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Vitamin C as a modulator of oxidative stress in erythrocytes of stored blood

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ABSTRACT

Aim: To determine the effects of Vitamin C (VC-ascorbic acid) as an additive on erythrocytes of stored blood. **Background:** Oxidative stress (OS) plays a major role in the formation of storage lesion of erythrocytes. Antioxidants, such as VC could be beneficial in combating oxidative damage during storage. **Materials and methods:** Blood obtained from male *Wistar* rats was stored at 4 °C in anticoagulant solution citrate-phosphate-dextrose-adenine solution. Blood samples were divided into 3 groups – (i) Controls, (ii) VC 10 (VC at a concentration of 10 mM), (iii) VC 30 (VC at a concentration of 30 mM). Markers of OS in erythrocytes such as – hemoglobin, superoxides, antioxidant enzymes (superoxide dismutase; SOD, catalase and glutathione peroxidase), hemolysis, lipid peroxidation products (conjugate dienes and malondialdehyde), protein oxidation products and ascorbic acid were determined on days 0, 10 and 15 of storage. **Results:** Addition of ascorbic acid to the storage solution contributed to the protection of erythrocytes from oxidative damage. Ascorbic acid at a concentration of 30 mM decreased SOD levels and increased protein sulfhydryls (P-SH) levels on day 15 showing that higher concentration supplemented the inherent antioxidant defense system of erythrocytes during blood storage. **Conclusion:** VC proved to be effective in combating OS during blood storage. However, further exploration of antioxidants as additives and the erythrocyte storage lesion would result in better management of blood storage.

Introduction

Blood transfusion is an acute intervention, implemented to solve life- and health-threatening conditions on a short-term basis, and in general its long term effects tend to be of secondary importance [1]. Red blood cell (RBC) transfusion is a key element of modern medical care. The ability to store RBCs for reasonable times clearly improves their availability and lowers their cost [2]. Whole blood can be stored up to a period of 35 days in anticoagulant solution CPDA-1 (citrate, phosphate, dextrose and adenine) while erythrocytes (RBCs) possess a shelf life of 42 days at 4 °C in SAGM (saline, adenine, glucose and mannitol) solution [2].

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During storage, RBCs undergo structural and functional changes that may reduce function and viability after transfusion [3]. Changes accompanying the storage of RBCs are known as "storage lesion". During storage of blood and its components, in various additive solutions, it has been found that oxidative stress (OS), influences their functioning, efficacy and shelf life. OS is the fundamental phenomenon leading to the formation of storage lesion in erythrocytes [4–6]. Protein oxidation and lipid peroxidation (LPO) also occur, in turn causing vesiculation and loss of deformability [7]. OS plays an important role in the formation of storage lesions in blood and its components and there exists an inherent antioxidant system in blood to combat OS. However, during storage, there is a reduction in blood antioxidant capacity, leading to increased susceptibility to OS. Hence, there is a possibility of utilizing antioxidants to aid in protecting the erythrocytes against oxidative damage during storage.

Vitamin C (VC) or ascorbic acid is found in erythrocytes and can scavenge a wide variety of free radicals directly in the aqueous phase. VC reduces superoxide and lipid peroxyl radicals, a synergistic agent for Vitamin E [8–10] and protects membrane [11]. Ascorbic acid serves as both an antioxidant and a prooxidant. As a prooxidant, it generates cofactors of activated oxygen radicals during the promotion of LPO, in presence of Fe^{3+} and Cu^{2+} ions [12]. Studies on the effects of ascorbic acid on erythrocytes during storage have focused on biochemical parameters, hemolysis and fragility [13–19].

Rat models provide a platform for rapid experimentation, in turn forming a basis for further studies on human models. A comparative study between rat and human erythrocytes revealed that rat erythrocytes when stored for 1 week in CPDA-1 solution, develop a storage lesion equivalent to that of a lesion formed in human erythrocytes stored for 4 weeks [20]. Our previous work has elucidated that reactive oxygen species are maximum on days 10 and 15 of storage [21] and hence, our current study focuses on the storage lesion on days 0, 10 and 15 of storage. Therefore this study aims to evaluate the effects of VC on erythrocytes of stored rat blood through the following 3 aspects – (i) oxidant levels of erythrocytes in terms of hemolysis, LPO and protein oxidation products, (ii) antioxidant enzymes and (iii) VC as an additive in storage solution.

