FITC Annexin V Apoptosis Detection Kit I, BD Biosciences. The data was recorded with an excitation of 488 nm and emission of 530 nm [27], and analyzed by using the CytExpert software. Protein content was determined by Lowry et al. using bovine serum albumin as the standard [28].

Statistical analysis

Results are represented as mean $\pm$ standard error (SE) of 5 animals per group. Differences between the controls and antioxidant groups) and within the groups were analyzed by two-way ANOVA. Bonferroni post hoc test was performed. Pearson's correlation coefficient was measured for the relevant markers. The values were considered significant at $p < 0.05$. Statistical analysis was performed using the GraphPad Prism software (version 6, GraphPad Software Inc., Boston, MA, USA).

Results

The significant changes were represented with p-values between the controls and antioxidant groups as well as the sub-groups (storage days).

Hemoglobin (Hb)

Hb levels were significant in all the groups in comparison to day 1. Hb levels increased by 38% ($p < 0.05$) on day 42 in controls with day 1. VC + VE showed increments of 53%

($p < 0.001$) and 61% ($p < 0.0001$) on days 28 and 42 respectively, compared to day 1. Hb levels increased by 37% ($p < 0.05$) and 63% ($p < 0.0001$) on days 28 and 42 respectively, in VC + NAC in comparison to day 1. Hb levels increased by 17% on day 28 in VC + VE and VC + NAC, and 22% on day 42 in VC + NAC compared to controls (Figure 1).

Superoxide dismutase (SOD)

SOD activity was similar throughout the storage period in both control and VC + NAC groups, whereas decreased by 32% ($p < 0.01$) on day 14 in VC + VE compared to day 1. SOD activity increased by 23% on day 1 in VC + VE & VC + NAC compared to controls, and 25% on day 14 in VC + NAC compared to controls & VC + VE (Figure 2A).

Catalase (CAT)

Catalase activity was similar throughout the storage in controls, whereas varied in the antioxidant groups. CAT activity decreased by 35% and 46% on days 14 and 28 respectively, in the VC + VE compared to day 1. CAT activity showed decrements of 56%, 32%, and 45% on days 14, 28, and 42 respectively, in VC + NAC compared to day 1. CAT activity showed increments of 47% and 72% on day 1 in VC + VE and VC + NAC groups respectively, compared to controls. CAT activity increased by 35% and 47% on day 28 in VC + NAC compared to Control and VC + VE respectively (Figure 2B).

Glutathione Peroxidase (GPX)

GPX activity increased in antioxidant groups towards the end of storage period. GPX activity decreased by 27% on day 14 and normalized on day 42 in controls compared to day 1. GPX activity increased by 64% and 105% in VC + VE and 57%

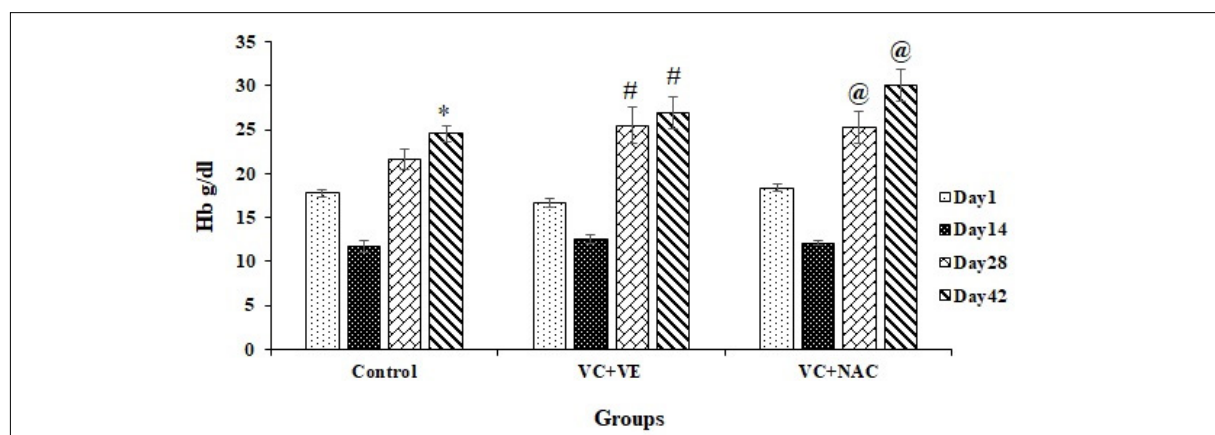


Figure 1. Hemoglobin levels in stored erythrocytes

*represents significant changes during storage in control with respect to day 1.

represents significant changes during storage in VC + VE with respect to day 1.

