European Journal of Clinical and Experimental Medicine

ONLINE FIRST

Authors: Magdaline Christina Rajanand, Anusha Berikai Ananthakrishna, Vani Rajashekaraiah

Article type: Original Article

Received: 20 January 2026

Accepted: 12 June 2026

Published online: 23 July 2026

eISSN: 2544-1361

Eur J Clin Exp Med

doi:10.15584/ejcem.2026.3.7

Vitamin C mitigates oxidative stress and preserves platelet efficacy during prolonged storage – an in vitro study

Magdaline Christina Rajanand, Anusha Berikai Ananthakrishna, Vani Rajashekaraiah

Department of Biotechnology and Genetics, School of Sciences, JAIN (Deemed-to-be University), Bengaluru, Karnataka, India

Corresponding author: Vani Rajashekaraiah, e-mail: vani.rs@jainuniversity.ac.in

ORCID

MCR: https://orcid.org/0000-0001-7733-447X

ABA: https://orcid.org/0000-0002-1094-5310

VR: https://orcid.org/0000-0002-4155-0960

ABSTRACT

Introduction and aim. Platelets stored at 22–24°C beyond 3–5 days undergo oxidative changes leading to storage lesions (PSL). Platelet additive solutions (PAS) improve platelet shelf-life, and antioxidant additives in PAS can mitigate PSL. This in vitro study evaluated the impact of Vitamin C (VC), a potent free radical scavenger, as an antioxidant additive in PAS-SSP+ on the redox status and functional integrity of rat platelets during prolonged storage.

Material and methods. Platelets from male *Wistar* rats (n=5) were grouped into controls (SSP+) and 10-VC (10mM VC in SSP+) and stored at 22–24°C for 11 days. Markers of antioxidant and oxidative status, platelet function and metabolism were investigated on days 1, 4, 7 and 11.

Results. Superoxide radicals, thiobarbituric acid reactive substances, and advanced oxidation protein products were significantly lower in 10-VC than controls. Superoxide dismutase and catalase were maintained whereas, glutathione was higher by 27% ($p<0.01$) and protein sulfhydryls by 65% ($p<0.01$) on day 7 than controls. Total antioxidant capacity was significantly higher than controls throughout storage in 10-VC. P-selectin lowered by 58% ($p<0.001$) on day 7 than controls while aggregation and cell viability were preserved in 10-VC.

Conclusion. 10-VC reduced oxidative stress by scavenging reactive species, inhibiting lipid and protein oxidation, augmenting antioxidant capacity, and preserving platelet functions and viability. Thus, VC in SSP+ maintained redox status and improved platelet efficacy, highlighting its potential as an additive in PAS ‘for preserving platelet integrity during prolonged storage.

Keywords. antioxidants, blood platelets, oxidative stress, platelet banking, vitamin C

Introduction

Platelet transfusion is vital for treating thrombocytopenia. The development of platelet storage lesions (PSL) in plasma at 22–24°C limits platelet shelf-life to 3–5 days thereby, rendering them unfit for transfusion.[1,2] Replacement of plasma with platelet additive solutions (PAS) such as SSP+ have been proven to mitigate PSL and prolong shelf-life for up to 9 days.[3-6]

Platelet metabolism generates reactive oxygen species (ROS) leading to oxidative stress (OS) and suppressing endogenous antioxidant defenses.[7] OS promotes platelet activation, lipid and protein oxidation thereby, reducing platelet efficacy during storage.[8] It also contributes to shedding of platelet receptors such as glycoprotein VI (GPVI) affecting platelet adhesion to collagen while altered integrin function caused by OS, impairs platelet aggregation.[9-11] Studies have proven that antioxidant additives have mitigated OS and enhanced the quality of stored platelets.[12-20] Antioxidants/ROS scavengers have the potential to preserve platelet functions such as GPVI-mediated adhesion and platelet response to agonists during storage.[21,22] These studies have predominantly focused on the effects of antioxidants on metabolic stability, functional integrity, mitochondrial damage and apoptosis rather than maintaining redox homeostasis during platelet storage. Our previous studies have reported oxidative modulations occurring in platelets stored in PAS with antioxidant additives such as N-acetyl cysteine and caffeic acid.[23,24]

Vitamin C (VC) is a potent ROS scavenger which inhibits lipid peroxidation and regenerates vitamin E and glutathione.[25-27] VC is known to inhibit ROS-mediated platelet activation by specifically inhibiting CD40L ligand on platelets without affecting agonist-induced aggregation.[28] Mohammed et al. studied the impact of VC on functions of saline-stored platelets.[29] However, the influence of VC on oxidative modulations in stored platelets remains unclear. Continuous variations in platelet efficacy have not been studied in terms of redox status and antioxidant capacity to mitigate PSL at different time periods during storage. Previous studies have not assessed VC as an additive in SSP+ with continuous evaluation of redox status and platelet functions during extended storage. Thus, this study investigates the influence of VC on the continuous oxidative modulations and platelet efficacy during extended storage in PAS, SSP+. This is the first report on the responses of platelets to VC as an additive in SSP+ during extended storage period.

Wistar rats were studied as an exploratory model to gain insights into the platelet responses. Rat and human platelets share similar physiology although minor variations in receptor distribution may influence functional responses.

Aim

This study aimed to continuously monitor the progression of PSL, with the primary objective of evaluating the effects of VC on oxidative stress and redox status during extended storage, and the secondary objective of assessing its influence on platelet function, viability and metabolic status. To the best of authors'

knowledge, this is the first in vitro study to evaluate VC as an additive in SSP+ for stored rat platelets during an extended storage period with continuous assessment of redox and functional parameters.

Material and methods

All experimental procedures were conducted in compliance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA) provided by the Institutional Animal Ethics Committee (IAEC), New Delhi, India. Whole blood samples were collected by cardiac puncture method under anesthesia in accordance with the approved animal care protocols of CCSEA. Animals were procured from Vertebrates (2138/PO/RcBiBt/S/21/CPCSEA) dated 1st August 2024. Blood sampling was performed at Nargund College of Pharmacy according to the institutional ethics regulations (1051/PO/Re/S/07/CPCSEA).

Chemicals

2′ 7′-dichlorodihydrofluorescein diacetate (DCFH-DA), cytochrome c, thiobarbituric acid and adenine triphosphate (ATP) standard were purchased from Sisco Research Laboratories Pvt Ltd, India. Epinephrine, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), bathocuproinedisulfonic acid disodium salt (BCS), nicotinamide adenine dinucleotide (NADH), Griess reagent and collagen were purchased from Sigma Aldrich chemicals, Mumbai, India. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide reagent (MTT), bovine serum albumin (BSA) and Vitamin C were purchased from HiMedia Laboratories Pvt Ltd, Mumbai, India. All other chemicals and solvents were reagent and spectral grade, respectively.