Materials and methods

Animal care and maintenance was in accordance with ethical committee regulations.

Chemicals

Hemoglobin (Hb) reagent was obtained from Coral Clinical Systems, Goa, India. Thiobarbituric acid (TBA), Epinephrine, Glutathione reductase (GR), Glutathione (GSH) and Bovine Serum Albumin (BSA) were purchased from Sigma–Aldrich Chemicals [St. Louis, MO, USA]. All other 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 [6]. In brief, a syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. Four to five milliliter of blood was carefully aspirated from the heart into 5 mL plastic collecting tubes with anticoagulant solution, citrate-phosphate-dextrose-adenine-1 (CPDA-1).

Experimental design

Blood was drawn from *Wistar* rats (4 months old) and stored at 4 °C in anticoagulant solution, CPDA-1. Blood samples were divided into 3 groups – (i) Controls, (ii) VC 10 (samples with VC as additive at a concentration of 10 mM), (iii) VC 30 (samples with VC as additive at a concentration of 30 mM). Erythrocytes were isolated from stored blood and the biomarkers of OS were studied.

Erythrocyte separation

Erythrocytes were isolated by centrifugation for 20 min at 1000 × *g* at 4 °C, plasma and buffy coat were removed using a micropipette. Cell pellet was washed 3 times and suspended in an equal volume of isotonic phosphate buffer [22]. This constituted the erythrocyte suspension.

Hb estimation

Hb was measured using Hemocor-D Kit [Coral Clinical Systems, Goa, India], which utilizes Cyanomethemoglobin method [23]. Whole blood was incubated with Hb reagent for 3 min at room temperature and absorbance was measured colorimetrically at 540 nm. Hb concentration was represented in terms of g/dL.

Superoxide

Superoxide generated was determined by the method of Olas and Wachowisz [24]. Cytochrome c (160 μM concentration) was added to equal volume of sample and incubated at 37 °C for 5–15 min. Samples were then centrifuged at 3500 rpm for 5 min. Absorbance of the supernatant was measured at 550 nm.

Antioxidant enzymes

Superoxide dismutase [SOD, EC 1.15.1.1]
SOD was measured by the method of Misra and Fridovich [25]. Hemolysate was added to carbonate buffer [0.05 M]. Epinephrine was added to the mixture and measured [ELICO, Model SL 159, India] at 480 nm. SOD activity is expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50%.

Catalase [CAT, EC 1.11.1.6]
CAT was determined by the method of Aebi [26]. Briefly, hemolysate with absolute alcohol was incubated at 0 °C. An aliquot was taken up with 6.6 mM hydrogen peroxide (H_2O_2)

and decrease in absorbance was measured at 240 nm. An extinction coefficient of 43.6 M cm^{-1} was used to determine enzyme activity.

Glutathione peroxidase [GSH-Px, EC.1.11.1.9]
GSH-Px was analyzed by the method of Flohe and Gunzler [27]. Fifty microliters of 0.1 M phosphate buffer (pH 7.0), 100 μL enzyme sample, 100 μL GR (0.24 units), and 100 μL of 10 mM GSH were mixed and pre-incubated for 10 min at 37 °C followed by addition of 100 μL of 1.5 mM β-nicotinamide adenine dinucleotide phosphate reduced tetra sodium salt (NADPH) in 0.1% sodium bicarbonate ($NaHCO_3$). The overall reaction was started by adding 100 μL of pre-warmed tert-butyl hydroperoxide and the decrease in absorption at 340 nm was monitored for 3 min.

Hemolysis

A 5% suspension of packed erythrocytes in buffer was mixed with equal volume of 8 mM H_2O_2. Mixtures were incubated at 37 °C in an incubator. Hemolysis was determined by measuring released Hb into the supernatant of induced samples at 540 nm and expressed on the basis of maximum absorbance [100%] in aliquots of erythrocytes completely hemolysed in distilled water [28].

