

Age associated changes in erythrocyte membrane surface charge: Modulatory role of grape seed proanthocyanidins

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Abstract

Aging, a multifactorial process of enormous complexity is characterized by impairment of physio-chemical and biological aspects of cellular functions. It is closely associated with increased free radical production, which situation ultimately leads to devastation of normal cell function and membrane integrity. The present study was aimed to determine the effect of proanthocyanidins rich grape seed extract (GSP) on membrane surface charge density in erythrocytes during animal age associated oxidative stress. GSP (100 mg/day/kg body weight) was administered orally for 15 and 30 days to young and aged rats. Significant decrease in surface charge levels with concomitant increase in protein carbonyls and decrease in glycoprotein, antioxidants status was noted in erythrocytes of aged rats when compared with young rat erythrocytes. Duration dependent supplementation of GSP increased the erythrocyte surface charge density to near normalcy in aged rats. Decrease in protein carbonyls level and increase in glycoproteins as well as antioxidant status was observed in aged rat erythrocytes on GSP treatment. Thus, from our results, we conclude that GSP is an effective anti-aging drug in preventing the oxidative stress associated loss of membrane surface charge, which thereby maintains the erythrocyte membrane integrity and functions in elderly.

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Keywords: Aging; Erythrocyte surface charge; Protein carbonyls; Glycoprotein; Antioxidants; Proanthocyanidins

1. Introduction

Aging is the sum total changes that occur in living organisms with passage of time leading to functional impairment, increased pathology and death. It is a ubiquitous biological phenomenon associated with biochemical and functional alterations of the cells and tissues. The consequence for such decline in biological function in most of the cells and organ is attributed mainly to the age-dependent increase in production of free radicals (Harman, 1992). The exposure of free radicals on biological systems leads in many cases to increase the cellular levels of oxidatively modified proteins, lipids

and nucleic acids, which coincidentally enhance the development of one or more well recognized, age related manifestations, including loss of physical activity, cellular functions and metabolic integrity. Reactive oxygen species (ROS) attack vital cellular protein component leading to the oxidation of amino acid side chains and protein backbones, formation of protein–protein cross linkages, inhibition of protein synthesis that ultimately results in protein fragmentation and increase of protein carbonyl levels, leading gradually to cell death (Bandyopadhyay et al., 1999).

Erythrocytes, the unique carriers of oxygen are highly susceptible to oxidative stress conditions. The rich polyunsaturated membrane lipids and iron, a potent catalyst for free radical reactions makes erythrocyte a good substrate for oxidative damages. Membrane oxidations do affect the intrinsic membrane properties as well, by altering membrane fluidity, ion transport and loss of enzymic activities of the cell (Chiu et al., 1989). As cell membrane is an important target for radical damage, and blood can reflect the liability of the whole animal to oxidative condition,

Abbreviations used GSP, grape seed proanthocyanidins; ROS, reactive oxygen species; PEG, polyethylene glycol; BSA, bovine serum albumin; DNPH, 2,4-dinitro-phenylhydrazine.

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erythrocytes have been used extensively for determining the effect of aging in studies concerning the possible involvement of free radicals. The erythrocyte cell membrane has a total negative electric charge, which determines the correct course of many processes like transport of metabolic substrates and products through ionic pumps, carriers and membrane channels, for the transfer of information (Nalecz, 1989) and mainly to prevent aggregation of erythrocytes from each other (Jovtchev et al., 2000). The aggregability of erythrocytes is mainly determined by glycocalyx on their membrane, in particular by the amount of the sialic acid residues bearing negative surface charge. Oxidative stress or other damaging effects to surface sialosaccharide may itself play role in aggregation of erythrocytes, increasing the adhesiveness to endothelial cells contributing for the development of various pathologies including diabetes mellitus, atherothrombotic complications (Wautier et al., 1981) and sequestration of circulating erythrocytes by macrophages (Beppu et al., 1995). In normal conditions, the amount of oxy-repair enzymes or antioxidants would possibly be sufficient to cope with the amount of damage produced. However, during aging an oxidant challenge exceeds the capacity of the cell's defense system, membrane damage may occur. During such conditions, dietary intake of antioxidants gains immense importance.

Plant derived flavonoids are suggested to be natural antioxidants associated with prevention of various diseases and pathological conditions. There has been increasing interest on grape seed flavonoid, as they are rich sources of proanthocyanidin oligomers, the flavan-3-ols that has number of biological and therapeutic properties. Recently, grape seed extract have shown to rejuvenate the antioxidant system in central nervous system of aged rats (Balu et al., 2005), have demonstrated cytotoxicity towards melanomas (Martinez Conesa et al., 2005), has beneficial effect on bone formation and bone strength for treating bone debilities (Yahara et al., 2005), and to protect renal tissues against toxicity (Mohanasundari et al., 2005). It also has antigenotoxic effect against H_2O_2 in human lymphoblastoid cells (Sugisawa et al., 2004), possess differential effects on insulin resistance, hypertension, and cardiac hypertrophy (Al-Awwadi et al., 2005) and even has favorable influence on vascular function (Clifton, 2004) and anti-thrombotic effects (Sano et al., 2005). Moreover, studies on erythrocytes have reported that proanthocyanidins from cocoa have strong inhibitory effect towards hydrogen peroxide, xanthine oxidase, organic hydroperoxide, dialuric acid, and 2,2'-azobis(2 amidinopropane)dihydrochloride (AAPH) induced hemolysis (Zhu et al., 2002). However, the effect of GSP on erythrocyte membrane surface charges during animal aging remains unexplored. Hence, the present study was designed to examine the effect of GSP on erythrocyte membrane surface charge, protein carbonyl level and antioxidant status during animal aging.