Autospan Liquid Gold Glucose kit, Arkray, Mumbai, India (Catalog no. 93LS100-75), Rat P-selectin GENLISA™ ELISA kit, KRISHGEN Biosystems, Mumbai, India (Ref: KLR2228), Rat Nicotinamide Adenine Dinucleotide Phosphate Oxidase 2 (NOX2) GENLISA™ ELISA kit, KRISHGEN Biosystems, Mumbai, India (Ref: KLR5069).

Blood sampling

Male *Wistar* rats (4 months old) were anesthetized with ketamine hydrochloride and approximately 4-5mL of whole blood was obtained by cardiac puncture and collected into tubes containing CPDA-1 (citrate phosphate dextrose adenine-1) anticoagulant.[30]

Platelet isolation

Whole blood was subjected to centrifugation at 190g for 15min at room temperature to obtain platelet-rich plasma (PRP). The PRP was subsequently centrifuged at 2500g for 15min at 22°C.[30] The platelet pellet obtained was resuspended in SSP+ (pH 7.4) with 30% residual plasma. Platelet concentrates were prepared by resuspending the platelet pellet in SSP+ (1:60).

Experimental design

VC was optimized using ROS assay using DCFH-DA method with different concentrations – 3, 5 and 10mM. VC at 10mM (10-VC) exhibited maximum inhibition of ROS (Fig. S1) and was selected for further analysis. Platelet preparations obtained from individual animals served as experimental units (n=5 in each group). Platelets were grouped into i) Control: SSP+ without VC and ii) Experimental: 10-VC in SSP+. The samples were stored in polypropylene tubes under mild agitation at 22°C for 11 days. Oxidative stress, antioxidant status, platelet function, viability and metabolism were analyzed on storage days 1, 4, 7 and 11.

ROS DCFH-DA assay

A stock solution, 20 mM DCFH-DA, was prepared in methanol and diluted in Tyrode's buffer to obtain a 75 µM working solution. Platelets were incubated at 37°C with 25 µM DCFH-DA for 30 min and the fluorescence was read at 485 nm (excitation) and 520 nm (emission).[31]

Antioxidant status

The antioxidant status of stored platelets was evaluated by measuring superoxide dismutase (SOD), catalase (CAT), glutathione (GSH) and total antioxidant capacity (TAC).

SOD activity was determined by monitoring the inhibition of epinephrine oxidation in carbonate buffer (0.05 M, pH 10.2) containing 0.1 mM ethylene diamine tetraacetic acid (EDTA) at 480nm and expressed as the amount of enzyme required to achieve 50% inhibition of epinephrine oxidation.[32]

CAT activity was determined by treating the samples with absolute ethanol followed by incubation in an ice bath for 30 min and addition of phosphate buffer and 0.066 M hydrogen peroxide (H_2O_2). The absorbance was read at 240 nm using a molar extinction coefficient of 43.6 $M^{-1}cm^{-1}$.[33]

GSH levels were estimated after treating the samples with 4% sulfosalicylic acid and centrifugation at 2500 g for 15 min; the resulting supernatant was treated with 10 mM DTNB, and absorbance was recorded at 412 nm.[34]

TAC was assessed using BCS (0.25 mM), copper sulphate (0.5 mM) and EDTA (0.01 M). The absorbance was read at 490 nm, using uric acid as standard.[35]

Oxidative stress

Oxidative status was evaluated by measuring superoxide and nitrite radicals, thiobarbituric acid reactive substances (TBARS), advanced oxidation protein products (AOPP) and protein sulfhydryls (SH).

Superoxide radicals were determined by monitoring cytochrome C reduction following incubation of stored platelets with cytochrome C (160 µM) at 37°C, with absorbance measured at 550 nm.[26]

Nitrite levels were estimated using Griess reagent after incubation in the dark at room temperature for 30 min. The absorbance was recorded at 548 nm using sodium nitrite as the standard.[36]

Lipid peroxidation was measured by assessing TBARS by treating platelets with 20% cold trichloroacetic acid in 0.6 M hydrochloric acid, followed by reaction with 0.12 M thiobarbituric acid. After incubation in a boiling water bath for 15min, absorbance was measured at 532 nm.[37]

Protein oxidation was evaluated by determining AOPP and SH levels.

AOPP was estimated by treating the samples with isotonic phosphate buffer, 1.16 M potassium iodide and glacial acetic acid. The absorbance was read at 340nm with an extinction coefficient of 26 $mM^{-1}cm^{-1}$.[38]

SH content was determined using the DTNB method where platelets were treated with 0.08 M sodium phosphate buffer containing sodium dodecyl sulphate (2%) and sodium EDTA (0.5 mg/mL). After addition of DTNB, the color developed was recorded at 412 nm and SH content was estimated using a molar extinction coefficient of 13,600 $M^{-1}cm^{-1}$.[39]

Platelet function

Platelet functions were evaluated by measuring NOX2 levels, P-selectin release (platelet activation), platelet aggregation and ATP secretion.

NOX2 and P-selectin were quantified using sandwich-ELISA kits specific for rat NOX2 (KRISHGEN Biosystems; Ref: KLR5069) and rat P-selectin (KRISHGEN Biosystems; Ref: KLR2228), respectively following the manufacturer's instructions. The absorbance was recorded at 450 nm.[23]

Platelet aggregation was determined by incubating stored platelets with and without collagen (2 µg/mL) at 37°C, followed by measurement of absorbance at 405 nm in shaking mode for 1.5–2 min.[40] % aggregation was calculated according to Tamang et al.[41]

ATP secretion was assessed after incubation of stored platelets with collagen (2 µg/mL) at 37°C, followed by treatment with 1.2 M perchloric acid. Absorbance was measured at 260 nm and ATP levels were quantified using an ATP standard.[42]

Platelet viability

Samples were incubated with MTT reagent (0.5 mg/mL) for 4 h at 37°C, followed by the addition of dimethyl sulfoxide and overnight incubation at 37°C. Absorbance was recorded at 570 nm.[43]

Protein estimation

Protein was estimated according to Lowry et al. to determine the protein content of the samples. Stored platelets were treated with alkaline copper sulphate and Folin's reagent. The absorbance was measured at 660 nm.[44]

Platelet metabolism

Platelet metabolism during storage was evaluated by measuring pH, levels of extracellular glucose and lactate dehydrogenase (LDH).