@ represents significant changes during storage in VC + NAC with respect to day 1.

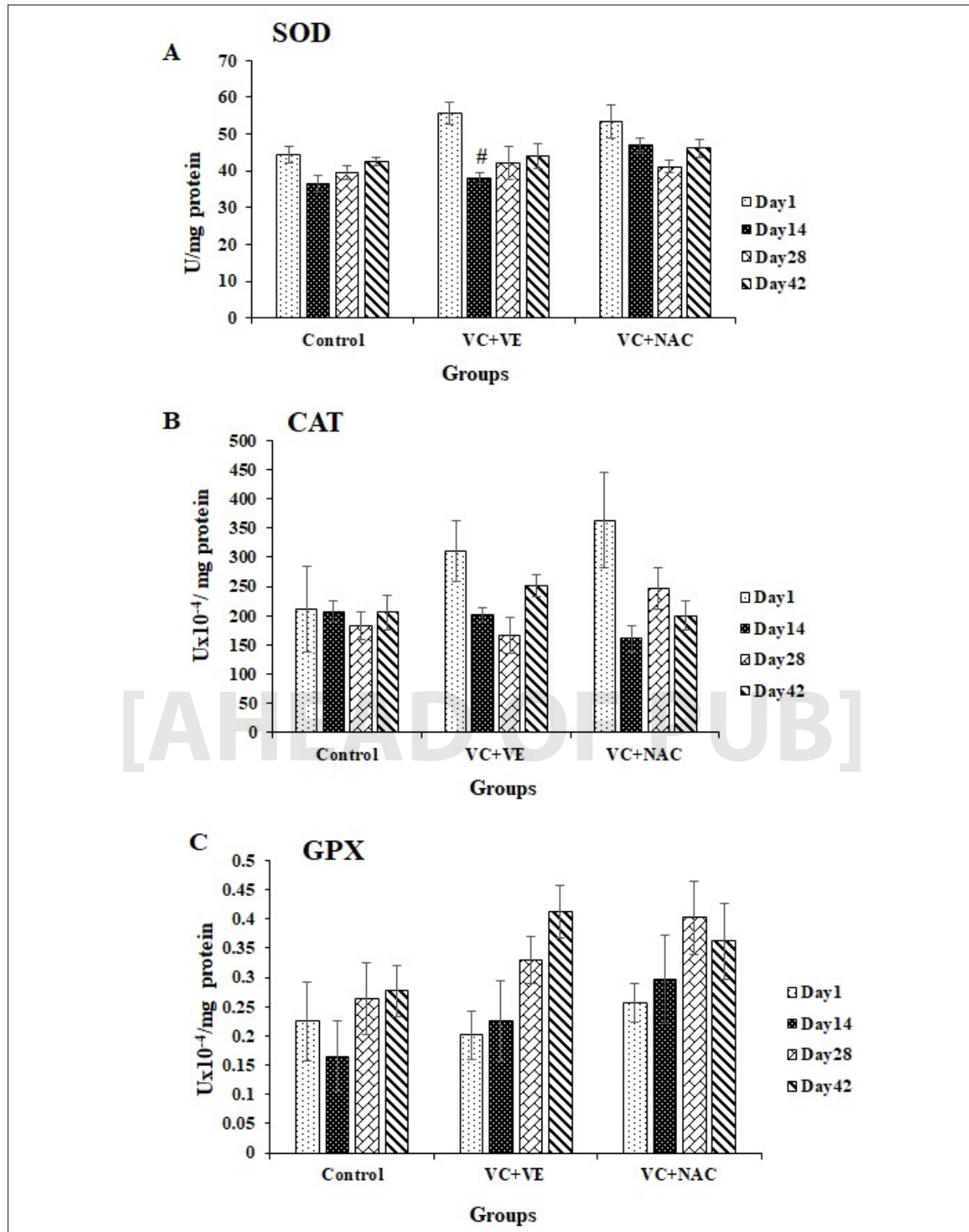


Figure. 2A. Superoxide dismutase activity in stored erythrocytes;

2B. Catalase activity in stored erythrocytes;

2C. Glutathione peroxidase activity in stored erythrocytes.