2. Materials and methods

2.1. Chemicals

Dextran 500 (500,000 molecular weight), Polyethylene glycol (PEG, 8000 molecular weight), Bovine serum albumin (BSA), and 2,4-dinitro-phenylhydrazine (DNPH) were purchased from Sigma Chemical and Company, Saint Louis, MO, USA. All other chemicals were of reagent grade.

2.2. Animals and treatment

Male albino rats of Wistar strain, weighing approximately 120–160 g (young) and 380–420 g (old) were used. The animals were obtained from King Institute of Preventive Medicine, Chennai. The animals were divided into two major groups namely, Group I: normal young rats (3–4 months old) and Group II: normal aged rats (about 24 months old). Each group was further sub-divided into three groups: one control group (Group Ia, IIa) and two experimental groups based on the duration of GSP administration for 15 days (Group Ib, IIb) and 30 days (Group Ic, IIc). Each group consisted of six animals. The animals were maintained on commercial rat feed containing 5% fat, 21% protein, 55% nitrogen free extract, 4% fiber (w/w) with adequate mineral and vitamin contents and had access to water ad libitum. GSP was supplemented orally at the dosage of 100 mg/kg body weight/day for 15, 30 days, whereas control young and aged rats received vehicle alone in a similar manner.

On completion of 15, 30 days of GSP supplementation, the blood was collected with 3.8% sodium citrate from jugular vein and used for the isolation of erythrocytes and erythrocyte membranes. The general physical health of rats with respect to body weight and movement abilities were monitored throughout the duration of GSP therapy and the changes were found to be insignificant.

2.3. Preparation of proanthocyanidins rich grape seed extract

Preparation of proanthocyanidins rich grape seed extract was performed as described before (Zhao et al., 1999). The total polyphenolic content of the extract obtained when subjected to high performance liquid chromatography contained 81.3% of proanthocyanidins, 6.7% of monomeric catechins and 5.4% of organic acids.

2.4. Preparation of erythrocytes and erythrocyte membranes

Isolation of erythrocytes and erythrocyte membranes was done according to Dodge et al. (1963) with slight modifications. Briefly, blood collected from animals with 3.8% sodium citrate was subjected to centrifugation at 3000 rpm for 10 min at 4 °C. The plasma and buffy coat

were removed by aspiration and the erythrocytes were washed three times with cold (4 °C) phosphate-buffered saline (PBS: 0.15 M NaCl, 1.9 mM NaH₂PO₄, 8.1 mM Na₂HPO₄, pH 7.4). Washed erythrocytes were hemolyzed in 40 volumes of 5 mM sodium phosphate buffer (pH 8.0) (containing 1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride, as protein inhibitor), and centrifuged for 20 min at 4 °C at 15,000 rpm. The supernatant (or hemolysate) was decanted carefully and saved, while the pellet were washed repeatedly and incubated for 45 min at 37 °C to reseal the membrane in the presence of 0.8 mM MgCl₂-ATP solution, for hemoglobin free ghosts. Hemoglobin amount was estimated by Drabkin and Austin (1932) method and erythrocyte membrane protein content by Lowry et al. (1951).

2.5. Determination of erythrocyte surface charge

Erythrocyte partition coefficients were determined at room temperature by a two-phase aqueous system containing 5% Dextran 500 and 4.3% PEG 8000 in isotonic phosphate buffer (pH 6.8) by the method of Walter (1985). The two-phase system used is 'charge sensitive' whose partition coefficient ratio indicates the level of erythrocyte surface charge.

2.6. Determination of protein carbonyls

Protein carbonyls content was determined by the reliable method based on the reaction of carbonyl groups with 2,4-dinitro-phenylhydrazine to form 2,4-dinitro-phenylhydrazone as suggested by Levine et al. (1994).

2.7. Determination of glycoproteins

Lipids were extracted from the erythrocyte membrane pellets according to the method of Folch et al. (1957) using chloroform-methanol mixture (2:1 v/v). The resulting defatted residue was suspended in sodium acetate buffer (containing 2 mg cysteine HCl/ml, final pH 7.0) and deproteinized by 4–5 volumes of ethanol, evaporated to dryness in the cold under a vacuum and subjected to hydrolysis by heating with 2 ml of 4 N HCl for 4–6 h. The hydrolyzed material was neutralized with 4 N sodium

hydroxide and used for estimating erythrocyte hexose (Niebes, 1972), hexosamine (Wagner, 1979) and sialic acid (Warren, 1959).

2.8. Determination of antioxidants

Superoxide dismutase (SOD) in hemolysate was assayed by the method of Marklund and Marklund (1974), while erythrocyte membranes were used to estimate catalase (CAT) (Beers and Seizer, 1952). Hemolysate glutathione peroxidase (GPx) was determined by Rotruck et al. (1973) method, glutathione reductase (GR) by the procedure of Staal et al. (1969), glucose-6-phosphate dehydrogenase (G6PD) by Zinkham et al. (1958) and glutathione-S-transferase (GST) according to Habig et al. (1974).

Hemolysate reduced glutathione (GSH) was estimated by Moron et al. (1979) and oxidized glutathione (GSSG) was assayed according to the method of Tietze (1969). Erythrocyte redox state was determined by the redox index: $(\text{GSH} + 2 \times \text{GSSG}) / (2 \times \text{GSSG} \times 100)$.

Blood ascorbic acid was assayed according to Omaye et al. (1979) and erythrocyte membrane α -tocopherol content by the method of Desai (1984).

2.9. Statistical analysis

The results are expressed as mean \pm standard deviation (SD) for six rats in each group. Differences between groups were assessed by one-way ANOVA using the SPSS version 7.5 software package for Windows, USA. Post hoc testing was performed for inter-group comparisons using the least significance difference (LSD) test; statistical significance at p -values <0.05 , <0.01 , <0.001 have been given respective symbols in the figures and tables.