The pH of the samples was assessed using Fisher Scientific pH indicator strips.[45]

Extracellular glucose present in the storage medium was quantified by the glucose oxidase-peroxidase (GOD-POD) method according to the manufacturer's instructions provided in the Autospan Gold kit, and absorbance was read at 546 nm.[46]

LDH was determined following incubation of stored platelets with a reaction mixture containing Reagent 1 (80 mM Tris, 200 mM sodium chloride and 1.6 mM pyruvate) and Reagent 2 (0.2 mM NADH) at 37°C. Absorbance was recorded at 340 nm.[47]

Statistical analyses

The data was assessed for normality (Shapiro-Wilk test) and passed the normality test. Results are presented as Mean with standard error from five independent biological samples (n=5). GraphPad Prism 8 software was used to perform two-way ANOVA (repeated-measures) and Bonferroni *post hoc* test to evaluate the effects between the storage days and the experimentals. Differences were considered statistically significant at $p<0.05$. Pearson's correlation analysis was also performed to determine the relationships between superoxide radicals and SOD, CAT and SOD, and GSH and SH. Correlation coefficients (r) and corresponding p-values were calculated.

Results

Results are presented as i) changes between the storage days and ii) changes between the experimentals on a particular day of storage.

Antioxidant defenses

SOD

SOD was maintained in 10-VC whereas, it increased by twofold ($p<0.05$) and ninefold ($p<0.0001$) on day 7 and day 11 with respect to day 1 in controls.

SOD was lower by 82% ($p<0.0001$) on day 11 in 10-VC compared to controls (Fig. 1A).

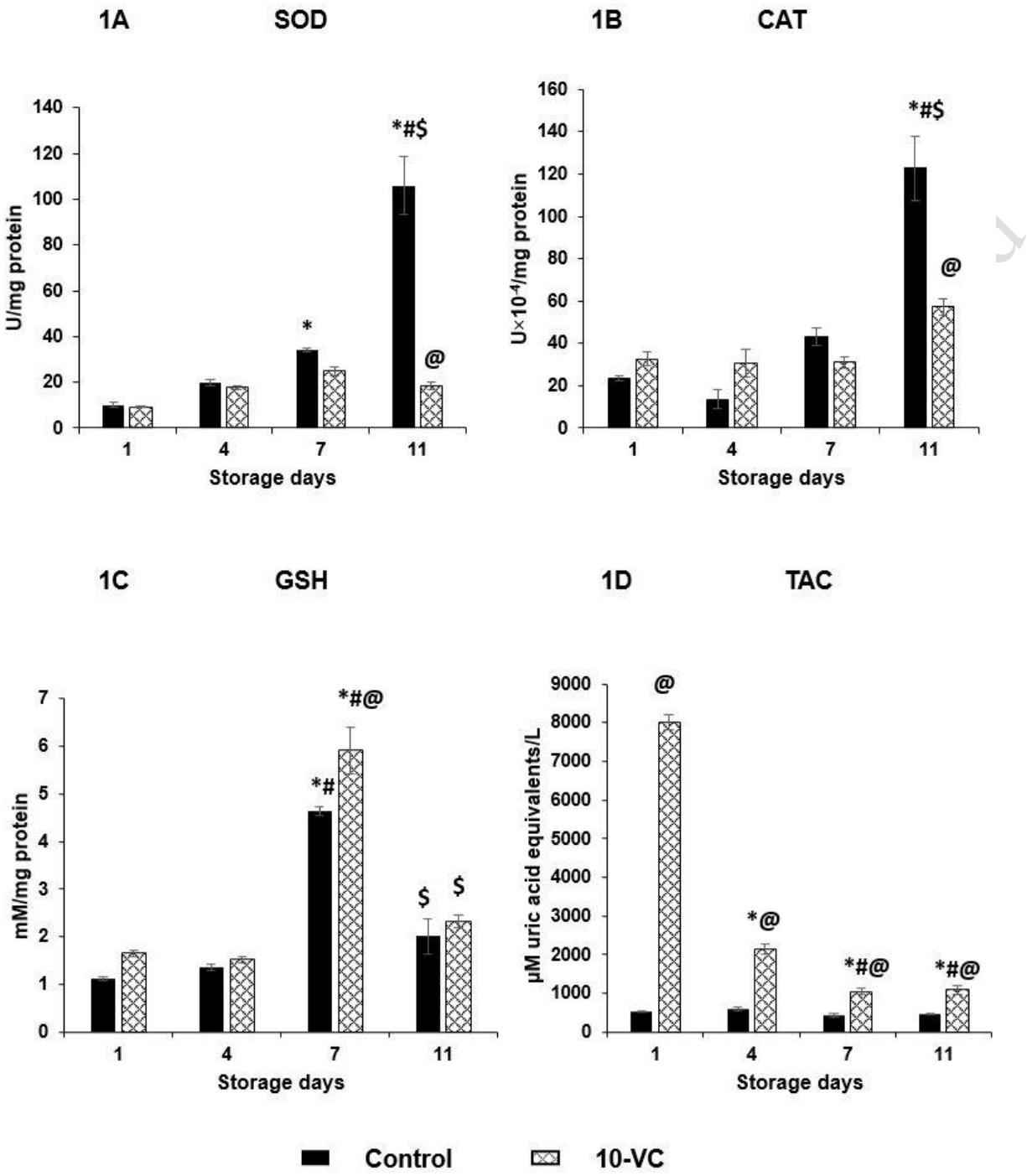

Fig. 1. Antioxidant status of platelets stored with VC, A: SOD, B: CAT, C: GSH, D: TAC (* – significant with day 1, # – significant with day 4, $ – significant with day 7, @ – significant in 10-VC against controls)

CAT

CAT was maintained throughout storage in 10-VC whereas it increased by fourfold ($p<0.0001$) on day 11 with day 1 in controls. CAT was lower by 50% ($p<0.0001$) on day 11 in 10-VC compared to controls (Fig. 1B).

GSH

GSH increased in 10-VC by 260% ($p<0.0001$) on day 7 with respect to day 1 and decreased by 60% ($p<0.0001$) on day 11 with day 7. GSH peaked 310% ($p<0.0001$) on day 7 with day 1 and decreased by 56% ($p<0.0001$) on day 11 with day 7 in controls.

GSH was higher by 27% ($p<0.01$) on day 7 in 10-VC compared to controls (Fig. 1C).

TAC

TAC decreased by 73% ($p<0.0001$) on day 4 with day 1 and decreased by 50% ($p<0.0001$) on day 7 and day 11 with day 4 in 10-VC whereas, it was maintained in controls.

TAC was higher by tenfold ($p<0.0001$) on day 1, twofold ($p<0.0001$) on day 4 and onefold ($p<0.01$) on days 7 and 11 in 10-VC compared to their respective controls (Fig. 1D).