Values are expressed as mean $\pm$ SE from 5 samples. Significance between the groups was analyzed by two-way ANOVA followed by Bonferroni post test, using GraphPad Prism 6 software.

represents significant changes during storage in VC + VE with respect to day 1.

and 41% in VC + NAC on days 28 and 42 respectively, compared to day 1. Increments of 36% & 80% (day 14), 25% & 52% (day 28), and 49% & 31% (day 42) were observed in VC + VE & VC + NAC respectively, compared to controls (Figure 2C).

Protein Carbonyls (PrC)

Carbonyls increased by 21% and 67% on days 28 and 42 respectively, in controls compared to day 1, whereas decreased towards the end of storage in both the antioxidant groups (Table 1).

Protein Sulfhydryls (P-SH)

Sulfhydryls increased by 139%, 85%, and 103% on days 14, 28, and 42 respectively, in controls compared to day 1. Sulfhydryls remained constant throughout storage, whereas lowered by 37% on day 28 and normalized on day 42 in VC + VE with respect to day 1. Sulfhydryls decreased by 25% on days 28 and 42 in VC + NAC in comparison to day 1. Sulfhydryls increased by 1-fold and 2-fold in VC + VE and VC + NAC groups respectively, on day 1 in comparison to controls. VC + NAC showed increments in sulfhydryls on day 1 (26%) and day 28 (55%) when compared to VC + VE (Table 1).

Malondialdehyde (MDA)

Controls showed increments of 32% and 68% on days 28 and 42 respectively, compared to day 1. MDA levels remained constant throughout the storage in VC + VE, whereas decreased by 23% and 30% on days 14 and 42 respectively, in VC + NAC with day 1. VC + NAC showed decrements of 39% and 26% on day 42 compared to controls and VC + VE respectively (Table 1).

Thiobarbituric acid reactive substances (TBARS)

Antioxidant groups had lower levels of TBARS in comparison to Controls. TBARS decreased by 14% on day 28 in VC + VE and VC + NAC respectively, compared to day 1. TBARS were lower by 23% on day 42 in VC + VE compared to Controls (Table 1).

Total Antioxidant Capacity (TAC)

TAC was higher in both the antioxidant groups compared to controls. TAC decreased by 23% on days 28 and 42 respectively, in controls with day 1. Decrements of 20% and 26%

Table 1. Antioxidant effects of melatonin [32]

Storage	Groups	Protein Carbonyls ($\mu\text{M}/\text{mg protein}$)	Protein Sulfhydryls ($\mu\text{M}/\text{mg protein}$)	Malondialdehyde ($\mu\text{M}/\text{mg protein}$)	TBARS ($\mu\text{M}/\text{mg protein}$)
Day 1	Control	2.49 $\pm$ 0.76	282.49 $\pm$ 98.74	3.72 $\pm$ 0.97	2.85 $\pm$ 0.20
	VC+VE	3.46 $\pm$ 0.36	691.56 $\pm$ 184.92	4.90 $\pm$ 1.08	2.62 $\pm$ 0.08
	VC+NAC	3.85 $\pm$ 0.15	873.63 $\pm$ 218.69	5.53 $\pm$ 0.169	2.80 $\pm$ 0.44
Day 14	Control	2.74 $\pm$ 0.31	675.91 $\pm$ 235	4.19 $\pm$ 0.45	2.38 $\pm$ 0.47
	VC+VE	3.78 $\pm$ 0.45	701.27 $\pm$ 176.13	4.94 $\pm$ 0.77	2.15 $\pm$ 0.43
	VC+NAC	3.54 $\pm$ 0.44	737.18 $\pm$ 228.76	4.27 $\pm$ 1.20	2.30 $\pm$ 0.07
Day 28	Control	3.01 $\pm$ 0.49	521.72 $\pm$ 173.14	4.91 $\pm$ 0.53	2.78 $\pm$ 0.49
	VC+VE	3.20 $\pm$ 0.49	432.78 $\pm$ 53.35	4.67 $\pm$ 0.97	2.24 $\pm$ 0.12
	VC+NAC	3.33 $\pm$ 0.80	668.83 $\pm$ 154.87	5.16 $\pm$ 0.84	2.43 $\pm$ 0.4
Day 42	Control	4.16 $\pm$ 0.32	572.13 $\pm$ 120.18	6.27 $\pm$ 1.18	3.93 $\pm$ 1.38
	VC+VE	3.27 $\pm$ 0.93	613.50 $\pm$ 143.67	5.19 $\pm$ 2.10	3.05 $\pm$ 0.26
	VC+NAC	3.36 $\pm$ 0.41	625.07 $\pm$ 113.34	3.85 $\pm$ 0.54	3.64 $\pm$ 0.40