3. Results

The young control (Group Ia) and aged control rats (Group IIa) received vehicle alone, whereas 15, 30 days treated young (Group Ib, Ic) and aged rats (Group IIb, IIc) received GSP orally at the dosage of 100 mg/kg body weight/day. The results obtained in erythrocytes of control

Table 1

Levels of partition coefficient ratio and protein carbonyls in erythrocytes of control and GSP treated young and aged rats

Parameters	Young rats			Aged rats		
	Ia (control)	Ib (15 days)	Ic (30 days)	IIa (control)	IIb (15 days)	IIc (30 days)
Partition coefficient ratio	0.82 \pm 0.08	0.83 \pm 0.08	0.84 \pm 0.09	0.48 \pm 0.04 A*	0.62 \pm 0.06 B#	0.74 \pm 0.07 B*CS
Protein carbonyls (μ moles DNPH/mg protein)	9.01 \pm 1.08	8.9 \pm 0.09	8.78 \pm 1.04	14.38 \pm 1.47 A*	11.55 \pm 1.46 B*	10.08 \pm 1.02 B*CS

Values are expressed as mean \pm SD for six rats. Comparisons are between A-Group Ia with IIa, B-Group IIb, IIc with Group IIa, C-Group IIb with Group IIc. Values are statistically significant at $p < 0.05$, $\#p < 0.01$, $*p < 0.001$.

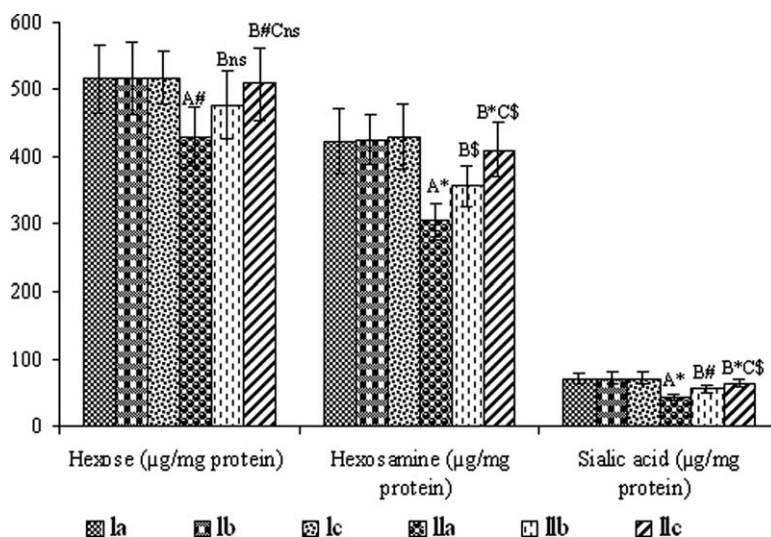


Fig. 1. Levels of glycoproteins in erythrocytes of control and GSP treated young and aged rats. Control young (Group Ia) and aged rats (Group IIa) received vehicle alone. 15, 30 days treated young (Group Ib, Ic) and aged rats (Group IIb, IIc) received GSP orally at the dosage of 100 mg/kg body weight/day. Values are expressed as mean \pm SD for six rats. Comparisons are between A-Group Ia with IIa, B-Group IIb, IIc with Group IIa, C-Group IIb with Group IIc. Values are statistically significant at \$ $p < 0.05$; # $p < 0.01$; * $p < 0.001$; ns, non-significant.

and experimental young and aged rats are described in detail below.

Table 1 shows the age related changes in partition coefficient ratio and erythrocyte membrane protein carbonyls level. Normal partition coefficient ratio and normal protein carbonyls level were observed in erythrocytes of control and GSP treated young rats. These demonstrate membrane integrity and functions are maintained at normal levels in erythrocytes of young rat. However, significant decrease ($p < 0.001$) in the partition coefficient ratio and increase in protein carbonyls level ($p < 0.001$) were observed in aged rat erythrocytes when compared with young rat erythrocytes. The result illustrated significant surface charge loss and increased free radical attack on membrane proteins in aged animal. GSP administered aged rats significantly increased partition coefficient ratio on 15 days ($p < 0.01$) and 30 days ($p < 0.001$) treatment. Protein carbonyls level in aged rats was also decreased ($p < 0.001$) on duration dependent GSP supplementation

with significant difference between 15 (Group IIb) and 30 days (Group IIc) treatment at $p < 0.05$.

Fig. 1 illustrates the levels of hexose, hexosamine and sialic acid in erythrocytes of control and GSP treated young and aged rats. The glycoprotein levels in erythrocytes of young rats were at normal level and did not show any significant alterations on GSP treatment. The aged control rats showed significant decrease in hexose, hexosamine and sialic acid by 17, 28, and 39%, respectively. GSP administration to aged rats for 15 days showed a significant increase in hexosamine ($p < 0.05$) and sialic acid ($p < 0.01$), while 30 days treatment showed increase of hexose at $p < 0.01$, hexosamine at $p < 0.001$ and sialic acid at $p < 0.001$.