Oxidative stress

Superoxide radicals

Superoxide radicals remained constant in 10-VC whereas it increased by ~ninefold ($p<0.0001$) on days 7 and 11 in controls.

Superoxide radicals were lower by 47% ($p<0.05$) and 83% ($p<0.0001$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2A).

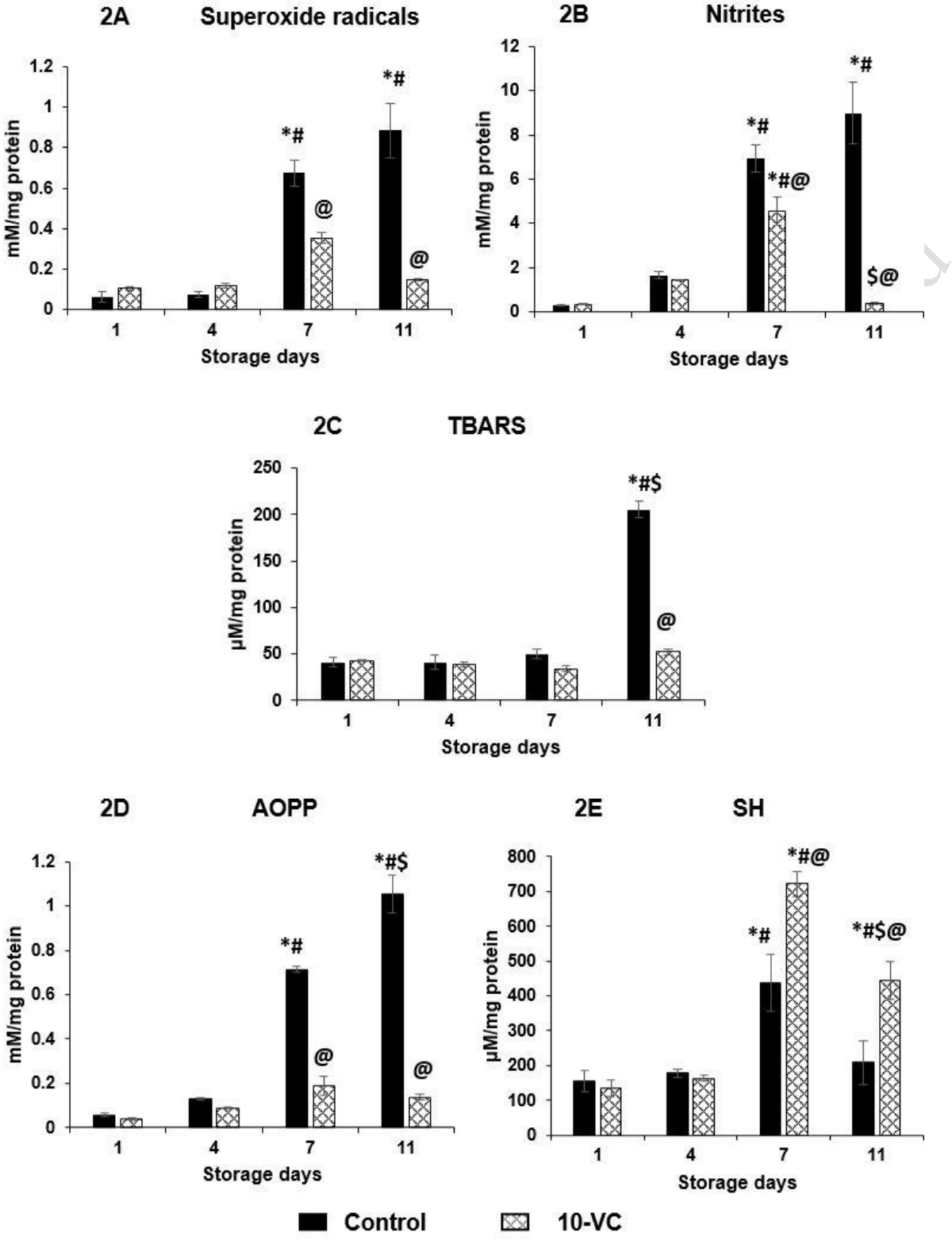

Fig. 2. Oxidative stress, lipid peroxidation and protein oxidation in platelets stored with VC, A: Superoxide radicals, B: Nitrites, C: TBARS, D: AOPP, E: SH (* – significant with day 1, # – significant with day 4, $ – significant with day 7, @ – significant in 10-VC against controls)

Nitrites

Nitrites increased in 10-VC by tenfold ($p<0.0001$) on day 7 which decreased by 91% ($p<0.0001$) on day 11 with day 7. Nitrites increased by twentyfold ($p<0.0001$) on day 7 and day 11 in controls. Nitrites were lower by 34% ($p<0.05$) and 95% ($p<0.0001$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2B).

TBARS

TBARS was maintained throughout storage in 10-VC whereas it increased by fourfold ($p<0.0001$) on day 11 in controls.

TBARS was lower by 74% ($p<0.0001$) on day 11 in 10-VC against controls (Fig. 2C).

AOPP

AOPP remained constant in 10-VC whereas controls exhibited increments of tenfold ($p<0.0001$) on day 7 and day 11.

AOPP was lower by ~80% ($p<0.0001$) on days 7 and 11 in 10-VC against controls (Fig. 2D)

SH

SH increased in 10-VC by 435% ($p<0.0001$) on day 7 and decreased by 38% ($p<0.01$) on day 11 with day 7. SH increased by 180% ($p<0.01$) on day 7 and normalized to day 1 levels on day 11 in controls.

SH was higher by 65% ($p<0.01$) and 110% ($p<0.05$) on days 7 and 11 respectively, in 10-VC against controls (Fig. 2E).

Platelet function, viability and metabolism

NADPH Oxidase-2 (NOX2)

10-VC exhibited increments of 75% ($p<0.001$) on day 7 whereas, it increased by 73% ($p<0.0001$) and 41% ($p<0.05$) on days 7 and 11 respectively, in controls. NOX2 was lower by 25% ($p<0.01$) on day 7 in 10-VC against controls (Table 1).

Table 1. Platelet function during storage with VC[a]

Storage days	Sub-groups	NOX2 (ng/mL)	P-selectin (pg/mL)	ATP secretion (µg/mL)
1	Control	35.10±2.37	2528.25±548.44	28760.00±2337.69
	10-VC	25.94±2.93	1214.69±312.89	34553.33±1359.35
4	Control	29.76±2.57	2570.62±754.86	30620.00±2427.03

	10-VC	26.48±1.28	1320.62±604.75	27960.00±857.54
7	Control	60.88±2.91*#	18008.47±1986.82*#	4153.33±132.33*#
	10-VC	45.34±1.74*#@	7531.78±222.46@	17593.33±2021.40*#@
11	Control	49.56±2.51*#	6709.04±1507.28$	4660.00±316.65*#
	10-VC	38.55±2.31	3820.62±908.49	19826.66±678.60*#@

[a] * – significant with day 1, # – significant with day 4, $ – significant with day 7, @ – significant in 10-VC against controls

P-selectin

P-selectin remained constant in 10-VC whereas, it increased by 600% ($p<0.0001$) on day 7 and decreased by 62% ($p<0.0001$) on day 11 with day 7 in controls.