Values are expressed as mean $\pm$ SE from 5 samples. Significance between the groups was analyzed by two-way ANOVA followed by Bonferroni post test, using GraphPad Prism 6 software. TBARS – Thiobarbituric acid reactive substances.

were observed in VC + VE and VC + NAC respectively, on day 42 compared to day 1. TAC showed increments of 20% (day 14) and 34% (day 28) in VC + VE and 43% (day 28) in VC + NAC compared to controls (Figure 3A).

Glutathione (GSH)

The GSH levels were significant towards the end of storage in VC + VE. GSH levels decreased by 17% on day 14 and normalized towards the end of storage in controls. GSH levels increased by 32% ($p < 0.01$) in VC + VE and 18% in VC + NAC on day 42 compared to day 1. VC + VE showed increments of 16% on days 28 and 42 compared to controls. GSH increased by 23% on day 14 in VC + NAC compared to controls and VC + VE respectively (Figure 3B).

Uric acid

Although the variations were similar, uric acid levels were higher in the antioxidant groups throughout the storage (Figure 3C).

Glucose

Glucose levels were similar throughout storage in controls, whereas they varied in the antioxidant groups. Glucose levels varied by 23% on day 14 and 26% on days 28 and 42 in VC + VE with day 1. Glucose levels decreased by 20% on days 14 and 28 in VC + NAC compared to day 1. Glucose levels increased by 20% (day 1) and 37% (day 14) in VC + VE compared to controls. Glucose levels elevated in VC + NAC by 41%, 18%, and 27% on days 1, 28, and 42 respectively, compared to controls and 26% and 46 % on days 28 and 42 compared to VC + VE (Figure 4A).

Lactate dehydrogenase (LDH)

LDH activity decreased by 60% and 77% on day 1 in VC + VE and VC + NAC respectively, compared to controls (Figure 4B).

Superoxides

Superoxide levels were similar in all the groups throughout the storage. Superoxides increased from 0.31 mM/mg protein (day 1) to 0.50 mM/mg protein (days 28 and 42) in controls. Superoxides increased from 0.34 mM/mg protein (day 1) to 0.48 mM/mg protein (day 14) and were stable until day 42 in VC + VE. Superoxides varied from 0.4 mM/mg protein (day 1) to 0.45 mM/mg protein (day 28), and 0.55 mM/mg protein (day 42) in VC + NAC.

Nitrites

Variations were similar in all the groups. Nitrites varied on day 14 (2.89 mM/mg protein) and day 28 (2.69 mM/mg protein) compared to day 1 (2.45 mM/mg protein) in controls. Nitrites increased on day 28 (3.13 mM/mg protein) in comparison to day 1 (2.62 mM/mg protein) and then normalized in VC + VE. Nitrite varied from 2.96 mM/mg protein (day 1) to 2.12 mM/mg protein (day 42) in VC + NAC.

Hemolysis

VC + VE showed a decrease of 43% on days 1, 14 and 28 compared to controls and 74%, 52%, and 25% on days 1, 14 and 28 compared to VC + NAC. VC + NAC showed decrements of 25% and 16% on days 28 and 42 in comparison to controls (Figure 5).