LPO

Conjugate dienes
Primary marker of LPO, conjugate dienes, was assessed by the method of Olas and Wachowisz [24]. Briefly, samples were diluted 5 times in ether:ethanol (1:3, v/v), vortexed for 1 min, centrifuged at 8000 rpm for 20 min and supernatant was analyzed in a spectrophotometer at 234 nm. Conjugate dienes produced was calculated using molar extinction coefficient of 29 500 M^{-1} cm^{-1}.

Malondialdehyde (MDA)
MDA, a product of LPO was determined according to the method of Ohkawa et al. [29]. In brief, hemolysate was added to 8.1% sodium dodecyl sulfate (SDS), vortexed and incubated at room temperature. This was followed by the addition of 20% acetic acid and 0.6% TBA, and placed in boiling water bath. Samples were allowed to cool and butanol-pyridine was added and centrifuged. Absorbance was measured at 532 nm with 1, 1, 3, 3-tetramethoxy propane as a standard. MDA concentration was expressed as nmol/mg protein.

Protein oxidation (protein sulfhydryls (P-SH))

P-SH was measured as described by Habeeb [30]. In brief, 0.08 mol/L sodium phosphate buffer containing 0.5 mg/mL of ethylene diamine tetra acetic acid disodium salt (Na_2-EDTA), and 2% SDS were added to each assay tube. 0.1 mL of 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) was also added. Absorbance was measured at 412 nm. P-SH was calculated from net absorbance and molar absorptivity, 13 600 mol/L cm^{-1}.

Ascorbic acid

Ascorbic acid levels were estimated by the method of Omaye et al. [31]. Briefly, Samples were mixed with equal volumes of 10% trichloroacetic acid (TCA) and centrifuged at 3500 × *g* for 20 min. Supernatant was mixed with DTC reagent (2,4-dinitrophenylhydrazine/thiourea/copper sulfate solution) and incubated for 3 h at 37 °C. Sixty five percent ice cold sulfuric acid was added and samples were allowed to stand at room temperature for 30 min. Absorbance was measured at 520 nm.

Protein determination

Protein was determined in lysate and membrane by the method of Lowry et al., using BSA as standard [32].

Statistical analyses

Results are represented as mean ± standard error (SE). Values between the groups were analyzed by two-way analysis of variance (ANOVA) and was considered significant at $P < 0.05$. Bonferroni's *Post* Test was performed for all assays using GraphPad Prism 5 software.

Results

Hb

Changes in Hb were insignificant during storage in all samples with respect to day 0.

Superoxide

Superoxide levels changed significantly as storage progressed. Superoxide levels reduced with storage by 70% (day 10) and 80% (day 15) against day 0 in controls. Decrements of 75% (day 10) and 90% (day 15) were observed in VC 10 with day 0. VC 30 also showed decreases in superoxides by 85% (days 10 and 15) against day 0.

Changes between antioxidants were insignificant (Fig. 1).

Antioxidant enzymes

SOD
Significant changes in SOD activity were observed in all groups with storage. Increments of 150% and 40% were observed in controls on days 10 and 15 (day 0). SOD activity in VC 10 increased by 83% and 110% on days 10 and 15 against day 0. VC 30 showed an increase in the SOD activity on day 10 by 63% with respect to day 0.

SOD varied significantly between antioxidants. SOD increased in VC 10 by 65% than control while VC 30 showed lower levels of SOD (40%) than VC 10 on day 15 (Fig. 2).

CAT
Our results showed significant changes in CAT activity with storage. An increase of 190% (day 10) and 43% (day 15) was observed in controls against day 0. CAT increased by 100%

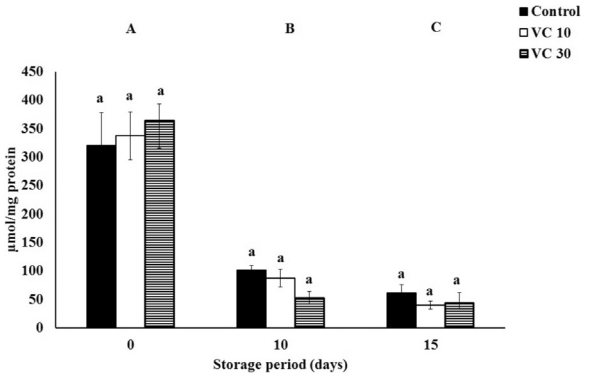

Fig. 1 – Superoxide in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. P < 0.05 was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