P-selectin was lower by 58% ($p<0.001$) on day 7 in 10-VC against controls (Table 1).

Aggregation

Aggregation levels in response to collagen were similar to day 1 throughout the storage period. (day 1: 85% in 10-VC and 53% in controls).

ATP secretion

ATP secretion declined by ~45% ($p<0.0001$) on day 7 and day 11 in 10-VC whereas, it decreased by ~85% ($p<0.0001$) on day 7 and day 11 in controls.

ATP levels were threefold higher ($p<0.0001$) on day 7 and day 11 in 10-VC against controls (Table 1).

Table 2. Platelet viability and metabolism during storage with VC[a]

Storage days	Subgroups	MTT (570 nm)	pH	Glucose (mmol/L)	LDH (U×10^{-6}/mg protein)
1	Control	0.45±0.03	7.0±0.0	2.32±0.26	10.73±1.20
	10-VC	0.42±0.04	7.0±0.0	1.80±0.17	9.35±0.29
4	Control	0.302±0.02*	7.0±0.0	0.67±0.08*	9.14±1.13
	10-VC	0.31±0.01	7.2±0.2	0.63±0.11	6.52±0.79
7	Control	0.18±0.01*#	7.5±0.0	0.45±0.13*	7.67±1.39
	10-VC	0.25±0.02*	7.2±0.2	0.53±0.09*	5.68±0.78
11	Control	0.11±0.02*#	7.6±0.16	0.59±0.14*	25.49±6.22*#$
	10-VC	0.17±0.005*#	7.25±0.32	0.53±0.21*	9.41±1.16@

[a] * – significant with day 1, # – significant with day 4, $ – significant with day 7, @ – significant in 10-VC against controls

Cell viability

Viable cells decreased by 40% ($p<0.001$) and 58% ($p<0.0001$) on day 7 and day 11 in 10-VC. Controls exhibited decrements of 33% ($p<0.01$) on day 4 and 40% ($p<0.01$) and 60% ($p<0.0001$) on day 7 and day 11 respectively, with day 4.

Changes in platelet viability were similar between controls and 10-VC on each storage day (Table 2).

pH

pH was maintained in both controls and 10-VC.

Variations in pH were similar between controls and 10-VC on each day of storage (Table 2).

Glucose

10-VC showed decrements of ~68% on day 4 ($p<0.001$), day 7 ($p<0.0001$) and day 11 ($p<0.001$). Glucose decreased by ~75% ($p<0.0001$) on days 4, 7 and 11 in controls.

Variations in glucose were similar between controls and 10-VC on each storage day (Table 2).

LDH

LDH remained constant in 10-VC whereas, it increased by twofold ($p<0.01$) on day 11 in controls. LDH was lower by 63% ($p<0.01$) on day 11 in 10-VC against controls (Table 2).

Correlation analysis

Pearson correlation analysis exhibited positive correlation between superoxide radicals and SOD ($r=0.83$, $p<0.05$) and GSH and SH ($r=0.93$. $p<0.05$) in 10-VC. Controls also showed positive correlation between superoxide radicals and SOD ($r=0.85$, $p<0.05$), SOD and CAT ($r=0.98$, $p<0.05$), GSH and SH ($r=0.99$, $p<0.01$).

Discussion

Storage of platelets in SSP+ has mitigated OS-induced storage lesions by augmenting antioxidant defenses and improving platelet viability and function during 7-day storage period.[30] Antioxidants as additive in PAS can attenuate OS. Hence, VC was used as an additive in SSP+ to investigate its effects on platelet quality, function and OS during storage.

10 mM VC was chosen as an antioxidant additive in SSP+ for platelet storage based on the ROS assay. Previous studies with VC at 10 mM concentration as an additive in erythrocyte storage solutions have mitigated OS by reducing superoxide radicals and elevating endogenous antioxidant defenses.[48,49] VC at 10

mM concentration has preserved erythrocyte efficacy during storage. Therefore, 10 mM VC was used in platelet storage solution as an additive in this study.

Platelets are equipped with endogenous antioxidant defenses, namely SOD and CAT, that are activated by high ROS levels i.e., superoxide radicals and H_2O_2) respectively.[27] SOD and CAT are proportional as the dismutation product of SOD (H_2O_2 at high concentrations) activate CAT. Superoxide radicals and SOD were maintained throughout storage (r=0.83) in 10-VC indicating the ROS scavenging property of VC. VC directly scavenges ROS by donating electrons/hydrogen atoms from its two ionizable -OH groups.[27] It is reported that VC reduced ROS levels induced by cisplatin in pig platelets and inhibited superoxide production by 90% compared to resveratrol.[25,26] VC being an efficient ROS scavenger limited ROS generation which resulted in lower SOD and CAT levels thereby, maintaining the redox status of the cells. SOD and CAT were proportional to the superoxide radicals in controls on days 7 and 11 (Superoxide radicals vs SOD, r=0.85; SOD vs CAT, r=0.98).

GSH is an endogenous antioxidant that directly scavenges ROS. The pentose phosphate pathway (PPP) contributes to GSH recycling by providing NADPH required for glutathione reductase (GR) activity.[27] The higher levels of GSH in 10-VC compared to controls on day 7 indicate that VC could maintain GSH in reduced state. This may be associated with improved GSH recycling thereby, maintaining redox balance. Similar observations have been reported by Puskas et al. who demonstrated that pretreatment with dehydroascorbate to T-lymphocytes increased GSH levels, potentially through the activation of PPP.[50] Antioxidant additives have been reported to improve platelet viability during storage.[17] This was evidenced in low LDH levels on day 11 in 10-VC than controls, suggesting reduced platelet damage. Variations of ~50% in 10-VC was observed compared to controls although the changes were insignificant. Thus, VC could lower storage lesions and preserve platelet integrity by reducing ROS levels during storage. TAC was higher than controls throughout storage in 10-VC which revealed the antioxidant potential of VC. However, it is reported that PPP is activated during platelet storage as a stress response in order to maintain the redox status.[51] This was evidenced in the elevated GSH levels on day 7 in controls which could be associated with enhanced GSH recycling. However, GSH decreased on day 11 in both 10-VC and controls due to high ROS. The GSH levels were also in line with SH content in controls (GSH vs SH, r=0.99). High metabolic rate evidenced by lower glucose levels throughout storage in controls and 10-VC may be indicative of an active glycolytic pathway/PPP. These results suggest that VC has worked in synergy with the endogenous antioxidant defenses as the SOD and CAT levels were maintained with augmentation of GSH thereby, resulting in mitigation of OS.