Phosphatidyl Serine (PS) externalization

The variations were observed in the PS exposure with storage. PS exposure increased by 9-fold on day 42 in controls compared to day 1 (0.51%). VC + VE and VC + NAC groups showed increments of 2-fold and 1-fold respectively, on day 42 in comparison to day 1 (VC + VE – 1.27%; VC + NAC – 1.46%).

Discussion

Storage lesions accumulate in erythrocytes when the oxidants overwhelm the antioxidant defenses. Hence, antioxidant augmentation could be a promising approach to maintain the microenvironment equilibrium.

VC + VE group

In this study the Hb levels increased towards the end of storage in the VC + VE group indicates the reverse conversion of methemoglobin (methHb) to Hb by vitamin C and vitamin E. We also noted this in our previous study, where the combination of VC and VE as additives increased Hb levels [29]. Atyabi et al. also reported that the combination of vitamin C and E was effective in reducing sodium nitrite-induced methHb formation [30].

SOD dismutates superoxide into H_2O_2 , and catalase scavenges H_2O_2 . This was evidenced by an inverse correlation between SOD activity and superoxide levels ($r = -0.9447$) and a positive correlation between SOD and catalase activity in the VC + VE group ($r = 0.8586$). SOD and catalase activities were higher in the VC + VE group on day 1, indicating that supplemented antioxidants upregulated the antioxidant

