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Vitamin C with N-acetylcysteine ameliorates the Antioxidant defenses in Erythrocytes during storage

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Abstract

Oxidative stress causes structural and biochemical alterations in stored erythrocytes. Antioxidants can be employed to prevent oxidative damage and enhance antioxidant defenses, thereby improving the efficacy of stored erythrocytes. N-acetylcysteine (a glutathione precursor), and Vitamin C (a free radical scavenger) can be potential candidates as additives. Hence, this study investigates the interactions of Vitamin C and N-acetylcysteine during erythrocyte storage. Blood was sampled from *Wistar* rats, and erythrocytes were grouped into (i) Controls and (ii) Experimentals [vitamin C-10 mM & N-acetylcysteine-0.5 mM]. The erythrocytes were stored in additive solution-7 (AS-7) at 4˚C for a duration of 35 days. Antioxidant and oxidative stress markers were analyzed at weekly intervals. Hemoglobin levels increased on day 14 in Experimentals compared to Controls and normalized on day 35. Catalase activity was maintained in Experimentals, whereas it decreased in Controls. Glutathione peroxidase activity decreased on day 14, whereas it increased on day 35 in Experimentals. Although thiobarbituric acid reactive substances decreased on days 14, 28 & 35 in both groups, there was a steep decline towards the end of storage in Experimentals. Endogenous antioxidant levels were higher throughout storage in Experimentals. Vitamin C with N-acetylcysteine reduced oxidative stress and enhanced antioxidant capacity during storage. This is the first report to elucidate the interactions of vitamin C and N-acetylcysteine in erythrocytes stored in AS-7.

Keywords: erythrocytes, N-acetylcysteine, oxidative stress, storage, vitamin C

1. Introduction

Erythrocytes exhibit an array of biochemical, and physiological changes during storage collectively referred to as “storage lesions”, which affect the survival and optimal function of transfused erythrocytes (1). Oxidative stress (an imbalance in redox homeostasis) has been considered a major cause of storage lesions. Redox imbalance is attributed to the formation of free radicals, alterations in the endogenous antioxidant systems, and oxidative modifications of proteins and lipids in erythrocytes (2-4).

Antioxidant interventions have been employed in various oxidative stress situations. Vitamin C is one of the most potent natural antioxidants in its oxidized form. It reduces superoxide and lipid peroxyl radicals and acts as a synergistic agent for vitamin E (5,6). N-acetylcysteine (NAC) is a thiol-containing antioxidant, that can stimulate glutathione [(GSH)-a tripeptide made up of cysteine, glutamate, and glycine] synthesis, by providing cysteine (7,8). NAC also scavenges reactive oxygen species (ROS) (9).

Erythrocyte storage solutions are formulated to supply additional nutrients necessary for anaerobic metabolism. Antioxidants significantly alleviate oxidative stress and increase the efficacy of stored erythrocytes. Hence, storage solutions with antioxidants can enhance endogenous antioxidant defenses and improve the efficacy of erythrocytes.

There are studies on the modifications of storage solutions with a single antioxidant, such as vitamin C or N-acetylcysteine. Vitamin C has been beneficial in terms of 2,3-diphosphoglyceric acid (2,3-DPG) levels (10), mechanical fragility & hemolysis (11), microparticle formation, post-transfusion recovery (12), lipid peroxidation and sulfhydryl content (13). NAC increases GSH levels until 21 days of storage and improves cell survival by decreasing cell lysis and fragility (14). Hence, NAC (glutathione precursor) and vitamin C (free radical scavenger), combined as additives, could maximize the effect. The selection of antioxidant concentrations, such as vitamin C, was based on our prior research conducted in our laboratory (13,15) and NAC from the literature by Pallotta et al. (16).

In this study, *Wistar rat* blood was used to mitigate human-specific variations, encompassing factors such as age, ethnicity, lifestyle, and underlying medical conditions. Rat models have been extensively employed for evaluating the effects of storage on blood and its components, as well as the safety and efficacy after blood transfusions, as evidenced by previous studies (12,17-19). d'Almeida et al. (18) reported that one week of rat blood storage is equivalent to four weeks of human blood storage, emphasizing the suitability of rat models for storage studies. Cavalcante et al. (20) have indicated that rat erythrocytes exhibit a decline in deformability, microvesiculation, and aggregability and an increase in membrane rigidity when compared to human erythrocytes.