Cellular lipids and proteins are prone to ROS attack during OS. Oxidation of cellular lipids and proteins disrupt membrane structure and function eventually leading to apoptosis. VC in synergy with VE inhibits lipid peroxidation.[52] This was evident in the constant levels of TBARS throughout storage in 10-VC whereas, it increased on day 11 in controls due to accumulation of ROS causing lipid peroxidation. Olas et

al. reported that VC reduced lipid peroxidation in pig platelets during OS conditions.[25,26] Proteins form dityrosine cross-linkages called AOPPs during OS which disrupt several cellular signaling cascades.[53] AOPPs were lower than controls in 10-VC which can be attributed to the ROS scavenging and antioxidant regenerating ability of VC. This was also evidenced in the levels of protein sulfhydryls. The disulfides (S-S) formed during oxidation can be reversed back to SH when ROS lowers or due to the interventions of antioxidant defenses. SH was higher than controls on days 7 and 11 due to the antioxidant potential of VC in maintaining thiols in reduced state which was also in line with GSH (r=0.93).

NOX2 is a major enzymatic source of ROS in platelets and is closely associated with platelet activation and functional responses.[54,55] NOX2 contributes to high ROS generation during storage which promotes undesirable platelet activation and impair responses to agonists.[8,10,56] The effects of VC was evidenced in low P-selectin levels in 10-VC group compared to controls although ATP secretion (granule release) was higher and aggregation response to collagen was maintained. VC has been reported to modulate platelet redox status and inhibit CD40 ligand expression (found on activated platelets) without affecting agonist-induced platelet aggregation.[28,29] This is in accordance with the results of platelet function markers. This suggests that VC could modulate redox balance and attenuate ROS-mediated activation pathways thereby, preserving platelet responses. Thus, VC could selectively inhibit platelet activation while maintaining functional integrity during storage.

Platelets primarily use glucose as their metabolic fuel. Glucose consumption increases lactate production which decrease the pH of the storage medium.[57] Glucose levels lowered in both controls and 10-VC throughout storage indicating its higher consumption although pH was maintained. Storage of platelets in an artificial medium or PAS with minimal residual plasma, preserves metabolic integrity.[57] The salts in SSP+ provide a buffering effect as well as the acetate component can act as a secondary metabolic fuel as evidenced in glucose and pH levels.[30] Platelet metabolic integrity was maintained by the components of SSP+ although, metabolic stress was observed (lower glucose levels) during storage. These results are in line with the findings of Rajanand et al. where pH was maintained despite lower glucose levels when stored in PAS-SPP+.[23] This was attributed to the salts present in SSP+ which preserved the metabolic status.

Study limitations

The present study used animal models as they are bred in controlled conditions. Thus, the variations in age, diet, genetics and microenvironment are minimized, allowing the observed effects to be more directly associated with the experimental interventions. However, the study was conducted using a limited number of biological samples (n=5) from male *Wistar* rats, which were selected to minimise potential influences of estrogen-associated antioxidant effects. Therefore, these findings require validation in human platelets.

Although the concentration of VC was initially optimized using ROS assay, only 10mM concentration was evaluated during extended storage. In addition, in vivo platelet recovery, post-transfusion survival and

clinical transfusion efficacy were not assessed. Therefore, the present findings should be interpreted within the context of an in vitro platelet storage model. Further studies using human platelets and in vivo validation are required to confirm the translational relevance of these findings.

Conclusion

10-VC reduced oxidative stress by scavenging superoxide and nitrite radicals, inhibiting lipid peroxidation, protein oxidation, augmenting antioxidant capacity and preserving platelet functions and viability during storage. Thus, VC (10mM) in SSP+ was able to maintain the redox status and improve selected markers of platelet efficacy during extended storage. This in vitro study highlights the prospects of employing vitamin C as an additive in PASs to preserve platelet efficacy during prolonged storage. However, further studies using human platelets are required to confirm the translational relevance of these findings.

Acknowledgments

The authors sincerely acknowledge JAIN (Deemed-to-be University) for financial support and facilities provided through the Minor Research Project. The authors also express their appreciation to Nargund College of Pharmacy, Bengaluru, India, for providing the necessary support towards this study. The authors further acknowledge Dr. Leela Iyengar, Dr. Sowmya Ravikumar, Dr. Manasa Mithun, Dr. Carl Hsieh, Dr. M. Pallavi, Ms. Dharmavaram Sravani and Ms. Jennifer Margaret D, for their assistance in this study.

Supplementary material

Fig. S1. Dose optimization of Vitamin C (VC) in stored platelets.

Declarations

Funding

Funding for the present study was provided through the Minor research project grant awarded by JAIN (Deemed-to-be University) (Reference No. JU/MRP/SOS/2022).

Author contributions

Conceptualization, V.R.; Methodology, M.C.R and V.R.; Software, M.C.R.; Validation, V.R.; Formal Analysis, M.C.R.; Investigation, M.C.R. and A.B.A.; Resources, V.R.; Data Curation, M.C.R; Writing – Original Draft Preparation, M.C.R.; Writing – Review & Editing, V.R.; Visualization, V.R.; Supervision, V.R.; Project Administration, V.R.; Funding Acquisition, V.R.

Conflicts of interest

The authors declare no conflicts of interest.

Competing interests

The authors declare no competing interests.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

Ethics approval

All experimental procedures were conducted in compliance with the guidelines of the Committee for Control and Supervision of Experiments on Animals (CCSEA) provided by the Institutional Animal Ethics Committee (IAEC), New Delhi, India. Whole blood samples were collected by cardiac puncture method under anesthesia in accordance with the approved animal care protocols of CCSEA. Animals were procured from Vertebrates (2138/PO/RcBiBt/S/21/CPCSEA) dated 1st August, 2024. Blood sampling was performed at Nargund College of Pharmacy according to the institutional ethics regulations (1051/PO/Re/S/07/CPCSEA).