Table 2 indicates the erythrocyte enzymatic antioxidant status of control and GSP treated young and aged rats. Young control rats had normal level of enzymatic antioxidants and showed insignificant changes on GSP supplementation. However, significant decrease in the activities of SOD, CAT, GPx, GR, GST and G6PD at

Table 2
Activities of enzymatic antioxidants in erythrocytes of control and GSP treated young and aged rats

Parameters	Young rats			Aged rats		
	Ia (control)	Ib (15 days)	Ic (30 days)	IIa (control)	IIb (15 days)	IIc (30 days)
SOD (U/mg Hb)	3.85 \pm 0.48	3.93 \pm 0.38	4.02 \pm 0.48	2.24 \pm 0.25 A*	3.00 \pm 0.34 B#	3.58 \pm 0.42 B*CS
CAT (μ moles/min/mg protein)	4.15 \pm 0.58	4.21 \pm 0.54	4.25 \pm 0.50	2.52 \pm 0.27 A*	3.12 \pm 0.36 B\$	3.75 \pm 0.40 B*CS
GPx (U/g Hb)	7.03 \pm 0.75	7.06 \pm 0.77	7.15 \pm 0.73	4.20 \pm 0.48 A*	5.41 \pm 0.61 B#	6.36 \pm 0.70 B*CS
GR (U/g Hb)	2.42 \pm 0.31	2.45 \pm 0.32	2.51 \pm 0.24	1.45 \pm 0.18 A*	1.87 \pm 0.13 B#	2.23 \pm 0.20 B*CS
GST (nmoles of CDNB conjugated/min/mg Hb)	1.13 \pm 0.18	1.17 \pm 0.16	1.23 \pm 0.13	0.74 \pm 0.07 A*	0.91 \pm 0.09 B\$	1.00 \pm 0.11B#Cns
G6PD (U/g Hb)	6.00 \pm 0.55	6.05 \pm 0.60	6.10 \pm 0.65	3.58 \pm 0.41 A*	4.81 \pm 0.47 B*	5.50 \pm 0.42 B*CS

Values are expressed as mean \pm SD for six rats. Comparisons are between A-Group Ia with IIa, B-Group IIb, IIc with Group IIa, C-Group IIb with Group IIc. Values are statistically significant at \$ $p < 0.05$; # $p < 0.01$; * $p < 0.001$; ns, non-significant.

Table 3
Levels of GSH, GSSG, GSH/GSSG ratio and redox index in erythrocytes of control and GSP treated young and aged rats

Parameters	Young rats			Aged rats		
	Ia (control)	Ib (15 days)	Ic (30 days)	IIa (control)	IIb (15 days)	IIc (30 days)
GSH (μ moles/g Hb)	3.21 \pm 0.31	3.25 \pm 0.32	3.28 \pm 0.34	2.00 \pm 0.26 A*	2.59 \pm 0.25 B*	2.98 \pm 0.24 B*CS
GSSG (μ moles/g Hb)	0.06 \pm 0.00	0.06 \pm 0.00	0.06 \pm 0.00	0.09 \pm 0.00 A*	0.07 \pm 0.00 B#	0.06 \pm 0.00 B*CS
GSH/GSSG ratio	53.5 \pm 5.01	54.16 \pm 5.88	54.66 \pm 6.40	22.22 \pm 2.13 A*	37.00 \pm 3.16 B*	49.65 \pm 5.01 B*CS
Redox index	0.27 \pm 0.02	0.28 \pm 0.02	0.28 \pm 0.02	0.12 \pm 0.01 A*	0.19 \pm 0.01 B*	0.26 \pm 0.02 B*CS

Values are expressed as mean \pm SD for six rats. Comparisons are between A-Group Ia with IIa, B-Group IIb, IIc with Group IIa, C-Group IIb with Group IIc. Values are statistically significant at \$ $p < 0.05$; # $p < 0.01$; * $p < 0.001$.

$p < 0.001$ were observed in control aged rat erythrocytes when compared with young control rats erythrocytes. 15 days GSP administration to aged rats showed increase in the activities of SOD ($p < 0.01$), CAT ($p < 0.05$), GPx ($p < 0.01$), GR ($p < 0.01$), GST ($p < 0.05$) and G6PD ($p < 0.001$). While 30 days treatment demonstrated a significant increase in all the enzymatic antioxidant at $p < 0.001$ except GST, which showed an increase at $p < 0.01$. Further, the difference between 15 and 30 days treatment for SOD, CAT, GPx, GR and G6PD was significant at $p < 0.05$. These results demonstrated that 30 days treatment is more efficacious than 15 days treatment in elevating the activities of decreased erythrocyte antioxidant status of aged rats to near normalcy.

Table 3 demonstrates the glutathione status in erythrocytes of control and experimental rats. Erythrocyte glutathione status was observed to be at normal levels in control and treated young rats. A significant decrease of GSH level with increase of GSSG levels in aged rats

erythrocytes indicates the elevated GSH oxidation with animal aging. These alterations ultimately decreased the GSH/GSSG ratio and redox index ($p < 0.001$) in aged rat erythrocytes. However, increase of GSH content and decrease of GSSG levels with subsequent increase of GSH/GSSG ratio and redox index ($p < 0.001$) was observed on duration dependent GSP treatment, demonstrating the glutathione improving activity of proanthocyanidins.

Fig. 2 illustrates the blood ascorbic acid status and erythrocyte membrane α -tocopherol levels in control and experimental rats. GSP supplemented young rats showed insignificant changes in ascorbate and α -tocopherol levels when compared with young control rats. However, ascorbate and α -tocopherol levels were significantly decreased ($p < 0.001$) in erythrocytes of aged rats. Administration of GSP to aged rats increased the ascorbate and α -tocopherol levels on 15 days treatment at $p < 0.01$ and on 30 days treatment at $p < 0.001$. This indicates the favorable

