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Glucose-Induced Oxidative Stress in Young and Old Erythrocytes: Modulatory Role of Grape Seed Proanthocyanidin Extract

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Abstract: ***Hyperglycemia is associated with increased oxidative stress (OS) and diabetes–related pathologies. Erythrocytes have a major role in the origin of various complications. We evaluated the responses of young and old erythrocytes from rats to induced-hyperglycemia along with the possible protection from grape seed proanthocyanidin extract (GSPE) against hyperglycemia. Erythrocytes obtained from percoll gradient were exposed to 10mM and 20mM glucose. Significant increases in fragility, ROS, Ca_i^{2+} and MDA along with decreases in P-SH, GSH and ATP were partially reversed by GSPE. In conclusion, a natural polyphenol could possibly protect young erythrocytes against OS-related shortened lifespan and perhaps prevent hemolytic anemia.***

Keywords: Erythrocyte Aging, Fragility, Grape seed proanthocyanidin extract, Hyperglycemia, Oxidative stress

1. Introduction

The lifespan of mammalian erythrocytes ranges from 120 days (humans) to 90-60 days in smaller animal models such as rats and mice. However, the lifespan is influenced by several environmental and stress factors including free radical generated oxidative stress (OS) [1], [2] in erythrocytes. Even if the mechanisms that regulate changes in RBC lifespan remain partially unclear, it is extensively accepted that RBC ageing and death are ultimately caused by OS [3], [4]. One of the main concerns regarding OS is its close association with pathological conditions such as diabetes mellitus (DM) wherein hyperglycaemia is a potent causative factor leading to hemolytic anemia.

Since the last decade, the antioxidant activity of natural phenolic compounds have drawn the attention of clinicians and pharmacologists in seeking their antihyperglycemic function to its metal chelating properties along with their effects on specific signaling pathways. Studies have emphasized on their free radical scavenging [5] mechanisms in restoring the erythrocyte properties against high glucose (HG)-mediated early eryptosis and shortened lifespan. Proanthocyanidin is a compound extracted from grape seed and its basic structure is made of monomer, dimer and trimer catechin, all of which are water-soluble molecules and contain a number of phenolic hydroxyls [6]. Grape seed proanthocyanidin extract (GSPE) can scavenge and protect the over-oxidative damage caused by free radicals [7].

Despite the availability of data regarding the role of GSPE on tissues, its effect in understanding the mechanisms using an *in vitro* hyperglycemia-induced OS model of young and old erythrocytes is limited. In the present study, we have evaluated the possible modulatory effects of GSPE on young and old erythrocytes exposed to varying glucose concentrations above the normal *in vitro*. We evaluated the cytosolic and membrane markers of OS-fragility, reactive oxygen species (ROS), lipid peroxidation (LPO), reduced glutathione (GSH), protein oxidation, intracellular calcium and ATP in the cells.

2. Materials and Methods

All procedures involving animals were approved by the Institutional Animal Ethics Committee (IAEC) of Bangalore University (Bangalore, India) and complied with the guidelines of the Control for the Purpose and Supervision of Experiments on Animals (CPCSEA). Healthy adult male rats were given access to water and food *ad libitum* (Amruth Feeds, Bengaluru). They were maintained in polyethylene cages at 22 ± 2 ^{o}C and were exposed to a 12-h light/dark cycle.

2.1 Blood sampling

Blood was drawn from the heart into 10% EDTA-coated tubes and erythrocytes were separated at 1000g and at 4^{o}C (Kubota, Model 6200, Japan). . The plasma and buffy coat were aspirated and isolated erythrocytes were washed thrice with cold GASP-buffer (pH 7.4) containing 5.5 mmol glucose, 9 mmol Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mmol NaCl and 0.8 g/L bovine serum albumin [8].

2.2 Density gradient fractionation of erythrocytes

Isolation of young and old erythrocytes was carried out using discontinuous Percoll/BSA density gradients [9] and by the method of Rennie et al. [10].

2.2.1 Grouping of erythrocytes.

Young and old erythrocytes were sub-grouped into i) controls (CON) with 5.5mM glucose (GLU), ii) controls with grape seed proanthocyanidin treatment (CON+GSPE), iii) cells incubated with 10mM GLU, iv) cells pre-treated with GSPE and incubated with 10mM GLU, v) cells incubated with 20mM GLU and vi) cell pre-treated with 1.5 mM GSPE and incubated with 20mM glucose. Our control

cells were incubated with 5.5mM/L of glucose since normal upper level of blood glucose level in non-diabetic healthy Wistar rats is 5.5 mM/L [11].