References

1. Rajashekaraiah V, Rajanand MC. Platelet storage: Progress so far. *J Thromb Thrombolysis*. 2023;55(1):9-17. doi:10.1007/s11239-022-02716-3
2. Shrivastava M. The platelet storage lesion. *Transfus Apher Sci*. 2009;41(2):105-113. doi:10.1016/j.transci.2009.07.002
3. Gulliksson H. Additive solutions for the storage of platelets for transfusion. *Transfus Med*. 2000;10(4):257-264. doi:10.1046/j.1365-3148.2000.00262.x
4. Ringwald J, Zimmermann R, Eckstein R. The new generation of platelet additive solution for storage at 22 C: development and current experience. *Transfus Med Rev.* 2006;20(2):158-164. doi:10.1016/j.tmrv.2005.11.003
5. Hornsey VS, McColl K, Drummond O, et al. Extended storage of platelets in SSP+ platelet additive solution. *Vox Sang*. 2006;91(1):41-46. doi:10.1111/j.1423-0410.2006.00771.x
6. Saunders C, Rowe G, Wilkins K, Holme S, Collins P. In vitro storage characteristics of platelet concentrates suspended in 70% SSP+ TM additive solution versus plasma over a 14-day storage period. *Vox Sang*. 2011;101(2):112-121. doi:10.1111/j.1423-0410.2011.01468.x
7. Alexandru N, Popov D, Georgescu A. Intraplatelet oxidative/nitrative stress: inductors, consequences, and control. *Trends Cardiovas Med*. 2010;20(7):232-238. doi:10.1016/j.tcm.2011.11.007

8. Rinalducci S, Zolla L. Biochemistry of storage lesions of red cell and platelet concentrates: A continuous fight implying oxidative/nitrosative/phosphorylative stress and signaling. *Transfus Apher Sci*. 2015;52(3):262-269. doi:10.1016/j.transci.2015.04.005
9. Arthur JF, Shen Y, Kahn ML, Berndt MC, Andrews RK, Gardiner EE. Ligand binding rapidly induces disulfide-dependent dimerization of glycoprotein VI on the platelet plasma membrane. *J Biol Chem*. 2007;282(42):30434-30441. doi:10.1074/jbc.M701330200
10. Qiao J, Arthur JF, Gardiner EE, Andrews RK, Zeng L, Xu K. Regulation of platelet activation and thrombus formation by reactive oxygen species. *Redox Biol*. 2018;14:126-130. doi:10.1016/j.redox.2017.08.021
11. Ray PD, Huang BW, Tsuji Y. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cell Signal*. 2012;24(5):981-990. doi:10.1016/j.cellsig.2012.01.008
12. Zhuang Y, Ren G, Li H, et al. In vitro properties of apheresis platelet during extended storage in plasma treated with anandamide. *Transfus Apher Sci*. 2014;51(1):58-64. doi:10.1016/j.transci.2014.03.009
13. Wang X, Fan Y, Shi R, Li J, Zhao S. Quality assessment of platelets stored in a modified platelet additive solution with trehalose at low temperature (10C) and in vivo effects on rabbit model of thrombocytopenia. *Platelets*. 2015;26(1):72-79. doi:10.3109/09537104.2013.872772
14. Handigund M, Bae TW, Lee J, Cho YG. Evaluation of in vitro storage characteristics of cold stored platelet concentrates with N acetylcysteine (NAC). *Transfus Apher Sci*. 2016;54(1):127-138. doi:10.1016/j.transci.2016.01.006
15. Mithun M, Rajashekaraiah. L-Carnitine as an additive in Tyrode's buffer during platelet storage. *Blood Coagul Fibrinolysis*. 2018;29(7):613-621. doi:10.1097/MBC.0000000000000760
16. Ekaney ML, Gray GG, McKillop IH, Evans SL. Enhanced platelet function in cold stored whole blood supplemented with resveratrol or cytochrome C. *J Trauma Acute Care Surg*. 2018;85(1):92-97. doi:10.1097/TA.0000000000001887
17. Hosseini E, Ghasemzadeh M, Atashibarg M, Haghshenas M. ROS scavenger, N-acetyl-l-cysteine and NOX specific inhibitor, VAS2870 reduce platelets apoptosis while enhancing their viability during storage. *Transfus*. 2019;59(4):1333-1343. doi:10.1111/ trf.15114
18. Wang L, Xie R, Fan Z, et al. The contribution of oxidative stress to platelet senescence during storage. Transfus 2019; 59(7):2389-2402. doi:10.1111/trf.15291
19. Amiri F, Dahaj MM, Siasi NH, Deyhim MR. Treatment of platelet concentrates with the L-carnitine modulates platelets oxidative stress and platelet apoptosis due to mitochondrial reactive oxygen species reduction and reducing cytochrome C release during storage. *J Thromb Thrombolysis*. 2021;51(2):277-285. doi:10.1007/s11239-020-02241-1

20. Hegde S, Wellendorf AM, Zheng Y, Cancelas JA. Antioxidant prevents clearance of hemostatically competent platelets after long-term cold storage. *Transfus*. 2021;61(2):557-567. doi:10.1111/trf.16200
21. Zhang P, Du J, Zhao L, et al. The role of intraplatelet reactive oxygen species in the regulation of platelet glycoprotein Ibα ectodomain shedding. *Thromb Res*. 2013:132(6):696-701. doi:10.1016/j.thromres.2013.09.034
22. Stef G, Csiszar A, Xiangmin Z, Ferdinandy P, Ungvari Z, Veress G. Inhibition of NAD (P) H oxidase attenuates aggregation of platelets from high-risk cardiac patients with aspirin resistance. *Pharmacol Rep*. 2007;59(4):428. PMID: 17901572
23. Rajanand MC, Ananthakrishna AB, Rajashekaraiah V. A laboratory study on N-acetyl cysteine in SSP+ modulating oxidative stress and delaying the progression of storage lesion in platelets. *Biomed Res Ther*. 2024;11(11):6950-6959. doi:0.15419/bmrat.v11i11.941
24. Rajanand MC, Ananthakrishna AB, Rajashekaraiah V. Caffeic Acid Alleviates Oxidative Stress and Prolongs the Shelf-Life of Rat Platelets. *Biopreserv Biobank.* 2026;24(1):41-51. doi:10.1089/bio.2025.0039
25. Olas B, Wachowicz B, Buczyn A. Vitamin C suppresses the cisplatin toxicity on blood platelets. *Anti-cancer drugs*. 2000;11(6):487-493. doi:10.1097/00001813-200007000-00010
26. Olas B, Wachowicz B. Resveratrol and vitamin C as antioxidants in blood platelets. *Thromb Res*. 2002;106(2):143-148. doi:10.1016/S0049-3848(02)00101-9
27. Halliwell B, Gutteridge JMC. Free Radicals in Biology and Medicine, 5th ed. New York: Oxford University Press; 2015.
28. Pignatelli P, Sanguigni V, Paola SG, Coco EL, Lenti L, Violi F. Vitamin C inhibits platelet expression of CD40 ligand. *Free Rad Biol Med*. 2005;38(12):1662-1666. doi:10.1016/j.freeradbiomed.2005.02.032
29. Mohammed BM, Sanford KW, Fisher BJ, et al. Impact of high dose vitamin C on platelet function. *World J Crit Care Med*. 2017;6(1):37. doi:10.5492/wjccm.v6.i1.37
30. Rajanand MC, Ananthakrishna AB, Rajashekaraiah V. Oxidative modulations in platelets stored in SSP+, PAS-G and Tyrode's buffer: a comparative analysis. *Hematol Transfus Cell Ther*. 2024;46:80-89. doi:10.1016/j.htct.2024.04.121
31. Blasa M, Angelino D, Gennari L, Ninfali P. The cellular antioxidant activity in red blood cells (CAA-RBC): a new approach to bioavailability and synergy of phytochemicals and botanical extracts. *Food Chem.* 2011;125(2):685-691. doi:10.1016/j.foodchem.2010.09.065
32. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*. 1972;247(10):3170-3175. doi:10.1016/S0021-9258(19)45228-9