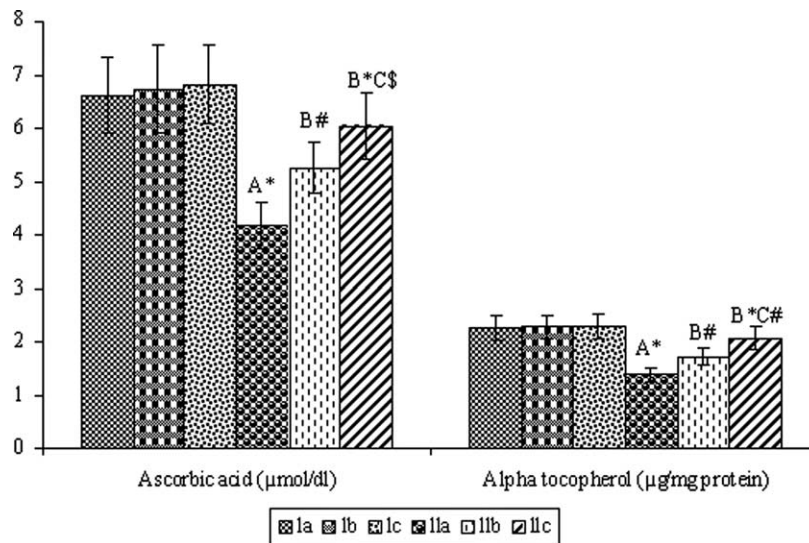


Fig. 2. Levels of non-enzymatic antioxidants in erythrocytes of control and GSP treated young and aged rats. Control young (Group Ia) and aged rats (Group IIa) received vehicle alone. 15, 30 days treated young (Group Ib, Ic) and aged rats (Group IIb, IIc) received GSP orally at the dosage of 100 mg/kg body weight/day. Values are expressed as mean \pm SD for six rats. Comparisons are between A-Group Ia with IIa, B-Group IIb, IIc with Group IIa, C-Group IIb with Group IIc. Values are statistically significant at \$ $p < 0.05$; # $p < 0.01$; * $p < 0.001$.

effect of GSP in improving the non-enzymatic antioxidant status as well in aged animals.

Correlation analysis study in aged control and treated rats showed, negative correlations between surface charge and protein carbonyls ($r = -0.678$, $p < 0.01$), GSSG levels ($r = -0.693$, $p < 0.01$). While positive correlations was observed between surface charge and activities of enzymatic antioxidants SOD ($r = 0.800$, $p < 0.001$), CAT ($r = 0.720$, $p < 0.001$), GPx ($r = 0.692$, $p < 0.01$), GR ($r = 0.662$, $p < 0.01$), GST ($r = 0.762$, $p < 0.001$), G6PD ($r = 0.891$, $p < 0.001$). Non-enzymatic antioxidants like ascorbic acid ($r = 0.795$, $p < 0.001$), α -tocopherol ($r = 0.650$, $p < 0.01$) and glutathione levels GSH ($r = 0.677$, $p < 0.01$), GSH/GSSG ($r = 0.892$, $p < 0.001$) also showed a positive correlation with surface charge levels. Glycoproteins like hexose ($r = 0.659$, $p < 0.01$), hexosamine ($r = 0.631$, $p < 0.01$) and sialic acids ($r = 0.761$, $p < 0.001$) when correlated with surface charge levels illustrated a positive correlation. Thus, except for protein carbonyls and GSSG levels all other parameter showed positive correlation with surface charge.

4. Discussion

Proper maintenance of membrane integrity and function is important for cell's survival. The membrane interacts with both intra and extracellular environment that could bring about membrane changes probably responsible for the selective removal of erythrocytes prematurely from circulation. Conservation of the cell membrane structure and proper charge levels on its surface determines the correct course of many processes. As surface charge is a measure of cell condition, changes in surface charge indicate increased risk of erythrocytes to form aggregates contributing to many pathologic conditions. Even changes in erythrocyte structure and functions in chronic renal insufficiency and purulent intoxication were paralleled by changes in negative surface charges (Samoilov et al., 2002). A significant decrease of erythrocyte surface charge in aged rats when compared with erythrocytes of young rats was noted in our study. This decrease could be possibly due to the increased protein oxidation leading to carbonyl formations in aged rats erythrocytes. As previous results have shown, skeletal protein of erythrocytes when treated with oxidative reagent like diamide, hydrogen peroxide and phenylhydrazine results in cross linkages of sulfhydryl group and surface charge modifications (Fung et al., 1996). Earlier, our laboratory results demonstrated significant changes in the metabolism of protein in number of organs during oxidative stress associated aging in rats (Arivazhagan and Panneerselvam, 1999). Thus, increased oxidative damage on membrane proteins during animal aging does lead to the diminution of negative surface charge in erythrocytes.

Further, decrease of glycoproteins observed in aged rat erythrocytes is also thought to play a major role in surface

charge determination. Studies have shown, both external and internal removal of glycoprotein, especially sialic acid on erythrocytes decreases surface charge level leading to the emergence of non-IgG covered epitopes on the surface of oxidized erythrocytes (Leb et al., 1987), consequently allowing the recognition of erythrocytes by macrophages (Beppu et al., 1995). Further, oxidation of glycophorin A, major sialoglycoprotein decreases sialic acid content that eventually decrease membrane surface charges, contributing to rouleaux formation (Beppu et al., 1994). In addition, our results are in accordance with the work of Vomel (1984) who also reported that the decrease of sialic acid in old individuals results in surface charge modifications.

GSP supplementation showed insignificant changes in erythrocytes of young rats probably because they are not affected by oxidative stress conditions with cellular potential to resist to ROS. However, significant decrease of protein carbonyls and increase of glycoproteins level to near normalcy in erythrocytes of GSP treated aged rats would have ultimately increased the membrane surface charge density in them. These alterations were probably due to the free radical scavenging and reduced thiol group elevating properties of GSP (Sen et al., 1998) that protects the membrane proteins from oxidative insults. Previous reports have also proposed, flavonoids maintain the redox state of sulfhydryl residues in membrane proteins of HL-20 cells by oxidoreductase activity (VanDuijn et al., 1998).