Cells suspensions (10% haematocrit, Hct) were incubated in sterile filtered D-glucose to simulate the *in vivo* hyperglycemia in diabetes [12]. Cells were pre-treated with 0.5 mL of 1.5mM GSPE in GASP buffer for 1h at 37 ^{o}C and 5% CO_2. This was followed by washing and incubating the cells with either 10mM or 20mM D-GLU for 2h.

2.3 Assays

2.3.1 Osmotic fragility
Osmotic fragility assay was carried out within 2h of fractionation of erythrocytes and by placing the cells in hypotonic saline solutions (pH 7.4) at 25 ^{o}C. After 15 min, samples were centrifuged at 1270g to pellet the cells and the extent of hemoglobin released into the supernatant in terms of hemolysis was measured at 540 nm by the method O'Dell et al. [13].

2.3.2 Measurement of intracellular calcium (Ca_i^{+2})
Young and old erythrocyte suspensions were incubated with Fluo-4/AM (Invitrogen, USA) and Ca_i^{+2} was quantified at Ex/Em-491/516 nm in a multimode plate reader (Infinite 200 PRO Tecan, Männedorf, Switzerland). Calcium is expressed in terms of RFU/µg protein.

2.3.3 ROS assay
Erythrocytes were incubated with H_2-DCFDA (Invitrogen, USA), according to the manufacturer's instructions in 96-well plates. Relative fluorescence intensity (RFU) of the DCF was measured at Ex/Em-494/528 nm in the multimode plate reader. ROS is expressed as RFU/µg protein

2.3.4 Lipid peroxidation (MDA)
Malondialdehyde, a product of lipid peroxidation was assayed by the method of Ohkawa et al. [14] using 1, 1, 3, 3-tetramethoxy propane as a standard in a spectrophotometer (ELICO, Model SL 159, India). MDA is expressed in terms of nmol/mg protein.

2.3.5 Protein oxidation (P-SH)
Membrane protein oxidation was measured in terms of protein thiol concentration as described by Habeeb [15]. Membrane suspensions of young and old cells containing approximately 120 µg protein were treated with 5, 5-dithio-bis (2-nitrobenzoic acid). After 15 min at room temperature, absorbance was measured in a spectrophotometer at 412 nm. Thiol concentration was calculated from the net absorbance using molar absorptivity, 13, 600 (mol/L.

2.3.6 Adenosine Triphosphate (ATP)
ATP was measured in the erythrocytes using ATP bioluminescence kit (Bio Vision Stay Brite ATP kit, Cat. K791-100). Relative luminescence was recorded in the multimode plate reader. ATP is expressed as RLU/µg protein.

2.3.7 Glutathione (GSH)
Glutathione (GSH) in the erythrocytes was measured by the method of Beutler et al. [16]. Young and old erythrocyte suspensions were prepared at 5% hematocrit, filtered and filtrates were treated 0.3M Na_2HPO_4 followed by 04% DTNB. Absorbency was measured at 412 nm and GSH level is expressed as µmoles/mg protein using an absorption coefficient of 1.36 x 10^4 L. mol^{-1} / cm^{-1}.

2.3.8 Protein Assay
Protein was determined in the erythrocytes by the method of Lowry et al. [17] (1951) with BSA as the standard.

2.4 Statistical Analyses

Results presented are mean ± SEM (n=3-5). Significance was calculated by one-way ANOVA followed by Tukey's multiple comparison using GraphPad Prism 6. To compare the significance between the two ages, two-way ANOVA followed by the post-hoc test was applied. Pearson's correlation (r) was calculated using GraphPad Prism 9, between the parameters between the young and old erythrocytes.

3. Results

3.1 Osmotic fragility

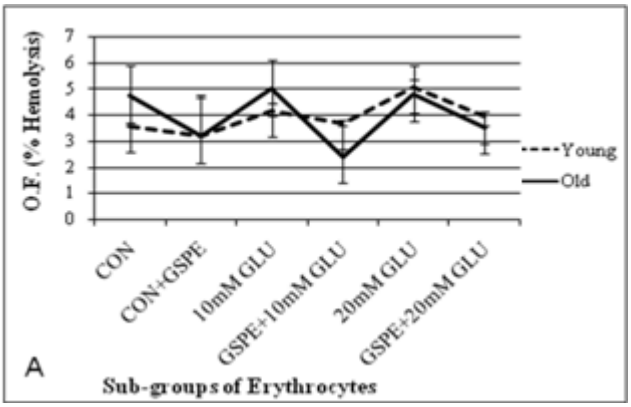
The results revealed that corpuscular fragility in terms of per cent haemolysis was highest in the erythrocytes at 0.3% followed by 0.4% while least in those at 0.9% saline. Old cells were more fragile compared to young cells. On exposure to 20mM glucose the extent of fragility was higher compared to 10mM glucose exposure irrespective of age. Pre-treated with GSPE at 10mM and 20mM glucose provided protection against GLU-induced fragility although to a lesser extent (Fig.1A-C).