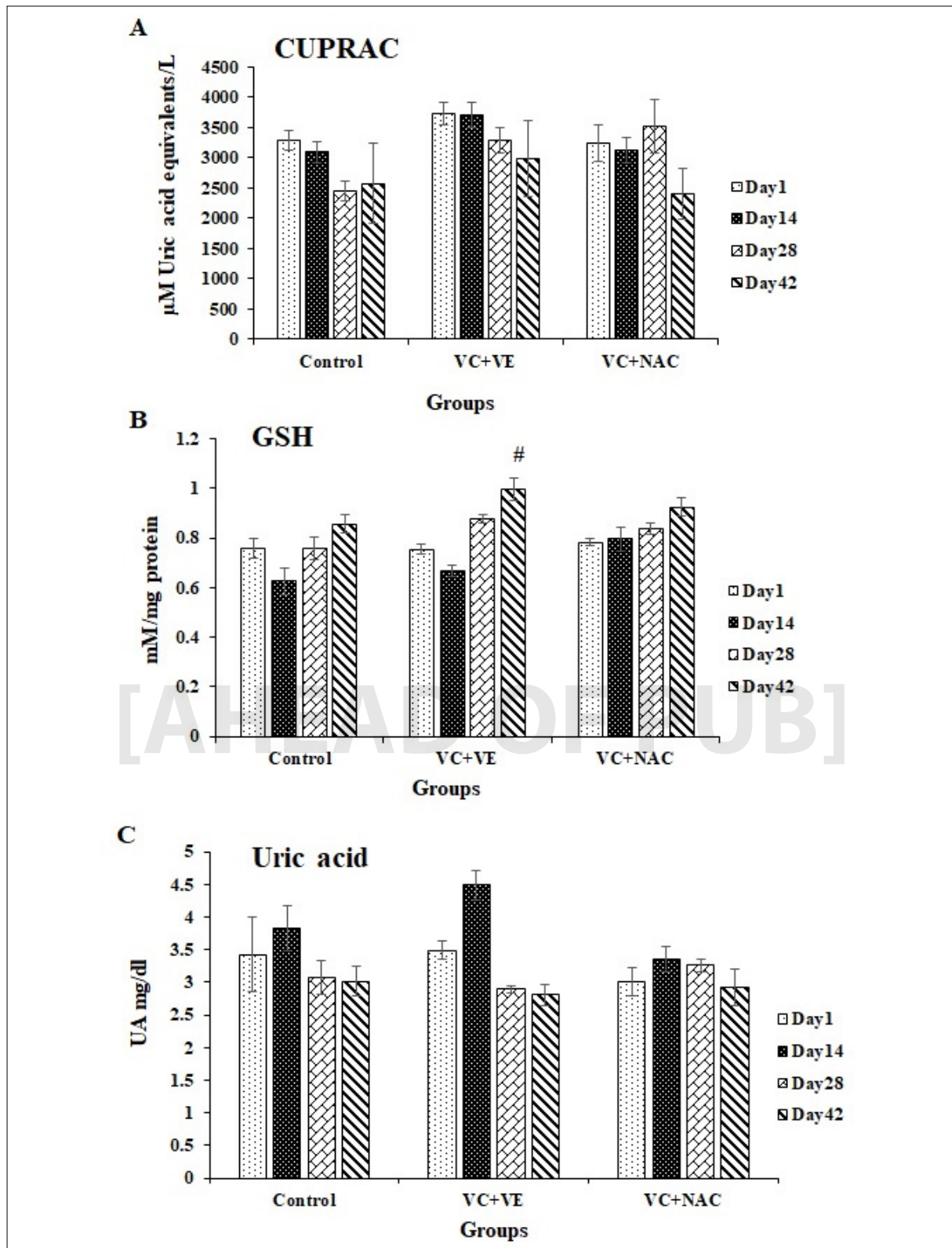


Figure 3A. CUPRAC in stored erythrocytes;

3B. Glutathione in stored erythrocytes;

3C. Uric acid in stored erythrocytes.

Values are expressed as mean $\pm$ SE from 5 samples. Significance between the groups was analyzed by Two-way ANOVA followed by Bonferroni post test, using GraphPad Prism 6 software.

represents significant changes during storage in VC + VE with respect to day 1.

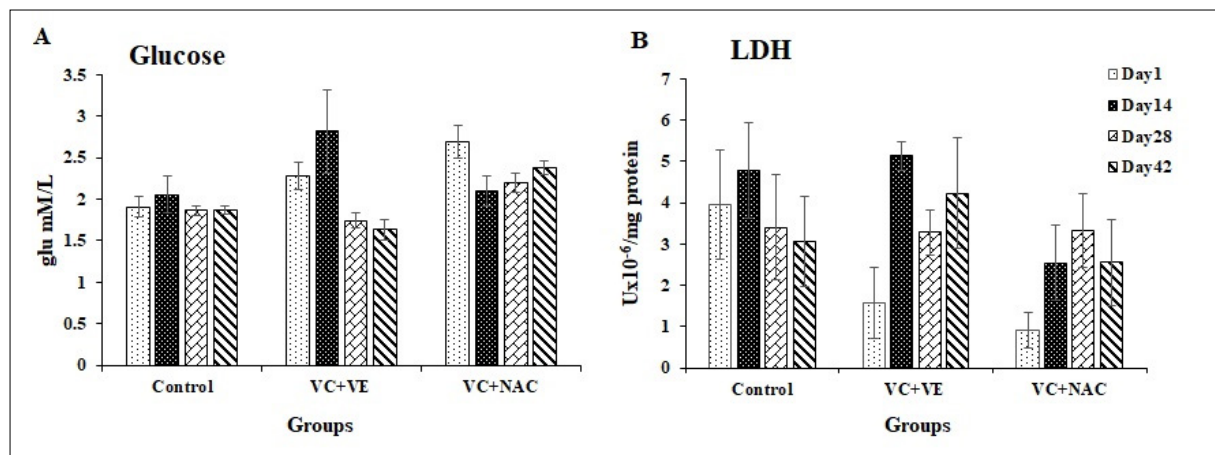


Figure 4A. Glucose in stored erythrocytes;
4B. Lactate dehydrogenase in stored erythrocytes.

Values are expressed as mean $\pm$ SE from 5 samples. Significance between the groups was analyzed by two-way ANOVA followed by Bonferroni post test, using GraphPad Prism 6 software.