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There is only one study on the combination of vitamin C and NAC as additives in CPD-SAGM (citrate-phosphate-dextrose-saline-adenine-glucose-mannitol), which reported on pH, hemolysis, ROS, and glucose metabolomics (16). However, the effect of these antioxidants on oxidative stress and antioxidant defenses in AS-7 (Additive solution-7) stored erythrocytes remains to be explored. AS-7 (SOLX) is designed to enhance the buffering capacity of solution and erythrocyte metabolism. AS-7 exhibits higher lactate production, adenosine triphosphate (ATP), 2,3-DPG, lower hemolysis, and conserves morphological properties (21). Therefore, this study aimed to assess the intermittent changes in antioxidant status and protein and lipid oxidative modifications in erythrocytes stored with vitamin C and N-acetylcysteine in AS-7. This is the first report to elucidate the interactions of vitamin C and N-acetylcysteine on erythrocytes stored in AS-7.

2. Materials and methods

Animal care and maintenance were according to ethical committee regulations (841/b/04/ CPCSEA). Ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC) at the Nargund College of Pharmacy, Bengaluru (IAEC/NCP/117/2022).

2.1. Blood Sampling

Animals were lightly anesthetized with ketamine hydrochloride, and blood was collected into polypropylene tubes with anticoagulant CPDA-1 (citrate, phosphate, dextrose, and adenine) (22).

2.2. Experimental design

Blood was drawn from 4-month-old male *Wistar* rats. Erythrocytes were isolated and grouped into (n=5/group): (i) Controls (in AS-7) [Sodium bicarbonate- 26 mM L−1, Di sodium hydrogen phosphate -12 mM L−1, Adenine - 2 mM L−1, Glucose - 80 mM L−1, Mannitol - 55 mM L−1; pH - 8.5; 237 imOsm]; and (ii) Experimentals- vitamin C (10 mM in AS-7) and N-acetylcysteine (0.5 mM in AS-7) [VC+NAC]. Markers of erythrocyte function, oxidative stress, and antioxidant defenses were analyzed at weekly intervals for a period of 35 days (Table 1).

Table 1. Experimental design

Study Groups	Storage solution	Storage Conditions	Analysis	Analysis Days
Controls (n=5)	AS-7	4˚C for 35 days	**Erythrocyte marker** (Hemoglobin) **Antioxidant defense markers** (Enzymatic antioxidants-SOD, CAT & GPX and Non-enzymatic antioxidants-GSH & Uric acid) **Oxidative stress markers** (Reactive oxygen species-Superoxides, Protein oxidation-AOPP& P-SH, and Lipid peroxidation- Conjugate dienes & TBARS) and **Metabolic marker** (Glucose)	1, 7, 14, 21, 28 & 35
Experimentals (n=5)	AS-7 with vitamin C (10 mM) & N-acetyl cysteine (0.5 mM)			

AS-7: Additive solution-7; Controls: Erythrocytes stored without any antioxidants (stored only in AS-7); Experimentals: Erythrocytes stored with antioxidants (vitamin C-10 mM and N-acetylcysteine-0.5 mM in AS-7); SOD: Superoxide dismutase; CAT: Catalase; GPX: Glutathione Peroxidase; GSH: Glutathione; AOPP: Advanced Oxidation Protein Products; P-SH: Protein sulfhydryls; TBARS: Thiobarbituric acid reactive substances.

2.3. Erythrocyte separation and hemolysate preparation

Blood was centrifuged at 1000 g at 4˚C for 20 min. The plasma and buffy coat were removed and the pellet was washed with isotonic phosphate buffer [(a) 0.155 M/L NaH_2PO_4 (Monobasic sodium phosphate), (b) 0.103 M/L Na_2HPO_4 (Dibasic sodium phosphate), pH 7.4, 310 mOsm] and resuspended in isotonic phosphate buffer to a final hematocrit of 50 %. The erythrocyte suspension was lysed in a hypotonic phosphate buffer [(a) 0.01 M/L NaH_2PO_4 (Monobasic sodium phosphate), (b) 6.6 mM/L Na_2HPO_4 (Dibasic sodium phosphate), pH 7.4, 20 mOsm] and the hemolysate was stored at -20˚C (23).

2.3.1. Erythrocyte marker

Hemoglobin (Hb)

Hemoglobin was measured using Hemocor-D Kit [Coral Clinical Systems, Goa, India], which utilizes the cyanomethemoglobin method (24). Erythrocytes were incubated with Hemocor-D reagent for 3 min at room temperature, and absorbance was measured at 540 nm.

2.3.2. Antioxidant markers

2.3.2.1. Enzymatic antioxidants

Superoxide Dismutase [SOD, EC 1.15.1.1]

SOD activity was measured by the method of Misra and Fridovich (25). Hemolysate was added to the carbonate buffer [(A) Sodium carbonate (0.05 M), (B) Sodium bicarbonate (4.2 g/L), pH 10.2]. Epinephrine was added to the above mixture and measured at 480 nm. SOD activity was calculated as the quantity of enzyme that inhibits the oxidation of epinephrine by 50%.