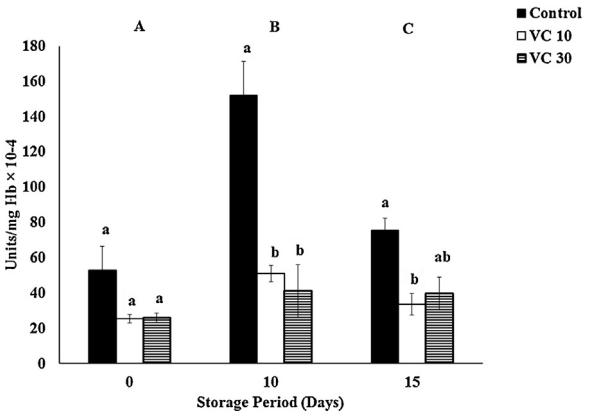

Fig. 3 – Catalase in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. P < 0.05 was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

and 32% in VC 10 while it incremented by 55% in VC 30 on days 10 and 15.

Changes in CAT between antioxidants were significant. Reductions of 60% (days 10 and 15) were observed in VC 10 and 70% (day 10) were observed in VC 30 against controls (Fig. 3).

GSH-Px

GSH-Px activity was significant with storage period. There was an elevation of 220% and 110% on days 10 and 15 respectively in controls (day 0). GSH-Px increased by 52% (day 10) and 280% (day 15) with respect to day 0 in VC 10. GSH-Px activity was higher in VC 30 by 30% on days 10 and 15.

Changes between antioxidants were significant. VC 10 showed a reduction in GSH-Px by 65%, 85% and 40% in comparison to respective controls on days 0, 10 and 15. Levels of GSH-Px reduced by 73% and 57% in VC 30 on days 10 and 15, against controls (Fig. 4).

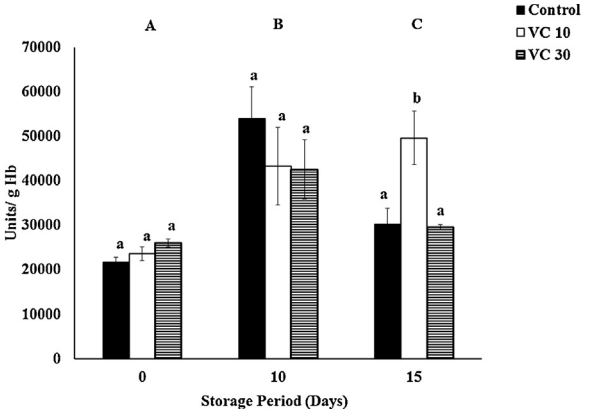

Fig. 2 – Superoxide dismutase in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. P < 0.05 was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

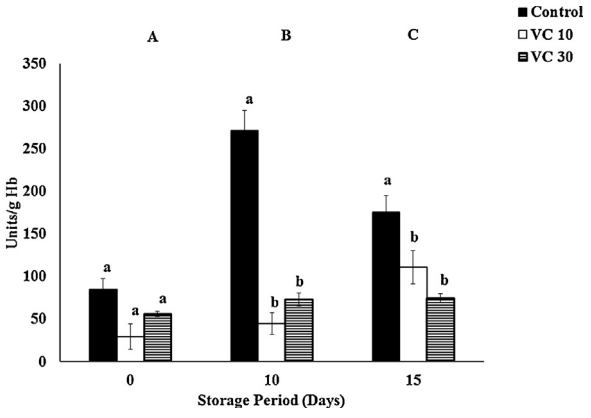

Fig. 4 – Glutathione peroxidase in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. P < 0.05 was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

Hemolysis

Significant changes in hemolysis were observed in all samples against storage. Hemolysis increased by 46% and 65% in controls on days 10 and 15 during storage. Levels of hemolysis increased in VC 10 and VC 30 by 39% and 43% on days 10 and 15 respectively.