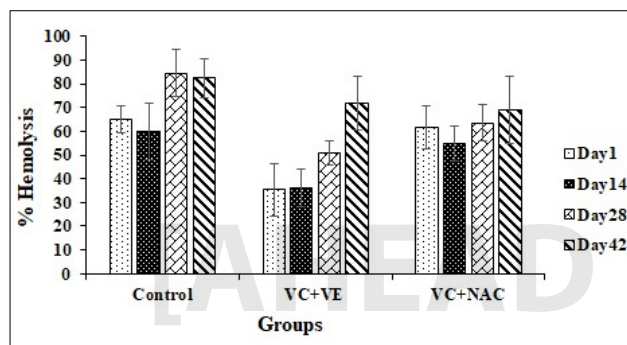


Figure 5. Hemolysis in stored erythrocytes

Values are expressed as mean $\pm$ SE from 5 samples. Significance between the groups was analyzed by two-way ANOVA followed by Bonferroni post test, using GraphPad Prism 6 software.

enzymes. Gultekin and Bhogade have also reported that vitamin C and E upregulates antioxidant enzymes [31-32]. However, SOD and catalase activity decreased on day 14 in VC + VE reflecting the superoxide and H₂O₂ scavenging activity of vitamin C and vitamin E [5, 33-34]. Therefore, superoxide levels reached a plateau from day 14 onwards in the VC + VE group.

GPX scavenges H₂O₂ at lower levels by using GSH as a co-factor and oxidizes GSH (reduced glutathione) to GSSG (oxidized glutathione) [35]. Supplementation of vitamin C and vitamin E increases cellular glutathione levels, thereby upregulating the GPX activity [36-37]. Previous studies also reported that supplementation of vitamin C and vitamin E increases GPX activity [38-39]. Similarly in this study, glutathione levels and GPX activity elevated towards the end of storage in the VC + VE group with a significant correlation between GSH and GPX activity ($r = 0.9389$) indicating that VC + VE enhanced antioxidant defenses.

Protein carbonyls declined in the VC + VE group with storage, suggesting the protective influence of Vitamin C and Vitamin E against protein oxidation, evidenced in our earlier in vitro study [29]. Goldfarb et al. and Marino et al. have also reported that vitamin C and E supplementation reduced protein carbonyl formation during exercise-induced and alcohol-induced oxidative stress, respectively [40-41]. Vitamin C and E synergistically quench the free radicals generated in the lipid core of the erythrocyte membrane and protect from protein oxidation, which was also evidenced in our findings [42]. Carbonyl levels increased towards the end of the storage in controls, indicating protein damage resulting from oxidative insult, which was also observed by Jana et al. [43].

Higher sulfhydryl levels in the VC + VE group until 2nd week of storage suggest the protection of sulfhydryl groups against oxidation or the reversible conversion of disulfides to sulfhydryls [44]. Vitamin C and vitamin E supplementation have been associated with elevated cellular GSH and sulfhydryls [36-37]. However, the lower levels of sulfhydryls on day 28 in the VC + VE group imply the conversion of GSH to GSSG, evidenced by an elevation in GPX and nitrites. Sulfhydryls normalized on day 42, due to the reversible conversion of GSSG to GSH due to the action of VC and VE [45].

The MDA levels were similar throughout the storage in the VC + VE group, indicating the lipid peroxyl radical scavenging activity of vitamin C and vitamin E [35, 46-47]. Vitamin C and vitamin E synergistically impede the progression of lipid peroxidation reactions in plasma lipoproteins and membranes [35]. Nonetheless, MDA levels peaked towards the end of storage in controls, indicating that inherent antioxidant defenses were inadequate to mitigate ROS attack on membrane lipids.

Oxidative damage to membrane lipids and proteins contributes to elevations in LDH activity [48]. LDH increased rapidly during the first 4 weeks of storage in controls. Verma et al. and Latham et al. also reported an increase in LDH activity during erythrocyte storage [49–50]. However, the VC + VE protected erythrocytes from LDH leakage until two weeks of storage. This indicates reduced oxidative damage to membrane proteins and lipids, resulting in lower hemolysis. Czubak et al., also demonstrated that VC and VE synergistically protect human erythrocytes from LDH leakage up to 20 days [51].

Total antioxidant capacity (TAC) was relatively higher in the VC + VE group, depicting that vitamin C and vitamin E synergistically enhanced antioxidant capacity. TAC was directly proportional to glutathione and uric acid (endogenous antioxidants). However, TAC decreased towards the end of storage due to oxidative stress. Similarly, Czubak et al. reported a storage-dependent decrease in antioxidant capacity during erythrocyte storage [51].

