European Journal of Clinical and Experimental Medicine

ONLINE FIRST

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Article type: Original Article

Received: 20 March 2023

Accepted: 24 April 2023

Published online: 5 May 2023

eISSN: 2544-1361

Eur J Clin Exp Med

doi: 10.15584/ejcem.2023.2.24

p-Coumaric acid as a potent additive in blood storage solution

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ABSTRACT

Introduction and aim. Stored erythrocytes develop lesions involving changes in their structure and function reducing their efficacy. Oxidative Stress (OS) being one of the main causes of storage lesion, can be attenuated by antioxidants as additives in the storage solution. This study aims to evaluate the effect of *p*-Coumaric acid (CA) on erythrocytes during whole blood storage.

Material and methods. Blood collected from Male Wistar rats was stored at 4°C in CPDA-1 solution for 21 days. Blood samples were stored with and without 1mM CA (CA 1) and 10 mM CA (CA 10). The erythrocytes were isolated every week during storage and the biomarkers for OS and antioxidant status were analysed.

Results. Superoxide dismutase and catalase elevated on day 14. Conjugate dienes decreased in CA 10 on day 14. Thiobarbituric acid reactive substances increased on day 7 and decreased on day 14 in CA groups. Protein sulfhydryls decreased in controls and CA 1 on day 14 whereas, it was maintained in CA 10.

Conclusion. Coumaric acid upregulated the antioxidant enzymes and protected the cells from oxidative damage. Thus, coumaric acid can be employed as a potent additive during storage and opens new avenues of employing it in similar OS situations in erythrocytes.

Keywords. antioxidants, blood storage, oxidative stress, *p*-coumaric acid

Introduction

Erythrocytes or red blood cells (RBCs) undergo several changes during storage, collectively referred to as the storage lesion, which reduce their effectiveness following transfusion.[1] The rheological properties undergo changes due to the RBC membrane loss with storage. Hemolysis and formation of microparticles

occur due to loss of RBC integrity, which further contribute to the complications associated with transfusion.[2]

Erythrocytes undergo changes like morphologic and metabolic alterations during storage which include loss of biconcave disc shape, depletion of potassium, 2, 3-diphosphoglycerate, adenosine triphosphate, and lipids, increased erythrocyte rigidity and impaired oxygen delivery.[3] Erythrocytes are subjected to oxidative stress due to exposure to oxygen during storage[4] resulting in diminished antioxidants and increase in reactive species. Erythrocytes are well-equipped with endogenous antioxidant systems (superoxide dismutase, catalase, glutathione peroxidase, glutathione, ascorbic acid).[5] Changes in ion permeability on the erythrocyte membrane, increase in lipid peroxidation, oxidation of protein sulfhydryl groups, inactivation of membrane-bound receptors and enzymes, degradation of proteins, and activation of proteolysis have been reported following the challenge of erythrocytes with different oxygen radical-generating systems.[6,7] Erythrocytes are prone to storage lesions due to oxidative stress (OS). A mixed population of young and old erythrocytes are present in circulation. OS influences the aging of erythrocytes during the storage, thereby diminishing the quality of stored blood.[8] Young erythrocytes can endure OS efficiently than the old erythrocytes.[9] Antioxidant intervention has proven to reduce the effect of aging on erythrocytes.[10]

Antioxidants as additives such as Trolox, curcumin, carnosine, spermine, phloretin and ascorbic acid have proven to attenuate the oxidative insult during erythrocyte storage.[11-14]

Studies have reported the preventive effects of *p*-coumaric acid (3-(4-hydroxyphenyl)-2-propenoic acid; CA), a phenolic acid which is found widely distributed in plants and as a part of human diet.[15-18] Coumaric acid has antiproliferative, antiapoptotic, antimicrobial, anti-inflammatory effects and free radical scavenging capacity.[17-22] The effects of CA on erythrocytes during storage have not been explored.

Aim

Hence, this study was carried out to study the effect of CA as an additive during storage.

Material and methods

Animals

Male Wistar rats were obtained from Shri Raghavendra Enterprises. Animal care and maintenance were in accordance with the ethical committee regulations (841/b/04/CPCSEA).

Chemicals

Hemoglobin reagent was obtained from Coral Clinical Systems, Goa, India. Acrylamide, bis thiobarbituric acid (TBA), sodium dodecyl sulphate (SDS), and bovine serum albumin (BSA) stock were purchased from

Sigma-Aldrich Chemicals (St. Louis, MO, USA). All other chemicals were of reagent grade and organic solvents of spectral grade obtained from HiMedia, Mumbai, India.