Changes between antioxidants were insignificant.

LPO

Conjugate dienes

Changes between controls and experimentals during storage were insignificant.

MDA

MDA was assayed as a measure of LPO and insignificant changes were observed during storage period. Changes between groups were insignificant.

Protein oxidation (protein oxidation (P-SH))

Lysate

P-SH was significant with storage. Increases of 86% and 130% were observed on days 10 and 15 in controls. P-SH reduced by 40% on day 10 while it increased by 35% on day 15 in VC 10 and VC 30.

VC 30 showed rises of 370% (day 0) with control. P-SH incremented in VC 30 by 200% and 95% on day 15 against control and VC 10 respectively (Fig. 5).

Membrane

P-SH was insignificant with storage and between groups in membrane.

Ascorbic acid

Ascorbic acid varied significantly with storage period. Controls showed increased levels of ascorbic acid on day 10 (150%) and day 15 (30%) against day 0. Ascorbic acid levels were higher in VC 10 by 50% on day 10. VC 30 showed higher ascorbic acid on days 10 and 15 (70% and 160% respectively) against day 0.

Ascorbic acid levels were significant among antioxidants. Ascorbic acid decremented by 35% in VC 30 against control on day 10 while it incremented by 85% on day 15 against respective control and VC 10 (Fig. 6).

Discussion

Hb, the oxygen carrying molecule present in erythrocytes, is exposed to a constant state of oxygen. Iron (ferrous) present in Hb, upon exposure to oxygen undergoes autoxidation to MetHb, which is unable to bind and carry oxygen [33]. This reaction is a reversible one and Hb can be restored by several antioxidant mechanisms [34]. Changes in Hb are indicative of successful conversion of MetHb (formed during storage) to Hb.

Superoxides ($O_2^{\bullet -}$) are produced in erythrocytes either by auto-oxidation of Hb or by exogenous sources [35, 36]. These radicals can directly or indirectly affect red cell membranes [37]. Levels of $O_2^{\bullet -}$ decreased in all groups with storage, indicative of successful scavenging by SOD and interaction of superoxide in reactive oxygen species (ROS) cascade to form H_2O_2, hydroxyl and peroxynitrite [11].

Various endogenous and exogenous mechanisms are present in order to combat the deleterious effects of OS by ROS [38]. SOD, CAT and GSH-Px play an important role in the protection of erythrocytes against oxygen toxicity [39]. The first barrier of defense against superoxide radicals is SOD – an enzyme which facilitates the dismutation of

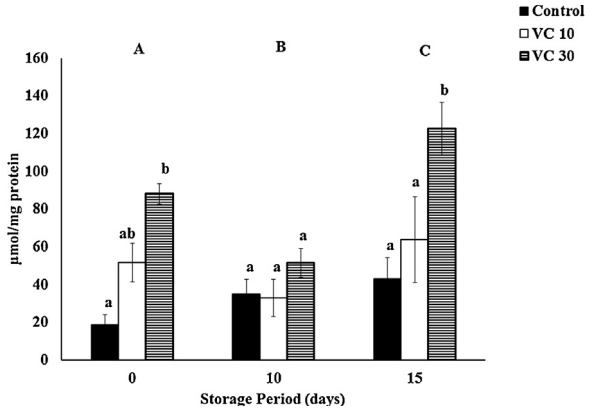

Fig. 5 – Protein sulfhydryls (hemolysate) in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. $P < 0.05$ was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