Catalase [CAT, EC 1.11.1.6]

Hemolysate was treated with absolute alcohol and incubated at 0˚C for 30 min. Hydrogen peroxide (H_2O_2) (6.6 mM) and phosphate buffer were added to the above mixture, and the decrease in absorbance was detected at 240 nm. Catalase activity was estimated using the extinction coefficient, 43.6 $M.cm^{-1}$(26).

Glutathione Peroxidase [GPX, EC.1.11.1.9]

GPX was analyzed using the method of Flohe and Gunzler (27). 50 µL of 0.1 M phosphate buffer (pH 7.0), 100 µL of enzyme sample, 100 µL of glutathione reductase (0.24 units), and 100 µL of 10 mM GSH were mixed and preincubated for 10 min at 37˚C, followed by addition of 100 µL of 1.5 mM nicotinamide adenine dinucleotide phosphate hydrogen (NADPH) in 0.1% Sodium bicarbonate. The overall reaction was started by adding 100 µL of prewarmed hydrogen peroxide, and a decrease in absorption at 340 nm was monitored for 3 min. Enzyme activity was expressed as Units/mg protein; 1 Unit corresponds to 1 mM NADPH oxidized/min.

2.3.2.2. Non-enzymatic antioxidants

Glutathione (GSH)

Glutathione was measured by the method described by Beutler et al. (28). Hemolysate was treated with 4% sulphosalicylic acid, vortexed, and centrifuged at 2500 *g* for 15 min. The supernatant was treated with 10 mM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB/Ellman's reagent), and the absorbance was measured at 412 nm.

Uric acid

Uric acid levels were determined by the Uricase/POD method kit, Aspen Laboratories (29). The hemolysate was treated with 1 mL of the reagent, mixed well, and incubated at 37˚C. The absorbance was measured at 492 nm.

2.3.3. Oxidative stress markers

2.3.3.1. Reactive oxygen species

Superoxide radicals

Superoxide levels were estimated by the method of Olas and Wachowicz (30). Hemolysate was treated with cytochrome C (160 µM), incubated at 37˚C, and the absorbance was measured at 550 nm.

2.3.3.2. Protein oxidation markers

Advanced Oxidation Protein Products (AOPP)

Hemolysate was diluted in phosphate buffer, and 1.16 mol.L^{-1} potassium iodide was added, followed by acetic acid. The absorbance was immediately read at 340 nm and calculated using the extinction coefficient, 26 $mM^{-1}cm^{-1}$ (31).

Protein Sulfhydryls (P-SH)

P-SH content in the proteins was measured as described by Habeeb (32). In brief, 0.08 M sodium phosphate buffer containing 0.5 mg/mL of Na_2-EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate, and 2% sodium dodecyl sulfate (SDS) were added to samples. 0.1 mL of 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) (20 mg in 10 mL) was then added and vortexed. Colour was allowed to develop at room temperature, and absorbance was measured at 412 nm. P-SH content was estimated with an extinction coefficient of 13,600 $M^{-1}L^{-1}$ cm^{-1}.

2.3.3.3. Lipid Peroxidation markers

Conjugate dienes (CD)

Conjugate dienes were assessed by the method of Olas and Wachowicz (30). The samples were diluted with ether: ethanol (1:3 v/v), vortexed for 1 min, and centrifuged at 8000 rpm for 20 min. The absorbance was measured at 234 nm. The amount of conjugate dienes produced was calculated using the molar extinction coefficient, 29,500 $M^{-1}cm^{-1}$.

Thiobarbituric acid reactive substances (TBARS)

Hemolysate with 0.9% sodium chloride was incubated at 37˚C for 20 min. 0.8 M hydrochloric acid containing 12.5% Trichloroacetic acid and 1% thiobarbituric acid (TBA) was added to the above lysate, boiled for 20 min, and cooled at 4˚C. Then it was centrifuged at 1500 g, and the absorbance was measured at 532 nm. TBARS were calculated with an extinction coefficient of 1.56 $x10^5$ $M^{-1}.cm^{-1}$ (33).

2.3.4. Metabolic marker

Glucose

Glucose levels were measured using the enzymatic glucose oxidase and peroxidase (GOD-POD) method as described in the Auto span Gold kit, and the absorbance was measured at 546 nm (34).

Protein estimation

Proteins were determined in the erythrocyte samples by the method of Lowry et al. (35), with bovine serum albumin as the standard.