Blood sampling

Animals were lightly anaesthetized and restrained in dorsal recumbency as described earlier.[23] In brief, the syringe needle was inserted just below the xyphoid cartilage and slightly to the left of midline. Blood was carefully aspirated from the heart into plastic collecting tubes with citrate-phosphate-dextrose-adenine-1 (CPDA-1) solution.

Experimental design

Blood was drawn from male adult Wistar rats (4 months old) and stored in CPDA-1 solution. Blood was collected from 6 animals and stored at 4°C for a period of 21 days. Sample from each animal was divided into the following three groups 1) control group; 2) CA 1-samples with CA as additive at a concentration of 1 mM; 3) CA 10-samples with CA as additive at a concentration of 10 mM. RBCs were isolated from stored blood at regular intervals (every seventh day) and the biomarkers of oxidative stress (OS) were studied.

RBCs separation

RBCs were isolated by centrifugation for 20 min at 1000 *g* at 4°C. Plasma and buffy coat were removed using a micropipette. The cell pellet was washed three times with isotonic phosphate buffer, pH 7.4, centrifuged at 1000 *g* for 10 min, and finally suspended in an equal volume of isotonic phosphate buffer to a final hematocrit of 50%.[24] This constituted of the erythrocyte suspension.

Hemoglobin (Hb)

Hb was measured using Hemocor-D Kit (Coral Clinical Systems), which utilizes the cyanmethemoglobin method.[25] Erythrocytes were incubated with Hb reagent for 3 minutes at room temperature and absorbance was measured at 540 nm (PC-based Double Beam Spectrophotometer 2202, Systronics, Gujarat, India). Hb concentration was represented in terms of g dL^{-1}.

Superoxide dismutase (SOD)

Carbonate buffer was added to the samples (0.05 M, pH 10.2, 0.1 mM ethylenediaminetetraacetic acid (EDTA)) followed by which Epinephrine (30 mM in 0.05 % acetic acid) was added to the mixture. The absorbance was measured at 480 nm for 4 min.[26] SOD activity was expressed as the amount of enzyme that inhibits oxidation of epinephrine by 50% which is equal to 1 unit.

Catalase (CAT)

Absolute ethanol was added to the samples and incubated in ice bath for 30 min. After incubation, phosphate buffer was added to the above mixture. 6 mM hydrogen peroxide (H_2O_2) was added just before reading the absorbance at 240 nm.[27] The molar extinction coefficient of 43.6 Mcm^{-1} was used to determine the catalase activity.

Conjugate dienes

Samples were transferred to ether/ethanol, 1:3 (v/v) mixture and vortexed. The mixture was centrifuged and the level of conjugate dienes was measured spectrophotometrically at 235 nm.[28] Conjugate dienes produced was calculated using the molar extinction coefficient of 29,500 M^{-1} cm^{-1}.

Thiobarbituric acid reactive substances (TBARS)

Sample with 0.9% NaCl was incubated at 37°C for 20 min. 0.8 M hydrochloric acid (HCl) containing 12.5% trichloroacetic acid and 1% thiobarbituric acid was added and kept in boiling water bath for 20 min and cooled at 4°C. Centrifugation was carried out at 1500g and absorbance was measured at 532 nm. TBARS was calculated by using the extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.[29]

Protein sulfhydryls (P-SH)

In brief, 0.08 mol/L sodium phosphate buffer (pH 8.0) containing Na_2-EDTA, and SDS was added to each assay tube containing the membrane protein. 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) was added and the solution was vortexed. Color was allowed to develop for 15 min at room temperature and absorbance was measured at 412 nm, using an equivalent concentration of protein as the blank.[30] Sulfhydryl concentration was calculated from the net absorbance and molar absorptivity, 13,600 mol/L^{-1} cm^{-1}.

Hemolysis

A 5% suspension of packed erythrocytes in buffered saline was mixed with an equal volume of 1% H_2O_2 solution. The mixtures were incubated at 37°C for 1 hour. Hemolysis was determined by measuring released Hb into the supernatant of the induced samples at 540 nm and expressed on the basis of the maximum absorbance [100 %] in the aliquots of erythrocytes completely hemolyzed in distilled water.[31]

Statistical analysis

Results are represented as mean ± SE. Values between the groups were analyzed by two-way ANOVA and $P < 0.05$ was considered significant. Bonferroni's post-test was performed for all the assays using GraphPad Prism 8 software (GraphPad Software, Inc., Dotmatics, San Diego, California).