33. Aebi H. Catalase in vitro. *Methods Enzymol*. 1984;105:121-126. doi:10.1016/s0076-6879(84)05016-3
34. Beutler E, Duron O, Kelly BM. Improved method for the determination of blood glutathione. *J Lab Clin Med*. 1963;61:882-888. PMID: 13967893.
35. Da Cruz G. Use of bathocuproine for the evaluation of the antioxidant power in liquids and solutions. 2003;US patent 6613577.
36. Chen LY, Mehta P, Mehta JL. Oxidized LDL decreases L-arginine uptake and nitric oxide synthase protein expression in human platelets: relevance of the effect of oxidized LDL on platelet function. *Circ*. 1996;93(9):1740-1746. doi:10.1161/01.CIR.93.9.1740
37. Olas B, Nowak P, Kolodziejczyk J, Ponczek M, Wachowicz B. Protective effects of resveratrol against oxidative/nitrative modifications of plasma proteins and lipids exposed to peroxynitrite. *J Nutr Biochem*. 2006;17(2):96-102. doi:10.1016/j.jnutbio.2005.05.010
38. Witko-Sarsat V, Friedlander M, Capeillère-Blandin C, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int*. 1996;49(5):1304-1313. doi:10.1038/ki.1996.186
39. Habeeb AFSA. Reaction of protein sulfhydryl groups with Ellman's reagent. *Methods Enzymol*. 1972;25(25):457-464. doi:10.1016/S0076-6879(72)25041-8
40. Born GVR, Cross MOJ. The aggregation of blood platelets. *J Physiol*. 1963;168(1):178. doi:10.1113/jphysiol.1963.sp007185
41. Tamang HK, Stringham EN, Tourdot BE. Platelet functional testing via high-throughput microtiter plate-based assays. *Curr Protoc*. 2023;3(2):668. doi:10.1002/cpz1.668
42. Wachowicz B, Olas B, Zbikowska HM, Buczyński A. Generation of reactive oxygen species in blood platelets. *Platelets*. 2002;13(3):175-182. doi:10.1080/09533710022149395
43. Shiri R, Yari F, Ahmadinejad M, Vaeli S, Tabatabaei MR. The caspase-3 inhibitor (peptide Z-DEVD-FMK) affects the survival and function of platelets in platelet concentrate during storage. *Blood Res*. 2014;49(1):49. doi:10.5045/br.2014.49.1.49
44. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem*. 1951;193(1):265-275. doi:10.1016/S0021-9258(19)52451-6
45. Amorini AM, Tuttobene M, Lazzarino G, Denti G. Evaluation of biochemical parameters in platelet concentrates stored in glucose solution. *Blood Transfus*. 2007;5(1):24. doi:10.2450/2007.0019-06
46. Basak A. Development of a rapid and inexpensive plasma glucose estimation by two-point kinetic method based on glucose oxidase-peroxidase enzymes. *Indian J Clin Biochem*. 2007;22(1):156-160. doi:10.1007/BF02912902

47. Buhl SN, Jackson KY. Optimal conditions and comparison of lactate dehydrogenase catalysis of the lactate-to-pyruvate and pyruvate-to-lactate reactions in human serum at 25, 30, and 37 degrees C. *Clin Chem*. 1978;24(5):828-831. doi:10.1093/clinchem/24.5.828
48. Soumya R, Vani R. Vitamin C as a modulator of oxidative stress in erythrocytes of stored blood. *Acta Haematol Pol*. 2017;48(4):350-356. doi:10.1016/j.achaem.2017.08.005
49. Pallavi M, Rajashekaraiah V. Vitamin C with N-acetylcysteine ameliorates the antioxidant defenses in erythrocytes during storage. *J Exp Clin Med*. 2024;41(2):302-310. doi:10.52142/omujecm.41.2.14
50. Puskas F, Gergely Jr P, Banki K, Perl A. Stimulation of the pentose phosphate pathway and glutathione levels by dehydroascorbate, the oxidized form of vitamin C. *FASEB J*. 2000;14(10):1352-1361. doi:10.1096/fasebj.14.10.1352
51. Guo L, Qian C, Gao C, et al. Activation of the pentose phosphate pathway mitigates platelet storage lesions and improves platelet preservation quality. *Thromb Res*. 2025;252:109377. doi:10.1016/j.thromres.2025.109377
52. Çimen MB. Free radical metabolism in human erythrocytes. *Clin Chim Acta*. 2008;390(1-2):1-1. doi:10.1016/j.cca.2007.12.025
53. Ou H, Huang Z, Mo Z, Xiao J. The characteristics and roles of advanced oxidation protein products in atherosclerosis. *Cardiovasc Toxicol*. 2017;17(1):1-12. doi:10.1007/s12012-016-9377-8
54. Masselli E, Pozzi G, Vaccarezza M, et al. ROS in platelet biology: functional aspects and methodological insights. *Int J Mol Sci*. 2020;21(14):4866. doi:10.3390/ijms21144866
55. Dionisio LM, Zheng Y, Cancelas JA. Redox Control in Platelet Activity and Therapy. *Antioxidants.* 2025;14(11):1286. doi:10.3390/antiox14111286
56. Freedman JE. Oxidative stress and platelets. *Arterioscler Thromb Vasc Biol*. 2008;28(3):11-16. doi:10.1161/ATVBAHA.107.15917
57. Gulliksson H. Defining the optimal storage conditions for the long-term storage of platelets. *Transfus Med Rev*. 2003;17(3):209-215. doi:10.1016/S0887-7963(03)00020-8