2.4. Statistical analysis

Results are represented as mean ± standard error (SE) of five animals /group (n=5). Differences between the groups (storage days) and within the groups (subgroups-Controls and Antioxidant groups) were analyzed by two-way ANOVA. Bonferroni Post-test was performed. The values were considered significant at $p < 0.05$. Statistical analysis was performed using the statistical software GraphPad Prism version 6.

3. Results

The results describe the changes in Controls and VC+NAC during storage followed by Control v/s VC+NAC on a particular storage day.

3.1. Erythrocyte markers

3.1.1. Hemoglobin (Hb)

The variations in Hb levels were significant in the VC+NAC group. Hb levels decreased by an average of 20% on days 7, 14, 21 & 35 in Controls with respect to day 1.

VC+NAC group showed an increment of 18% on day 14 and declined by 18%, 33% & 41% ($p<0.05$) on days 21, 28 & 35, respectively, in comparison to day 1.

Hb levels increased significantly by 83% ($p<0.001$) on day 14 in the VC+NAC group compared to Controls (Fig. 1).

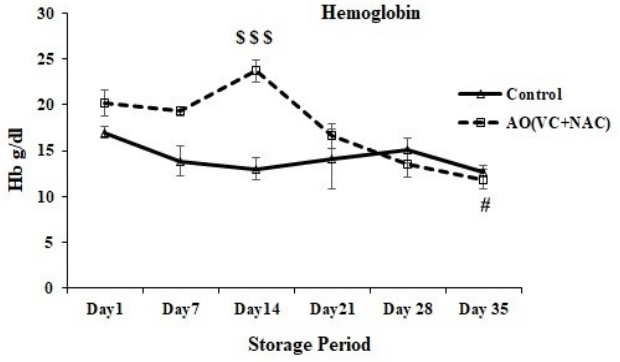

Fig. 1. Hemoglobin levels in stored erythrocytes. Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software. # represents significant changes during storage in VC+NAC group with respect to day 1 (P<0.05). $ $ $ represents significant changes during storage in VC+NAC group compared to Control on the same day (P<0.001)

3.2. Antioxidant markers

3.2.1. Enzymatic antioxidants

Superoxide Dismutase (SOD)

SOD activity showed variations throughout the storage in both groups; however, the changes were statistically insignificant.

SOD activity was 81 U/mg protein on day 1, 55 U/mg protein on day 21, and normalized to 72 U/mg protein on day 35 in Controls, whereas SOD activity remained similar throughout storage in VC+NAC (approximately 71 U/mg protein).

SOD activity was higher in the VC+NAC group from day 7 (19%) to day 21 (33%) in comparison with Controls (Fig. 2a).

Catalase

The changes in catalase activity were significant in capitals. Catalase activity significantly decreased on day 14 (62%) (p<0.05), peaked on day 21, and then decreased on day 28 (70%) (p<0.01) and day 35 (85%) (p<0.001) in Controls compared to day 1.

Catalase activity decreased by 33% (day 14), increased by 64% (day 21), and then decreased by 59% (day 28) & 72% (day 35) in the VC+NAC group in comparison to day 1 (456 Ux10^{-4}/mg protein), although the changes are statistically insignificant.

Catalase increased by 25% on day 21 in VC+NAC compared to the Control group (Fig. 2b).

Glutathione Peroxidase (GPX)

GPX activity showed significant variations in the VC+NAC group.

GPX activity decreased throughout storage by 24% (day 7), 84% (day 14), 49% (day 21), 52% (day 28), and 33% (day 35) in Controls, compared to day 1.

GPX decreased by 95% (p<0.01) on day 14 and then normalized to day 1 levels towards the end of storage. GPX increased by 88% (p<0.01) on day 35 against day 14 in the VC+NAC group.

GPX activity was higher by 41%, 83%, and 100% on days 1, 28, and 35 in the VC+NAC group than in Controls, although statistically insignificant (Fig. 2c).