Results

Result analysis have been depicted as changes during storage i.e., with respect to day 0 and also changes on a particular day of storage between the sub groups (control, CA 1 and CA 10).

Hemoglobin

Changes in Hb were significant ($p<0.001$) during the storage period. Controls showed an increase of 30% on day 14 against day 0. Significant changes were observed with sub groups (CA 1 and CA 10). Hb increased by 38% in CA 10 on day 7 against control (Fig. 1).

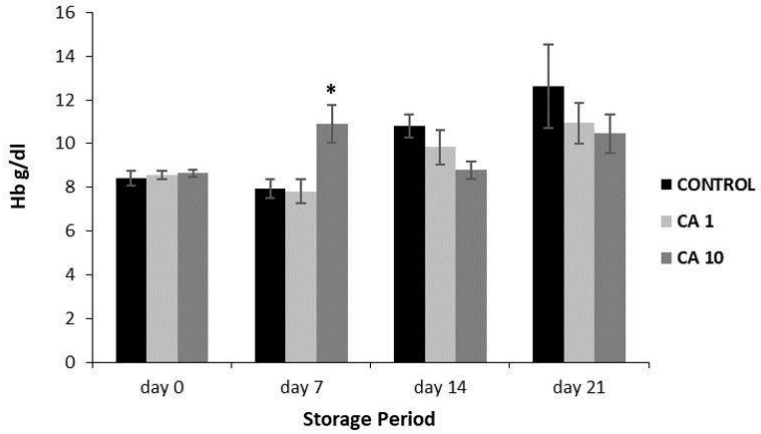

Fig. 1. Hemoglobin in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant at $p<0.001$; ***** represents significance between the subgroups (CA 1 and CA 10) against control)

Superoxide dismutase

Significant changes in SOD activity ($p<0.0001$) were observed in different groups with storage. SOD increased by 15% in control on day 21 compared to day 0. SOD also increased in CA groups (CA 1 and CA 10) on day 14 and day 21 by 17% compared to day 0. SOD activity increased by 10% in CA 10 against control on day 14 (Fig. 2).

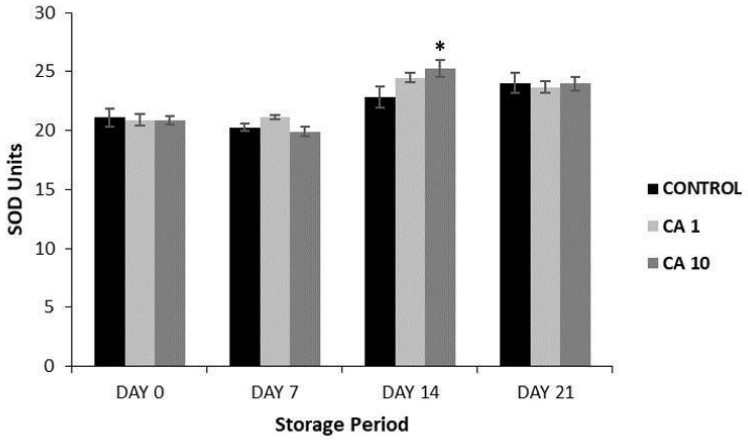

Fig. 2. Superoxide dismutase activity in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); $p<0.05$ was considered significant. Changes between groups (storage) are significant at $p<0.0001$; ***** represents significance between the subgroups (CA 1 and CA 10)

Catalase

CAT activity varied significantly with storage ($p<0.001$). CAT increased in CA 1 by 35% on day 14 against day 0. CAT activity increased by 93% in CA 1 and 54% in CA 10 on day 14 ($p<0.0001$) against the control (Fig. 3).

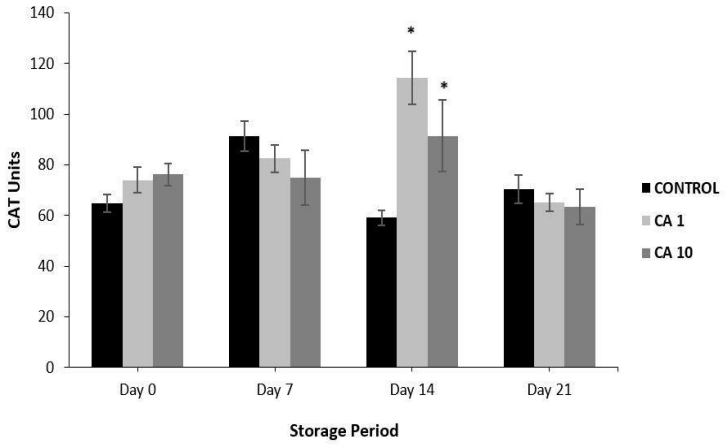

Fig. 3. Catalase activity in erythrocytes of stored blood (values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between the groups (storage) are significant at $p<0.0001$; *****represents significance between the sub groups (CA 1 and CA 10)