Glucose levels were higher on day 14 and declined towards the end of storage in the VC + VE group, which can be attributed to the higher glucose consumption by erythrocytes due to an increase in their metabolic activity [52]. Glucose serves as a crucial substrate for both glycolysis and the pentose phosphate pathway to generate NADH and NADPH essential to reduce methemoglobin, which correlated with higher hemoglobin levels towards the end of storage [53]. The effective glucose metabolism plays a role in regulating glutathione levels, thereby protecting the sulfhydryl groups of hemoglobin from oxidation, which was evidenced with higher glutathione levels [35].

Phosphatidyl serine (PS) exposure on the outer leaflet of the erythrocyte membrane indicates storage lesions and the signals for apoptotic senescence [54]. The percentage of PS-exposing cells increases with storage, which was evidenced in our results [48, 55]. However, PS externalization was comparatively lower in VC + VE suggesting that supplementation of vitamin C and vitamin E reduced apoptosis, which also corroborates with the findings of Huang et al. [56].

Hemolysis is a result of protein and lipid oxidation. Vitamin C decreases the tocopheroxyl radicals in the membrane during the lipid–aqueous phase transition [57]. Vitamin C and vitamin E act synergistically to prevent the propagation of oxidation in erythrocyte membranes and hemolysis, which was evident in the hemolysis results [48, 51, 58]. Sakarya et al. and Claro et al., also reported that vitamin C and E attenuates the oxidation of erythrocyte membrane [50, 59].

VC + NAC group

The elevations in Hb levels towards the end of storage in VC + NAC could be due to the reverse conversion of metHb

to Hb by the antioxidants. Vitamin C and NAC reduce metHb formation, which was evidenced in the Hb results [60–61].

SOD and catalase activities increased on day 1 in the VC + NAC group, indicating the upregulation of the antioxidant enzymes by vitamin C and NAC supplementation [31, 62]. Catalase activity decreased from day 14 in VC + NAC indicating the H_2O_2 scavenging activity of vitamin C and NAC [32, 63]. Consequently, the lower levels of H_2O_2 towards the end of storage were further mitigated by GPX. There is a positive correlation between GSH and GPX activity towards the end of storage ($r = 0.660$).

Protein carbonyls declined towards the end of storage in VC + NAC, indicating the protective effect of vitamin C and NAC against protein oxidation. Goldfarb et al. and Banach et al. reported that Vitamin C and NAC decrease protein carbonyl formation against exercise and age-related oxidative stress, respectively [40, 64]. MDA levels also decreased towards the end of storage in the VC + NAC group which is consistent with the study by Pallotta et al. [65]. NAC indirectly contributes to lipid peroxyl radical scavenging activity by providing GSH for the reduction of ascorbate radicals to ascorbic acid [66].

Sulfhydryl levels were higher in VC + NAC compared to VC + VE suggesting GSH replenishment due to supplementation of NAC. Pallotta et al. reported that the storage of human erythrocytes with vitamin C and NAC resulted in higher GSH levels till 42 days [65]. Grinberg et al. also demonstrated that NAC protected erythrocytes from oxidative stress by restoring cellular glutathione [67]. VC + NAC protected lipids from oxidation as evidenced by enhanced endogenous antioxidants such as uric acid, glutathione and total antioxidant capacity.

LDH activity increased rapidly during the first 4 weeks of storage in controls whereas, the VC + NAC group showed a lower LDH release reflecting decreased erythrocyte damage as evidenced by reduced hemolysis and PS exposure. Glucose levels were higher in the VC + NAC group compared to controls. Glucose metabolism efficiently maintained GSH and thus protected hemoglobin and membrane from oxidation [35, 53].

Conclusion

Antioxidant combination (VC + VE and VC + NAC) supplements protected hemoglobin, blood proteins, and lipids from oxidation, and enhanced antioxidant defenses. VC + VE elevated glutathione levels and lowered hemolysis. VC + VE was effective against hemolysis compared to VC + NAC due to their synergistic action. VC + NAC was beneficial as it lowered LDH leakage and apoptosis. Both the antioxidant combinations have a positive influence on the efficacy of stored erythrocytes. Our results imply that prior supplementation

augments the antioxidant status and influences the quality of stored blood.

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Conflict of Interest

There are no conflicts of interest to declare.

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