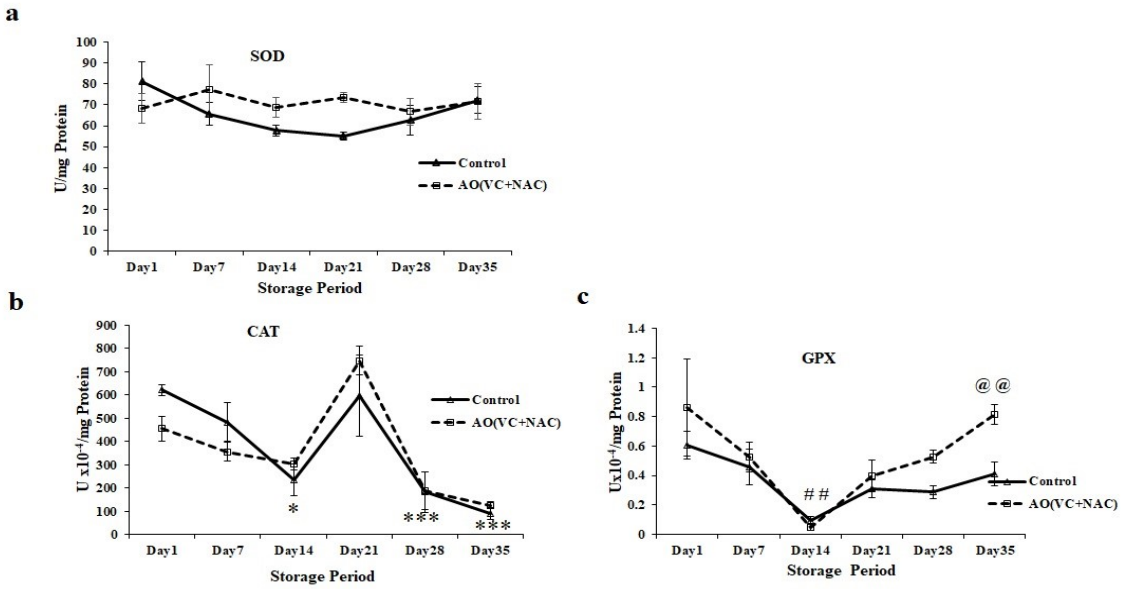

Fig. 2a. Superoxide dismutase activity in stored erythrocytes**; 2b.** Catalase activity in stored erythrocytes; **2c.** Glutathione peroxidase activity in stored erythrocytes Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software. * represents significant changes during storage in Controls with respect to day 1 (P<0.05). (**represents P<0.01), (*** represents P<0.001). # represents significant changes during storage in VC+NAC group with respect to day 1 (P<0.05), (# #represents p< 0.01); @ @ represents significant changes during storage in the VC+NAC group on day 35 with respect to day 14 (P<0.01).

3.2.2. Non-enzymatic antioxidants

Glutathione

Glutathione levels showed variations in both groups, although the changes were statistically insignificant.

Glutathione levels showed a range from 0.86 mM/mg protein (day 1), 0.9 mM/ mg protein (day 14), and 1 mM mg protein (day 35) in Controls, whereas in VC+NAC ranged from 0.95 mM/mg protein (day 1), 1 mM/ mg protein (day 14) and 1.2 mM/ mg protein (day 35). However, these changes are statistically similar in both groups.

VC+NAC group had higher levels of glutathione than the Controls throughout the storage period (Fig. 3a).

Uric acid

Though uric acid levels were maintained in both groups, the VC+NAC group had higher uric acid levels throughout the storage (Fig. 3b).

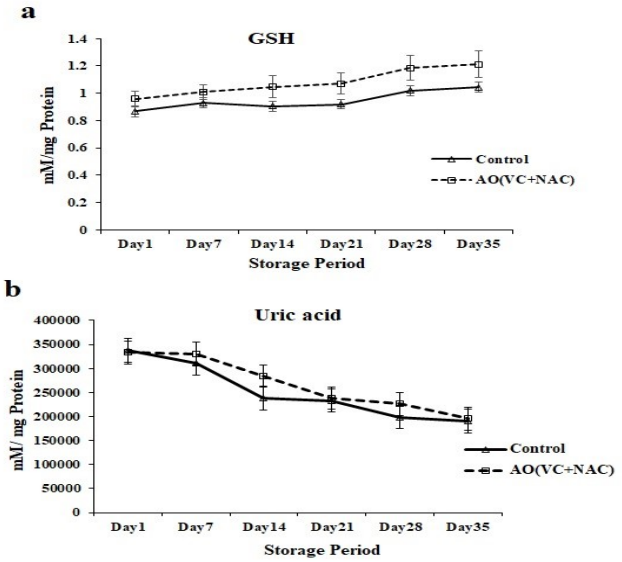

Fig. 3a. Glutathione in stored erythrocytes; **3b.** Uric acid levels in stored erythrocytes. Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software.

3.3. Oxidative stress markers

3.3.1. Reactive oxygen species

Superoxide radicals

Variations in superoxide levels were similar throughout the storage period.

Superoxide levels increased towards the end of storage in Controls, ranging from 0.57 mM/mg protein (day 1), 0.37 mM/mg protein (day 21) to 0.60 mM/mg protein (day 35). However, the VC+NAC group showed a decline towards the end of storage (0.69 mM/mg protein (day 1) to 0.38 mM/mg protein (day 35).

Superoxide radicals decreased by 46% and 36% on days 28 and 35, respectively, in the VC+NAC group in comparison to Controls (Fig. 4).