Conjugate dienes

Variations in conjugate dienes were significant during storage ($p<0.0001$). Conjugate dienes decreased in controls by 27% on day 7, 35% on day 14 & day 21; while in CA 10 it decreased by 23% on day 14 with respect to day 0. Conjugate dienes decreased against the control by 35% in CA 1 and 26% in CA 10 on day 0 (Table 1).

Table 1. Conjugate dienes, TBARS and protein sulfhydryls in erythrocytes of stored blood [a]

Storage days	Groups	Conjugate dienes	TBARS (µmol/mg protein)	Protein sulfhydryls

		(mM/mg protein)		(Umol/mg protein)
Day 0	Control	0.77±0.08	8.39±1.84	218.52±30.72
	CA 1	0.49±0.03*	2.57±0.31	211.91±18.24
	CA 10	0.57±0.02*	2.70±0.31	81.76±30.18*
Day 7	Control	0.56±0.05	4.91±1.06	105.88±39.66
	CA 1	0.49±0.02	3.85±1.45	103.67±21.76
	CA 10	0.45±0.02	9.5±0.55*	163.52±37.12
Day 14	Control	0.48±0.03	4.67±1.05	78.92±30.07
	CA 1	0.44±0.02	3.70±0.53	80.51±11.82
	CA 10	0.44±0.02	3.54±0.78	70.15±13.45
Day 21	Control	0.52±0.02	2.43±1.12	155.00±41.00
	CA 1	0.48±0.01	3.44±1.21	119.85±33.97
	CA 10	0.47±0.01	7.25±1.96	114.12±39.61

[a] values are expressed as mean ± SE from 6 samples; CA 1 – *p*-coumaric acid (1 mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant at $p<0.01$; ***** represents significance between the subgroups (CA 1 and CA 10).

Thiobarbituric acid reactive substances

Variations in TBARS were significant ($p<0.01$) during storage. TBARS increased in CA 10 by 200% on day 7 and declined to day 0 levels on day 14. TBARS increased by 98% in CA 10 on day 7 against control (Table 1).

Protein sulfhydryls

P-SH varied significantly ($p<0.01$) in different groups with storage. Control and CA 1 showed decrements of 63% on day 14 with day 0 whereas, in CA 10 it was maintained. CA 10 showed decrements of 63% and 54% against control and CA 1 on day 0 (Table 1).

Hemolysis

Significant changes ($p<0.0001$) in hemolysis were observed in all groups with storage. Hemolysis increased in CA 1 by 85% on day 7 and 90% in CA 10 on day 21 when compared to day 0. Hemolysis increased on day 21 by 84% in CA 10 against control (Fig. 4).

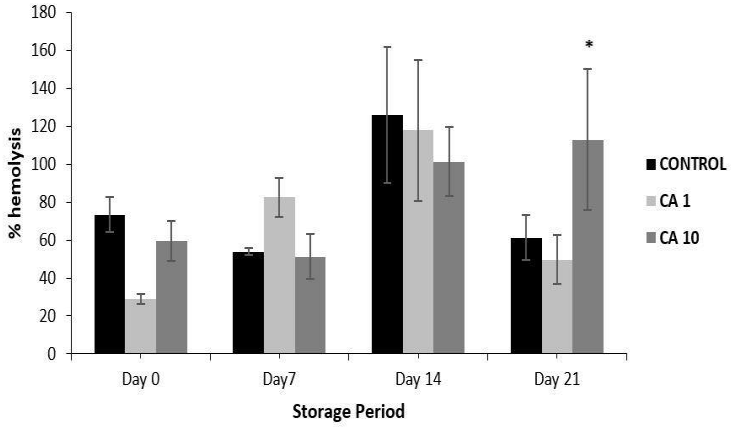

Fig. 4. Hemolysis in erythrocytes of stored blood (values are expressed as mean ±SE from 6 samples. CA 1 – *p*-coumaric acid (1mM); CA 10 – *p*-coumaric acid (10 mM); changes between groups (storage) are significant $p<0.0001$; ***** represents significance between the sub groups (CA 1 and CA 10)