Imbalance between radical production and catabolism of oxidant during aging would shift the cells towards oxidative stress resulting in alterations of membrane properties and cell dysfunction. Since, earlier studies have reported, stressed reticulocytes never mature to erythrocytes having normal membrane surface charges and gradually leads to the premature death of erythrocytes (Walter et al., 1975). SOD plays a key role in detoxifying superoxide anions, which otherwise damages the cell membranes and macromolecules. A significant decrease in SOD activity was observed in aged rats erythrocytes compared to young rats. This might be due to the increased superoxide radical formation via the Fenton reaction in aged animals. The increase of SOD activity in aged rat erythrocytes on GSP treatment may be due to the potential quenching of free radicals by proanthocyanidins through the formation of resonance-stabilized phenoxyl radical, which significantly decrease the superoxide radicals level (Rice-Evans et al., 1996). Other investigation by ESR techniques has also reported proanthocyanidins, as SOD equivalents in scavenging superoxide radicals very effectively (Virgili et al., 1998). Catalase, a hemoprotein that catalyzes the decomposition of H_2O_2 , protects the cell from oxidative damage was decreased in our present study in erythrocytes of aged rats. Our data was analogous to prior reports, which showed decrease of erythrocyte CAT activities in old rats (Glass and Gershon, 1984). The reduction of CAT with animal aging could be because of the decrease in NADH, since NADH is necessary for the conversion of CAT from its inactive state.

GSP treatment increased CAT activity in aged rats via the increase of G6PD activity, which produces NADH by pentose pathway in erythrocytes.

GPx efficiently protects the cell membrane from lipid peroxidation and catalyzes the reaction of hydroperoxides with GSH to form GSSG. As glutathione is a reducing agent because of its sulphhydryl groups against oxidative stress, depletion of glutathione causes a proportional decrease of GPx activity (Spector et al., 1993). Thus, the decreased GSH in erythrocytes of aged rats could be the reason for the inhibited activities of GPx and GST, since these enzymes act only at the expense of GSH. Further, decreased G6PD activity with decrease of GR in aged rat erythrocytes shows the inability of the system to maintain GSH/GSSG ratio. Liu and Choi (2000) has also reported rat erythrocyte GSH levels are lower in 24 months old rats when compared to 3 month old rats. Moreover, earlier studies have shown decrease in GR activities and GSH content with animal age (Stio et al., 1994). These reasons could possibly explain for the altered GSH/GSSG and redox index in erythrocytes of aged rats. However, GSP treatment increased the GSH level that ultimately increased the GPx, GST GR, G6PD levels to near normalcy in aged rats. The reason was probably due to the protection of sulphhydryl groups in glutathione from oxidative damages via free radical quenching action of di-OH (catechol) structure in the B ring of proanthocyanidin in GSP (Ishige et al., 2001).

The decrease of ascorbic acid in aged rats compared to young rat erythrocytes was because of the increased oxidative stress with animal aging. The decrease of ascorbate ultimately damages the erythrocyte membrane, since they are involved in regeneration of α -tocopherols, the lipophilic membrane antioxidant from its oxidized form. Our results showed that GSP were capable of reversing the decline of vitamin C in the aged rats. This would have been possibly due to the (i) increased ascorbic acid absorption, (ii) stabilization of ascorbic acid, (iii) reduction of dehydroascorbate to ascorbate, and (iv) metabolic sparing of ascorbic acid by the flavonoids (Hughes and Wilson, 1977) in GSP. In addition, based on the former information of Yamasaki and Grace (1998), proanthocyanidin prolong ascorbate radical lifetime by one-electron reduction of dehydroascorbic acid and regenerate ascorbic acid from its corresponding radical. Further, the increased oxidisability of fatty acids in aged rat erythrocytes decreases vitamin E, as they protect the cellular bilayer from free radical attack. Elevation of vitamin E levels in GSP administered aged rats was probably through the chain breaking action of proanthocyanidins within the core of phospholipids bilayer, which save vitamin E and recycle them from the tocopheroxyl radical through an H-transferring mechanism, thereby behaving as a sacrificial antioxidant (Maffei Facino et al., 1998). Moreover, the metal chelating property of GSP increase vitamin E content by preventing the involvement of hemoglobin iron in lipid peroxidation processes (Maffei Facino et al., 1996). Taken together,

GSP potentially increase the enzymatic and non-enzymatic antioxidants levels in aged rat erythrocytes. These antioxidants protect erythrocyte membrane from free radical attack eventually preventing the loss of surface charge levels during age associated oxidative stress.

In summary, we found that erythrocyte membrane surface charge is decreased in aged rats possibly due to the increased protein oxidation and decreased enzymatic and non-enzymatic antioxidant levels with advancement of animal age. GSP treatment significantly decreased protein oxidation and increased antioxidant status that concomitantly increased the surface charge density in erythrocytes of aged rats in duration dependent manner. The outcome of the present study thus indicated, GSP supplementation could be a possible therapeutic measure and an exact remedy for minimizing the age related alterations in erythrocytes for the existing population, as the health care and welfare of the aged have been attracting considerably as a social problem. Thereby, GSP acts as a potent anti-aging drug for preventing health disorders and for improving the well being of elderly to lead a normal life span free of ailments.