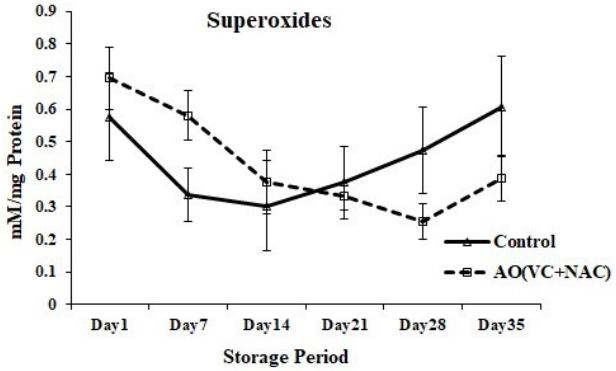

Fig. 4. Superoxide radicals in stored erythrocytes Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software.

3.3.2. Protein Oxidation markers

Protein oxidation products- advanced oxidation protein products and protein sulfhydryls showed variations throughout the storage. Nonetheless, these changes were statistically insignificant.

AOPP levels varied from 0.36 mM/mg protein (day 1) to 0.38 mM/mg protein (day 35) in Controls. AOPP levels remained constant (0.30 mM/ mg protein) until day 21 and increased slightly at the end of storage (0.38 mM/ mg protein) in VC+NAC (Fig. 5a).

Protein sulfhydryls varied from 303 µM/ mg protein (day 1) to 314 µM/ mg protein (day 35) in Controls. However, sulfhydryls ranged from 442 µM/ mg protein (day 1) to 563 µM/ mg protein (day 35) in the VC+NAC group. P-SH were higher by 46% and 79% on days 1 and 35 in the VC+NAC group than in Controls (Fig. 5b).

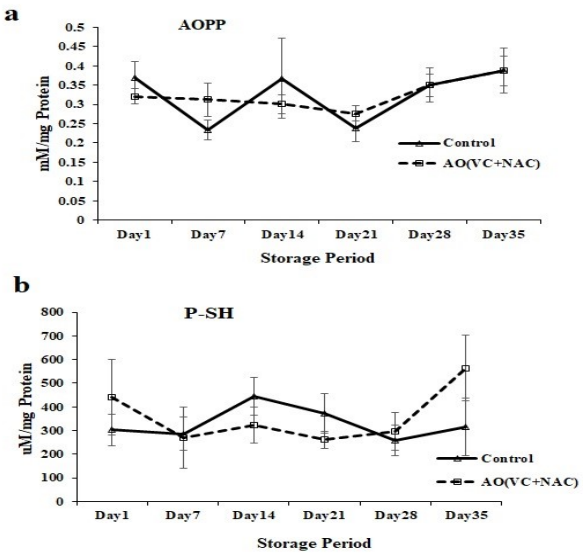

Fig. 5a. Advanced oxidation protein products in stored erythrocytes; **5b.** Protein sulfhydryls in stored erythrocytes Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software.

3.3.3. Lipid Peroxidation markers

Conjugate dienes showed similar variations throughout the storage in both groups.

Conjugate dienes showed insignificant variations in both Control and VC+NAC groups.

Conjugate dienes decreased from 324 µM on day 1 to 304 µM on day 21 and normalized to 308 µM on day 35 in Controls.

Conjugate dienes declined from 377 µM (day 1) to 298 µM (day 21) and normalized to 315 µM (day 35) in VC+NAC.

Changes in TBARS were significant in both groups.

TBARS decreased by 54% ($p<0.01$), 78% ($p<0.0001$) & 63% ($p<0.0001$) in Controls and 48% ($p<0.05$), 90% ($p<0.0001$) & 84% ($p<0.0001$) in VC+NAC respectively, on days 14, 28 & 35.

TBARS levels were lower by 53% and 56% on days 28 and 35, respectively, in the VC+NAC group in comparison to Controls (Fig. 6).

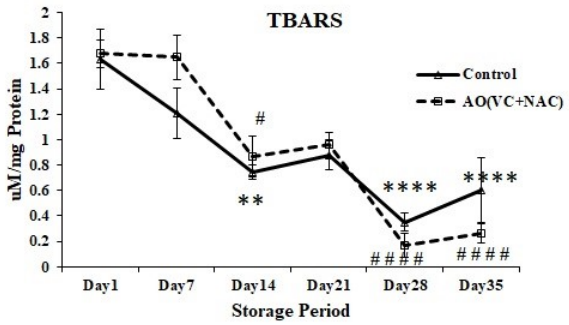

Fig. 6. Thiobarbituric acid reactive substances in stored erythrocytes Values are expressed as Mean ± SE from 5 samples. Changes between the groups and subgroups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software. * represents significant changes during storage in Controls with respect to day 1 (**represents P<0.01). (**** represents P<0.0001). # represents significant changes during storage in VC+NAC group with respect to day 1 (P<0.05). (# # # # represents P<0.0001).