Discussion

The continual changes in erythrocytes during storage and their response to CA as an additive has been assessed to gain insights into the interaction between reactive species and antioxidants. Hb undergoes autoxidation to form MetHb which can be restored by the antioxidants to its reduced state.[32,33] Hb increased on day 7 as OS usually sets in from the second week of storage, resulting in hemolysis.[34] An increase in the hemolysis on day 21 demonstrates the elevations in OS levels towards the end of storage. CA maintained Hb up to day 21, signifying the antioxidant property of coumaric acid through direct scavenging of reactive oxygen species.[35]

SOD catalyzes superoxide radicals to H_2O_2 and O_2.[36] CAT degrades H_2O_2 to H_2O and O_2.[5] SOD reduced in controls during early storage due to ROS levels being lower than the threshold to activate SOD. Urfalioglu et al. reported an increase in the SOD activity in the lung tissue treated with CA.[37] Shen et al. have reported the ability of CA to increase the response of antioxidant genes to ROS.[15] SOD levels elevated on days 14 and 21 in CA samples, suggesting that coumaric acid upregulated SOD which was in accordance with the earlier studies. CAT elevations on day 14 was in accordance with SOD levels as ROS is at its peak during the second week of storage.[23] CAT elevations in CA groups are also in response to coumaric acid's ability to upregulate antioxidant enzyme activity.[15]

The results of the primary products of lipid peroxidation, i.e., conjugate dienes, are indicative of the protective effect of coumaric acid on lipids. CA inhibits low-density lipoprotein thereby, reducing the production of malondialdehyde, a product of lipid peroxidation.[38]

Conjugate dienes showed decrements from day 7 indicating the efficient scavenging activities of antioxidant enzymes. Conjugate dienes also reduced in CA 10 reflecting the antioxidant activity of

coumaric acid. CA can prevent excessive lipid peroxidation with a specific scavenging activity for OH^- radicals as observed in the results of conjugate dienes.[36] TBARS were significant on day 7 and normalised to day 0 levels on day 14 due to the activation of antioxidant enzymes on day 14.

P-SH can be reversibly or irreversibly modified based on the extent of oxidative modifications.[39] P-SH variations correlated with the oxidative insult as observed in the lipid peroxidation. Higher concentrations of reactive oxygen species (ROS) oxidizes sulfhydryls (SH) to disulphides. Since there is high ROS on day 14, CA10 has increased SOD activity which is also reflected in the results of conjugate dienes.[34] CA scavenges hydroxyl radical, superoxide anion and H_2O_2 thereby, normalizing oxidative stress on day 21.[40] Though the changes are insignificant, the variations are due to CA which is reflected in SOD levels. The activation of antioxidant defenses has led to homeostasis and thereby, maintained sulfhydryls in reduced state.

Hemolysis is a result of lipid peroxidation and protein oxidations. Hemolysis increased on day 7 and day 21. A significant increase on day 7 is due to the production of free radicals as reflected in TBARS results. Hemolysis increased on day 21 leading to the evidence that antioxidants and coumaric acid could not scavenge the free radicals completely towards the end of storage, although it could attenuate lipid peroxidation and protein oxidation.

CA at 10 mM concentration was more beneficial than Coumaric acid at 1 mM concentration. CA10 augmented antioxidant defenses such as SOD and CAT, retained sulfhydryls in reduced state and hemolysis was observed only towards the end of the storage period whereas, hemolysis was evident on day 7 in CA1.

Conclusion

Antioxidant defenses of erythrocytes play a major role in attenuating OS during storage. Coumaric acid upregulated the antioxidant enzymes and thereby protected the cells from lipid peroxidation and protein oxidation. Coumaric acid at 10 mM concentration was more beneficial than Coumaric acid at 1 mM concentration. Thus, these results substantiate the potential of coumaric acid as an antioxidant additive during storage and opens new avenues of employing it in similar oxidative stress situations in erythrocytes.

Acknowledgments

The authors acknowledge Dr. Leela Iyengar, Dr. Sowmya Ravikumar, Dr. Manasa Mithun, Ms. Anusha BA and JAIN (Deemed-to-be University) for their support.

Declaration

Funding

No funding was received for conducting this study.

Author contributions

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

Conflicts of interest

The authors declare that they have no conflicts of interest.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical approval

Animal care and maintenance were in accordance with the ethical committee regulations (841/b/04/CPCSEA).

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