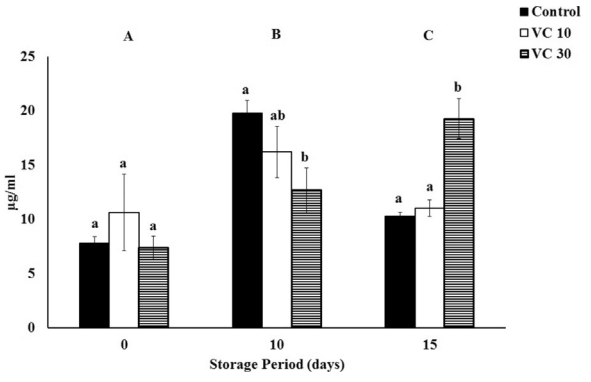

Fig. 6 – Ascorbic acid in erythrocytes of stored blood. Values are expressed as mean ± SE from 5 animals. VC 10 – Vitamin C (10 mM); VC 30 – Vitamin C (30 mM). Changes were analyzed by two-way ANOVA followed by Bonferroni's post-test using GraphPad Prism Software. $P < 0.05$ was considered significant. Changes between the groups are represented in upper case, while changes within a group are represented in lower case. Those not sharing the same letters are significant

superoxide radicals using the metal ion located in its active site [40]. SOD activity was higher on day 10 in accordance with higher levels of ROS. Decrease of SOD activity upon progression of storage period can be attributed to reduction in free radicals which can be due to effective antioxidant system of erythrocytes. Ascorbic acid itself reacts with superoxide radicals [41], hence assisting SOD in quenching superoxide radicals, as observed in the results of VC 30 on days 10 and 15.

GSH-Px efficiently protects the cell membrane from LPO and catalyzes reactions of hydroperoxides with GSH to form glutathione disulfide (GSSG) [42]. GSH-Px enzyme competes with CAT for H_2O_2 as a substrate and is a major source of protection against low levels of OS [43]. CAT mediates the decomposition of H_2O_2 through catalytic or peroxidative mechanisms at high levels of OS [44]. Our study shows activation of CAT and GSH-Px in all groups in accordance with SOD activity, to combat H_2O_2. High levels of CAT and GSH-Px were observed on day 10 in controls in accordance with high levels of ROS [21]. Decrease in antioxidant enzyme activity in control on day 15, may be due to free radical scavenging activity of antioxidant enzymes and their inhibition by free radical species [45, 46]. GSH-Px and CAT were significantly lower in experimental groups and this could be attributed to radical scavenging effects of VC [8–10], thereby reducing H_2O_2 formation.

Erythrocyte membrane is highly susceptible to peroxidative modifications due to large amounts of polyunsaturated fatty acids, oxygen rich environment and iron rich Hb [11]. Hemolysis is a reliable marker of erythrocyte membrane stability and increased in all groups indicating that VC could not protect the membrane from oxidation completely. LPO is a process that is accelerated by ROS cascade [47] and causes loss of membrane deformability and integrity, in turn leading to cell death [11]. Conjugate dienes and MDA are primary and secondary markers of LPO respectively. As storage progressed, LPO was insignificant demonstrating that VC maintained conjugate dienes and MDA.

Protein oxidation occurs due to OS and in erythrocytes, P-SH are one of the protein oxidation products of great importance. P-SH can be reversibly or irreversibly modified based on the extent of oxidative modifications [48]. P-SH levels increased on day 15 of storage in VC 30, indicative of the antioxidant effect of ascorbic acid. VC successfully maintained sulfhydryl levels during storage.

Ascorbic acid was analyzed to determine the utilization of the added VC during storage. Ascorbic acid levels increased on day 15 in VC 30, showing early utilization of VC in erythrocyte antioxidant defense.

Conclusion

VC assisted erythrocytes by scavenging free radicals, activating antioxidant enzymes and maintaining Hb, LPO and protein oxidation products. VC at a concentration of 30 mM decreased SOD levels and increased P-SH levels on day 15 showing that higher concentration supplemented the inherent antioxidant defense system of erythrocytes and protected them against OS during storage. Our findings give an insight into the effects of VC as an additive in blood storage solutions. This study forms a basis for further investigations of effective blood storage solutions.

Authors' contributions/Wkład autorów

According to order.

Conflict of interest/Konflikt interesu

None declared.

Financial support/Finansowanie

None declared.

Ethics/Etyka

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals.