3.4. Metabolic marker

Glucose

Glucose levels showed variations in Controls, whereas remained constant in VC+NAC.

Glucose levels decreased on day 7 (47%), peaked on days 14 & 21, and normalized on days 28 (54%) and 35 (42%) when compared to day 1 in Controls. However, glucose levels were maintained till day 28 and decreased on day 35 (44%) in the VC+NAC group (Fig. 7).

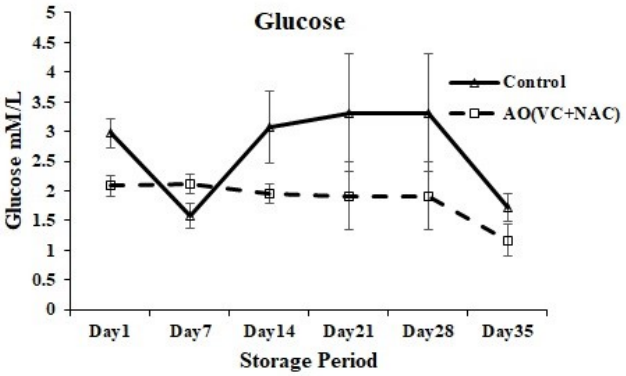

Fig. 7. Glucose levels in stored erythrocytes. Values are expressed as Mean ± SE from 5 samples. Changes between the groups and sub groups were analyzed by Two-way ANOVA followed by Bonferroni Post Test, using GraphPad Prism 6 software.

4. Discussion

Hemoglobin levels in the VC+NAC group elucidate the reverse conversion of methemoglobin to hemoglobin, which could be due to the reduction of methemoglobin by the antioxidants vitamin C (36) and N-acetylcysteine (37). These antioxidants were able to protect Hb from oxidation, even with higher levels of reactive species, on day 14 (6).

Superoxide dismutase catalyzes the conversion of superoxide radicals ($O_2^{\bullet-}$) into H_2O_2 and O_2 (38). SOD activity varied similarly in both groups. However, higher SOD activity was observed in the VC+NAC group from day 7 till day 21. Similar results were observed with NAC supplementation, where SOD activity ameliorated with low levels of glutathione (39) and cisplatin-induced oxidative stress conditions (40). Vitamin C also elevated SOD activity in Dimethoate-induced oxidative stress (41).

Superoxide radicals are generated in erythrocytes due to the autoxidation of oxyhemoglobin (oxyHb) (38). Superoxide levels increased towards the end of the storage in Controls, whereas declined in the VC+NAC group, indicating the superoxide scavenging activity of NAC and vitamin C (9,42,43).

Catalase and glutathione peroxidase (GPX) form vital defense mechanisms against H_2O_2. Low levels of H_2O_2 (10^{-9} M) are scavenged by GPX. The GSH-dependent activity of GPX has been regarded as the primary defense against H_2O_2 (38). Catalase activity significantly decreased in Controls due to its susceptibility to oxidation, which also corroborates with the results of Huyut et al. (44). CAT activity was maintained in VC+NAC throughout the storage period, as VC and NAC directly scavenged reactive species (45,9). However, Catalase activity was higher in the VC+NAC group on day 21, in comparison to Control suggesting that VC and NAC upregulated the catalase activity (46,47). GPX activity decreased on day 14 due to the H_2O_2 scavenging activity of NAC and VC (42,43), whereas it increased towards the end of the storage to combat oxidative stress.

Glutathione and Uric acid are the major endogenous

antioxidant systems in erythrocytes. GSH directly scavenges free radicals and acts as an important sulfhydryl buffer by maintaining P-SH groups in the reduced state (38). GSH synthesis in erythrocytes is limited by the availability of the substrate amino acids, especially cysteine (47). This was substantiated by GSH results, where the VC+NAC group exhibited GSH-replenishment till the end of storage. NAC protects erythrocytes from GSH depletion until 21 days of storage (14). Uric acid in erythrocytes can be attributed to its direct antioxidant activity by quenching free radicals and reactive oxygen species. Uric acid levels were comparatively higher in the VC+NAC group, indicating the activation of endogenous antioxidant defenses.