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References

- Al-Awwadi, N.A., Araiz, C., Bornet, A., Delbosc, S., Cristol, J.P., Linck, N., Azay, J., Teissedre, P.L., Cros, G., 2005. Extracts enriched in different polyphenolic families normalize increased cardiac NADPH oxidase expression while having differential effects on insulin resistance, hypertension, and cardiac hypertrophy in high-fructose-fed rats. *J. Agric. Food Chem.* 53, 151–157.
- Arivazhagan, P., Panneerselvam, C., 1999. Favorable effect of L-Carnitine supplementation on glycoprotein status in aged rats. *J. Clin. Biochem. Nutr.* 26, 193–200.
- Balu, M., Sangeetha, P., Haripriya, D., Panneerselvam, C., 2005. Rejuvenation of antioxidant system in central nervous system of aged rats by grape seed extract. *Neurosci. Lett.* 383, 295–300.
- Bandyopadhyay, U., Das, D., Banerjee, R.K., 1999. Reactive oxygen species, oxidative stress and pathogenesis. *Curr. Sci.* 77, 658–666.
- Beers, R.F., Seizer, I.W., 1952. A spectrophotometric method for measuring breakdown of hydrogen peroxide by catalase. *J. Biol. Chem.* 195, 133–140.
- Beppu, M., Takanashi, M., Kashiwada, M., Masukawa, S., Kikugawa, K., 1994. Macrophage recognition of saccharide chains on the erythrocytes damaged by iron-catalyzed oxidation. *Arch. Biochem. Biophys.* 312, 189–197.
- Beppu, M., Hayashi, T., Hasegawa, T., Kikugawa, K., 1995. Recognition of sialosaccharide chain of glycophorin on damaged erythrocytes by macrophage scavenger receptors. *Biochim. Biophys. Acta* 1268, 9–19.
- Chiu, D., Kuypers, F., Lubin, B., 1989. Lipid peroxidation in human red cells. *Semin. Haematol.* 26, 257–276.