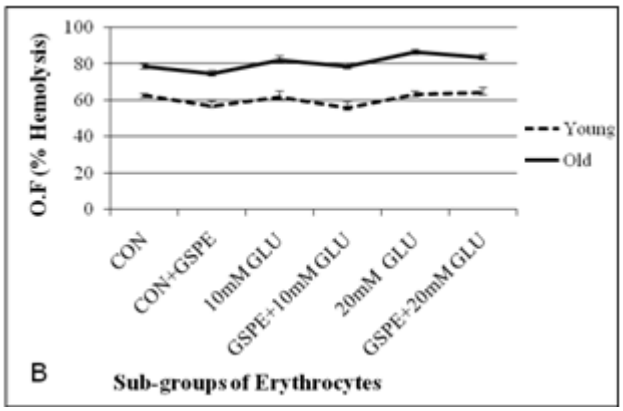
3.2 Intracellular calcium

The Ca_i^{+2} was measured as one of the associated markers of glucose-induced OS in young and old erythrocytes. Calcium level was reduced by 16% and 19% in GSPE treated young and old control cells (Table). Incubating young cells at 10mM and 20mM glucose resulted in significant ($p<0.0001$) increases (Fig.2A) by 111% and 120% compared to the control level. However, the extent of increase in GSPE pre-treated young cells was to a lesser extent, by 63% and 89% at 10mM and 20mM GLU (Table). Insignificant increases were noticed in old cells although the extent was greater than the young, and GSPE pre-treatment was ineffective in lowering calcium level.

3.3 Reactive oxygen species

The results showed increased ROS level in the young cells at 10mM ($P<0.01$) and 20mM (0.001) GLU exposure. GSPE was effective in lowering ROS in the GSPE pre-treated young cells ($p<0.01$) with respect to their untreated cells. Old cells displayed significant increase in ROS and unlike the young; the cells were less responsive to GSPE treatment (Fig.2B).





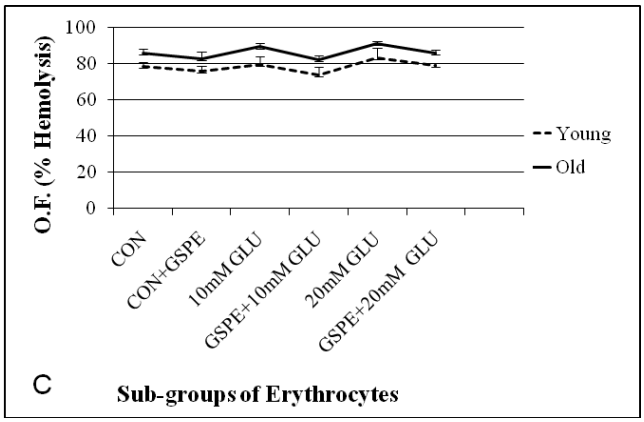
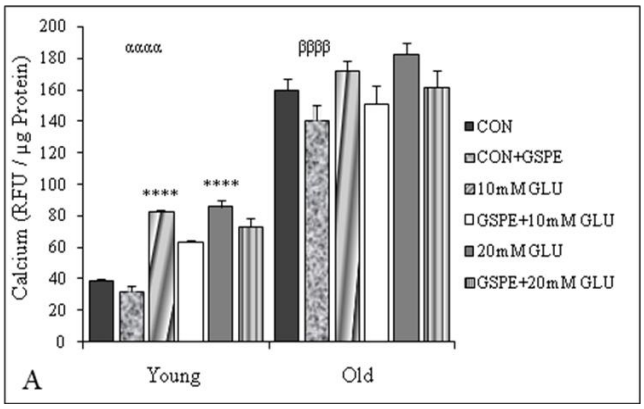


Figure 1: Variations in the hemolysis percentage of the young and old RBCs as a function of the NaCl concentration, (A), 0.9%; (B), 0.4%; (C), 0.3% percentage in the saline medium for different sub-groups of the study. Values are mean ± SEM (n=5).