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REFERENCES/PIŚMIENNICTWO

[1] Tsai AG, Hofmann A, Cabrales P, Intagliata M. Perfusion vs. oxygen delivery in transfusion with "fresh" and "old" red blood cells: the experimental evidence. Transfus Apher Sci 2010;43:69–78.
[2] Hess JR. Red cell storage. J Proteomics 2010;73:368–373.
[3] Kucukakin B, Kocak V, Lykkesfeldt J, Nielsen HJ, Magnussen K, Rosenberg J, et al. Storage-induced increase in biomarkers of oxidative stress and inflammation in red blood cell components. Scand J Clin Lab Invest 2011;71:299–303.
[4] Chaudhary R, Katharia R. Oxidative injury as contributory factor for red cells storage lesion during twenty eight days of storage. Blood Transfus 2011;10:59–62.
[5] D'Amici GM, Mirasole C, D'Alessandro A, Yoshida T, Dumont LJ, Zolla L. Red blood cell storage in SAGM and AS3: a comparison through the membrane two-dimensional electrophoresis proteome. Blood Transfus 2012;10:s46–s54.
[6] Vani R, Koshy AA, Koushik AK, Kaur H, Kumari K, Agrawal M, et al. The efficacy of erythrocytes isolated from blood stored under blood bank conditions. Transfus Apher Sci 2012;47:359–364.