- Clifton, P.M., 2004. Effect of grape seed extract and quercetin on cardiovascular and endothelial parameters in high-risk subjects. *J. Biomed. Biotechnol.* 2004, 272–278.
- Desai, I.D., 1984. Vitamin E analysis methods for animal tissues. *Methods Enzymol.* 105, 138–147.
- Dodge, J.T., Mitchell, C., Hanahan, D.J., 1963. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocyte. *Arch. Biochem. Biophys.* 100, 119–130.
- Drabkin, D.L., Austin, J.H., 1932. Spectrophotometric studies, spectrophotometric constants for common hemoglobin derivatives in human, dog and rabbit blood. *J. Biol. Chem.* 98, 719–733.
- Folch, J., Less, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497–509.
- Fung, L.W., Kalaw, B.O., Hatfield, R.M., Dias, M.N., 1996. Erythrocyte spectrin maintains its segmental motions on oxidation: a spin-label EPR study. *Biophys. J.* 70, 841–851.
- Glass, G.A., Gershon, D., 1984. Decreased enzymic protection and increased sensitivity to oxidative damage in erythrocytes as a function of cell and donor aging. *Biochem. J.* 218, 531–537.
- Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-transferases, the first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* 249, 7130–7139.
- Harman, D., 1992. Role of free radicals in ageing and disease. *Ann. N Y Acad. Sci.* 673, 598–620.
- Hughes, R.E., Wilson, H.K., 1977. Flavonoids: some physiological and nutritional considerations. *Prog. Med. Chem.* 14, 285–301.
- Ishige, K., Schubert, D., Sagara, Y., 2001. Flavonoids protect neuronal cells from oxidative stress by three distinct mechanisms. *Free Radic. Biol. Med.* 30, 433–446.
- Jovtchev, S., Djenev, I., Stoeff, S., Stoylov, S., 2000. Role of electrical and mechanical properties of red blood cells for their aggregation. *Colloids Surf. Physiochem.* 164, 95–104.
- Leb, L., Snyder, L.M., Fortier, N.L., Anderson, M., 1987. Antiglobulin serum mediated phagocytosis of normal senescent and oxidized RBC: role of anti-IgM immunoglobulins in phagocytic recognition. *Br. J. Haematol.* 66, 565–570.
- Levine, R.L., Williams, J.A., Stadtman, E.R., Shacter, E., 1994. Carbonyl assays for determination of oxidatively modified proteins. *Methods Enzymol.* 233, 346–357.
- Liu, R., Choi, J., 2000. Age-associated decline in γ -glutamylcysteine synthetase gene expression in rats. *Free Radic. Biol. Med.* 28, 566–574.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193, 265–275.
- Maffei Facino, R., Carini, M., Aldini, G., Berti, F., Rossoni, G., Bombardelli, E., Morazzoni, P., 1996. Procyanidins from *Vitis vinifera* seeds protect rabbit heart from ischemia/reperfusion injury: antioxidant intervention and/or iron and copper sequestering ability. *Planta Med.* 62, 495–502.
- Maffei Facino, R., Carini, M., Aldini, G., Calloni, M.T., Bombardelli, E., Morazzoni, P., 1998. Sparing effect of proanthocyanidins from *Vitis vinifera* on vitamin E: in vitro studies. *Planta Med.* 343, 347.
- Marklund, S., Marklund, G., 1974. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur. J. Biochem.* 47, 469–474.
- Martinez Conesa, C., Vicente Ortega, V., Yanez Gascon, M.J., Garcia Reverte, J.M., Canteras Jordana, M., Alcaraz Banos, M., 2005. Experimental model for treating pulmonary metastatic melanoma using grape-seed extract, red wine and ethanol. *Clin. Transl. Oncol.* 7, 115–121.
- Mohanasundari, M., Sabesan, M., Sethupathy, S., 2005. Renoprotective effect of grape seeds extract in ethylene glycol induced nephrotoxic mice. *Indian J. Exp. Biol.* 43, 356–359.
- Moron, M.S., Depierre, J.W., Mannervik, B., 1979. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. *Biochim. Biophys. Acta* 582, 67–78.
- Nalecz, K., 1989. Functional and structural aspects of transport of low molecular weight compounds through biological membranes. *Postepy Biochem.* 35, 437–467.
- Niebes, P., 1972. Determination of enzymes and degradation products of glycosaminoglycans metabolism in the serum of healthy and varicose subjects. *Clin. Chim. Acta.* 42, 399–408.
- Omaye, S.T., Turnbull, J.D., Sauberlich, H.E., 1979. Selected methods for the determination of ascorbic acid in animal cells, tissues, and fluids. *Methods Enzymol.* 62, 3–11.
- Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20, 933–956.
- Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G., 1973. Selenium: biochemical role as a component of glutathione peroxidase purification and assay. *Science* 179, 588–590.
- Samoilov, M.V., Mishnev, O.D., Kudriavtser, I.V., Naumor, A.G., Daniltov, A.P., 2002. Morphofunctional characteristics of erythrocytes in chronic kidney failure and purulent intoxication. *Klin. Lab. Diagn.* 6, 18–23.
- Sano, T., Oda, E., Yamashita, T., Naemura, A., Ijiri, Y., Yamakoshi, J., Yamamoto, J., 2005. Anti-thrombotic effect of proanthocyanidin, a purified ingredient of grape seeds. *Thromb. Res.* 115, 115–121.
- Sen, C.K., Roy, S., Packer, L., 1998. Flow cytometric determination of cellular thiols. *Methods Enzymol.* 299, 247–258.
- Spector, A., Wang, G.M., Wang, R.R., Garner, W.H., Moll, H., 1993. The prevention of cataract caused by oxidative stress in cultured rat lenses by H₂O₂ and photochemically induced cataract. *Curr. Eye Res.* 12, 163–179.
- Staal, G.E., Visser, J., Veeger, C., 1969. Purification and properties of glutathione reductase of human erythrocytes. *Biochim. Biophys. Acta* 185, 39–48.
- Stio, M., Iantomasi, T., Favilli, F., Marraccini, P., Lunghi, B., Vincenzini, M.T., Treves, C., 1994. Glutathione metabolism in heart and liver of the aging rat. *Biochem. Cell Biol.* 72, 58–61.
- Sugisawa, A., Inoue, S., Umegaki, K., 2004. Grape seed extract prevents H₂O₂-induced chromosomal damage in human lymphoblastoid cells. *Biol. Pharm. Bull.* 27, 1459–1461.
- Tietze, F., 1969. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal. Biochem.* 27, 502–522.
- VanDuijn, M.M., Van den Zee, J., Vansteveninck, J., Van den Broek, P.J., 1998. Ascorbate stimulates ferricyanide reduction in HL-60 cells through a mechanism distinct from the NADH-dependent plasma membrane reductase. *J. Biol. Chem.* 273, 13415–13420.
- Virgili, F., Kobuchi, H., Packer, L., 1998. Procyanidins extracted from *Pinus maritime* (Pycnogenol): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages. *Free Radic. Biol. Med.* 24, 1120–1129.
- Vomel, T., 1984. Properties of ATPases and energy-rich phosphate in erythrocyte of young and old individuals. *Gerontology* 30, 22–25.
- Wagner, W.D., 1979. A more sensitive assay discriminating galactosamine and glucosamine in mixtures. *Anal. Biochem.* 94, 394–396.
- Walter, H., 1985. Surface properties of cells reflected by partitioning red blood cells as a model. In: Walter, H., Brooks, D.E., Fisher, D. (Eds.), *Partitioning in Aqueous Two-Phase Systems*. Academic, Orlando, FL, pp. 327–376.
- Walter, H., Krob, E.J., Ascher, G.S., 1975. Abnormal membrane surface properties during maturation of rat reticulocytes elicited by bleeding as measured by partition in two polymer aqueous phases. *Br. J. Haematol.* 31, 149–157.
- Warren, L., 1959. The thiobarbituric acid assay of sialic acid. *J. Biol. Chem.* 234, 1971–1975.
- Wautier, J.L., Paton, R.C., Wautier, M.P., Pintigny, D., Abadie, E., Passa, P., Caen, J.P., 1981. Increased adhesion of erythrocytes to endothelial cells in diabetes mellitus and its relation to vascular complications. *N. Engl. J. Med.* 305, 237–242.

- Yahara, N., Tofani, I., Maki, K., Kojima, K., Kojima, Y., Kimura, M., 2005. Mechanical assessment of effects of grape seed proanthocyanidins extract on tibial bone diaphysis in rats. *J. Musculoskelet. Neuronal. Interact.* 5, 162–169.
- Yamasaki, H., Grace, C.G., 1998. EPR detection of phytophenoxyl radicals stabilized by zinc ions: evidence for the redox coupling of plant phenolics with ascorbate in H₂O₂-peroxidase system. *FEBS Lett.* 422, 377–380.
- Zhao, J., Wang, J., Chen, Y., Agarwal, R., 1999. Anti-tumour-promoting activity of a polyphenolic fraction isolated from grape seeds in the mouse skin two-stage initiation-promotion protocol and identification of procyanidin B5-3'-gallate as the most effective antioxidant constituent. *Carcinogenesis* 20, 1737–1745.
- Zhu, Q.Y., Holt, R.R., Lazarus, S.A., Orozco, T.J., Keen, C.L., 2002. Inhibitory effects of cocoa flavanols and procyanidin oligomers on free radical-induced erythrocyte hemolysis. *Exp. Biol. Med.* 227, 321–329.
- Zinkham, W.H., Lenhard, R.E., Childs, B., 1958. A deficiency of glucose-6-phosphate dehydrogenase activity in erythrocytes from patients with favism. *Bull. Johns Hopkins Hosp.* 102, 169–175.