Proteins are major targets due to their rapid reaction with radicals and oxidants. The action of hypochlorous acid and chloramines forms dityrosine containing cross-linked protein products-AOPP. Vitamin C and N-acetylcysteine scavenge the free radicals (45,9), evidenced in the AOPP results. AOPP levels were maintained in VC+NAC, whereas they were higher on days 14 and 21 in Controls. Protein sulfhydryls are found largely in cysteine components of proteins and at lower concentrations in glutathione. Protein sulfhydryls can be reversibly oxidized to disulfides (13). Protein sulfhydryls were maintained in the reduced state till the end of the storage in the VC+NAC group, as NAC replenishes glutathione (42).

The polyunsaturated fatty acids present in erythrocytes are prone to oxidative stress. Lipid peroxidation generates hydroperoxides and endoperoxides, which, in turn, produce aldehydes. The primary products and secondary products of lipid peroxidation, i.e., conjugate dienes, were maintained, and TBARS decreased, indicating the successful scavenging activity of antioxidant defenses in both groups. This was evidenced by the inverse correlation between GSH and TBARS in the VC+NAC group (r = - 0.9547). TBARS were lower at the end of storage in the VC+NAC group than in Controls. The decrease in TBARS is directly correlated with superoxide levels in the VC+NAC group (r = 0.8731), which emphasizes the free radical scavenging effect of vitamin C and NAC.

Glucose levels were conserved till day 28 in the VC+NAC group, in contrast to the Control group, where it varied throughout the storage. Glucose metabolism efficiently maintains glutathione in the reduced form, thereby protecting sulfhydryls from oxidation (42), which was evident in the results of glutathione and sulfhydryls.

Vitamin C and N-acetyl cysteine combination had a positive influence on stored erythrocytes and enhanced their ability to endure oxidative stress (Fig. 8). Therefore, vitamin C with N-acetylcysteine is a potential antioxidant combination that can be employed to develop effective storage solutions. Storage solutions augmented with antioxidants can influence the efficacy of erythrocytes, which have a direct impact on post-transfusion recovery.

However, the findings of this study need to be further validated in the context of human samples and their post-transfusion characteristics, such as viability and efficacy.

Vitamin C and N-acetylcysteine activated antioxidant defenses and protect erythrocytes from oxidative damage until day 21. Thus, vitamin C and N-acetylcysteine complement each other, and their synergistic action effectively modulates the oxidative changes during storage. This study provides new avenues for the development of additive solutions and blood banking practices to minimize the potential risks under various clinical conditions such as surgery, trauma, and anemia.

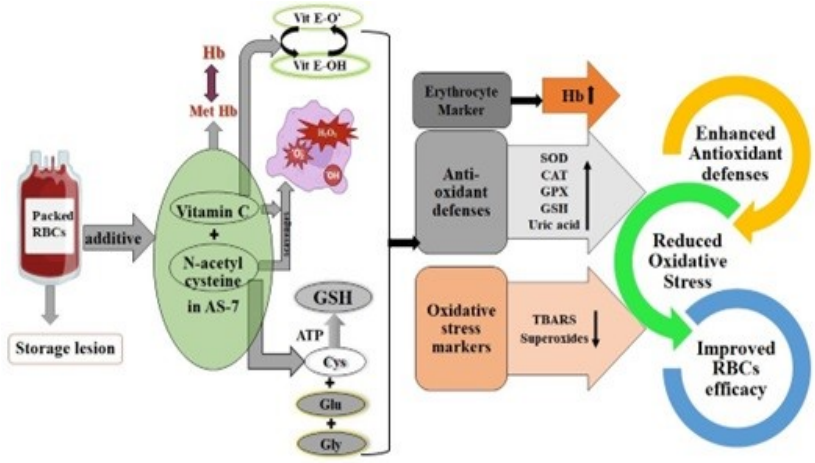

Fig. 8. Possible mechanism of vitamin C and N-acetylcysteine as additives to improve the efficacy of stored erythrocytes AS-7: Additive solution-7, Vit E'O: tocopheroxyl radical (oxidize form), Vit E-OH: α-tocopherol (reduced form), Gly: Glycine, Glu: Glutamate, Cys: Cysteine, GSH: Glutathione, ATP: Adenosine triphosphate, Hb: Hemoglobin, Met Hb: Methemoglobin, SOD: Superoxide dismutase, CAT: Catalase, GPX: Glutathione peroxidase, TBARS: Thiobarbituric acid reactive substances.

Conflict of interest
The author has no conflicts of interest to declare.

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Authors' contributions
Concept: V.R., Design: V.R., Data Collection or Processing: M.P., Analysis or Interpretation: M.P., Literature Search: M.P., Writing: V.R., M.P.

Ethical Statement
Animal care and maintenance were according to ethical committee regulations (841/b/04/ CPCSEA). Ethical approval was obtained from the Institutional Animal Ethics Committee (IAEC) at the Nargund College of Pharmacy, Bengaluru (IAEC/NCP/117/2022).

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