[7] Tinmouth A, Fergusson D, Yee IC, Herbert PC. Clinical consequences of red cell storage in the critically ill. Transfusion (Paris) 2006;46:2014–2027.
[8] Beyer RE. The role of ascorbate in antioxidant protection of biomembranes: interaction with Vitamin E and coenzyme Q. J Bioenerg Biomembr 1994;26:349–358.
[9] Halliwell B. Vitamin C: antioxidant or prooxidant *in vivo*? Free Radic Res 1996;25:439–454.
[10] Durak D, Kalendar S, Uzun FG, Ogutcu A, Uzunhisarcikli M, Kalender. Mercury chloride-induced oxidative stress in human erythrocytes and the effect of vitamins C and E in vitro. Afr J Biotechnol 2010;9:488–495.
[11] Cimen MYB. Free radical metabolism in human erythrocytes. Clin Chim Acta 2008;390:1–11.
[12] Yu BP. Cellular defenses against damage from reactive oxygen species. Immunol Rev 1994;14:139–162.
[13] Moore GL, Ledford M, Brummell MR. Improved red blood cell storage using optional additive systems (OAS) containing adenine, glucose and ascorbate-2-phosphate. Transfusion (Paris) 1981;21:723–731.
[14] Moore GL, Marks DH, Carmen RA, Ledford ME, Nelson EJ, Fishman RJ, et al. Ascorbate-2-phosphate in red cell preservation. Transfusion (Paris) 1988;28:221–225.
[15] Wood LA, Beutler E. The effect of periodic mixing on the preservation of 2, 3-diphosphoglycerate (2,3-DPG) levels in stored blood. Blood 1973;42:17–25.
[16] Zan T, Tao J, Tang RC, Liu IC, Liu Y, Huang B, et al. Effect of vitamin C antioxidative protection on human red blood cells. J Exp Hematol 2005;13:1106–1108.
[17] Raval JS, Fontes J, Banerjee U, Yazer MH, Mank E, Palmer AF. Ascorbic acid improves membrane fragility and decreases haemolysis during red blood cell storage. Transfus Med 2013;23:87–93.
[18] Pallotta V, Gevi F, D'Alessandro A, Zolla L. Storing red blood cells with vitamin C and N-acetylcysteine prevents oxidative stress-related lesions: a metabolomics overview. Blood Transfus 2014;12:376–387.
[19] Stowell SR, Smith NH, Zimring JC, Fu X, Palmer AF, Fontes J, et al. Addition of ascorbic acid solution to stored murine red blood cells increases posttransfusion recovery and decreases microparticles and alloimmunization. Transfusion (Paris) 2013;53:2248–2257.
[20] d'Almeida MS, Jagger J, Duggan M, White M, Ellis C, Chin-Yee IC. A comparison of biochemical and functional alterations of rat and human erythrocytes stored in CPDA-1 for 29 days: implications for animal models of transfusion. Transfus Med 2000;10:291–303.
[21] Soumya R, Vani R. CUPRAC-BCS and antioxidant activity assays as reliable markers of antioxidant capacity in erythrocytes. Hematology 2015;20:165–174.
[22] Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. Arch Biochem Biophys 1963;100:119–130.
[23] Hawk FB. Physiological chemistry. New York: Tata McGraw Hill; 1965.
[24] Olas B, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. Thrombosis Res 2002;106:143–148.
[25] Misra HP, Fridovich I. The role of superoxide anion in the auto oxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247:3170–3175.
[26] Aebi H. Catalase *in vitro*. Meth Enzymol 1984;105:121–126.
[27] Flohe L, Gunzler WA. Assays for glutathione peroxidase. Meth Enzymol 1984;105:114–121.
[28] Senturk UK, Gunduz F, Kuru O, Aktekin MR, Kipmen D, Yalçin O, et al. Exercise-induced oxidative stress affects erythrocyte in sedentary rats but not exercise-trained rats. J Appl Physiol 2001;91:1991–1994.
[29] Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxidation in animal tissues by thiobarbituric acid reaction. Anal Biochem 1979;95:351–358.
[30] Habeeb AF. Reaction of protein sulfhydryl groups with Ellman's reagent. Meth Enzymol 1972;34:457–464.
[31] Omaye ST, Turnbull JD, Sauberlich HE. [1] Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. Meth Enzymol 1979;62:3–11.
[32] Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin Phenol reagent. J Biol Chem 1951;193:265–275.
[33] Mansouri A, Lurie AA. Concise review: methemoglobinaemia. Am J Hematol 1993;42:7–12.
[34] Siems WG, Sommerburg O, Grune T. Erythrocyte free radical and energy metabolism. Clin Nephrol 2000;53:S9–S17.
[35] Giulivi C, Daviess KJA. A novel antioxidant role for hemoglobin. The co-proportionation of ferrylhemoglobin with oxyhemoglobin. J Biol Chem 1990;265:19453–19460.
[36] Hebbel RP, Eaton JW, Balasingam M, Steinberg MH. Spontaneous oxygen radical generation by sickle erythrocytes. J Clin Invest 1982;70:1253–1259.
[37] Shalev O, Leida MN, Hebbel RP, Steinberg MH. Abnormal erythrocyte calcium homeostasis in oxidant-induced hemolytic disease. Blood 1981;58:1232–1235.
[38] Pandey KB, Rizvi SI. Biomarkers of oxidative stress in red blood cells. Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub 2011;155:131–136.
[39] Stepanik TM, Ewing DD. Coisolation of glutathione peroxidase, catalase and superoxide dismutase from human erythrocytes. J Biochem Biophys Meth 1990;20:157–169.
[40] Fattman CL, Schaefer LM, Oury TD. Extracellular superoxide dismutase in biology and medicine. Free Rad Biol Med 2003;35:236–256.
[41] Niki E. Action of ascorbic acid as a scavenger of active and stable oxygen radicals. Am J Clin Nutr 1991;54. 1119S–24S.
[42] Sangeetha P, Balu M, Haripriya D, Paneerselvam C. Age associated changes in erythrocyte membrane surface charge: modulatory role of grape seed proanthocyanidins. Exp Gerontol 2005;40:820–828.
[43] Rahman K. Studies on free radicals, antioxidants, and co-factors. Clin Interv Aging 2007;2:219–236.
[44] Kurata M, Suzuki M, Agar NS. Antioxidant systems and erythrocyte life-span in mammals. Comp Biochem Physiol 1993;106B:1477–1487.
[45] Hodgson EK, Fridowich I. The interaction of bovine erythrocyte superoxide dismutase with hydrogen peroxide; inactivation of the enzyme. Biochemistry 1975;14:5294–5299.
[46] Dallak M. Lack of ameliorative effect of Vitamins E and C supplements to oxidative stress and erythrocytes deterioration after exhaustive exercise at high altitude in native rats. Afr J Biotechnol 2012;11:9835–9843.
[47] Rizvi SI, Maurya PK. Markers of oxidative stress in erythrocytes during aging in humans. Ann N Y Acad Sci 2007;1100:373–382.
[48] Thomas JA, Mallis RJ. Aging and oxidation of reactive protein sulfhydryls. Exp Gerontol 2001;36:1519–1526.