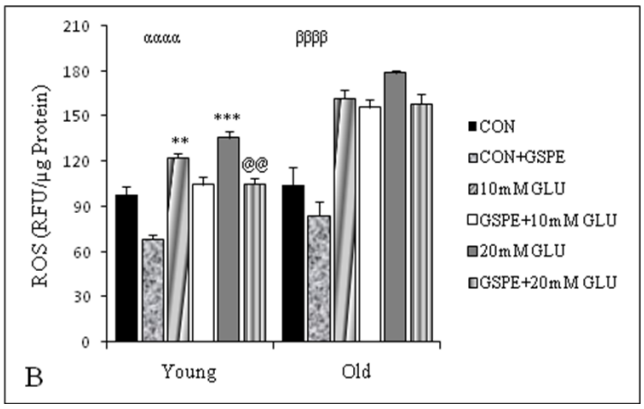


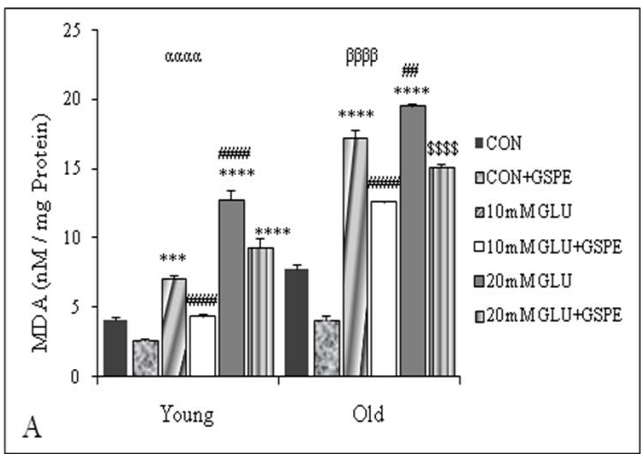
Figure 2: Alterations in intracellular calcium (A) and ROS (B) in young and old erythrocytes to glucose *in vitro* and effect of grape seed proanthocyanidin extract. Significance was calculated by one-way ANOVA and post-hoc test was in accordance with Tukey's multiple comparison using GraphPad Prism 6 and represented as ****P<0.0001, *** P< 0.001 and **P<0.01 *v/s* CON;[at][at]P<0.01 *v/s* 20mM GLU; Significance between age groups $^{\alpha\alpha\alpha\alpha}$ and $^{\beta\beta\beta\beta}$ P < 0.0001.

3.4 Lipid peroxidation

As indicated in Fig.3A, elevated levels of MDA in young erythrocytes were noticed in the 10mM (P<0.001) and 20mM (P<0.0001) GLU exposed compared to the control. A similar trend was noticed in the old erythrocytes. Among the young GSPE-treated cells, respective decreases in MDA was noticed in those at 10mM GLU exposures (p<0.0001). Similarly, lowered MDA levels were evident in response to GSPE treated 10mM and 20mM GLU-exposed old cells (p<0.0001).

3.5 Protein oxidation

There was a significant decrease in P-SH level in the young erythrocytes exposed to 20mM glucose (P<0.0001) in contrast to old cells wherein, decrease was insignificant. GSPE was effective in improving P-SH in the young cells although insignificant (Fig.3B).



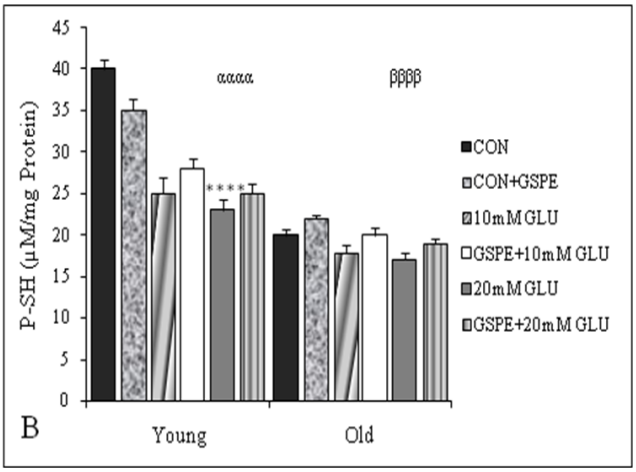


Figure 3: Changes in MDA (A) and protein sulfhydryl levels (B) in young and old erythrocyte membrane exposed to glucose *in vitro* and effect of grape seed proanthocyanidin extract. Values are mean ± SEM (n=5). GLU, glucose; GSPE; CON, control. Significance was calculated by one-way ANOVA and post-hoc test was in accordance with Tukey's multiple comparison using GraphPad Prism 6 and represented as ***P<0.001, ****P<0.0001 *v/s* CON; #### P<0.0001, ## P<0.001 *v/s* 10mM GLU; $$$$P<0.0001*v/s* 20mM GLU; Significance between age groups αααα and ββββ P < 0.0001.

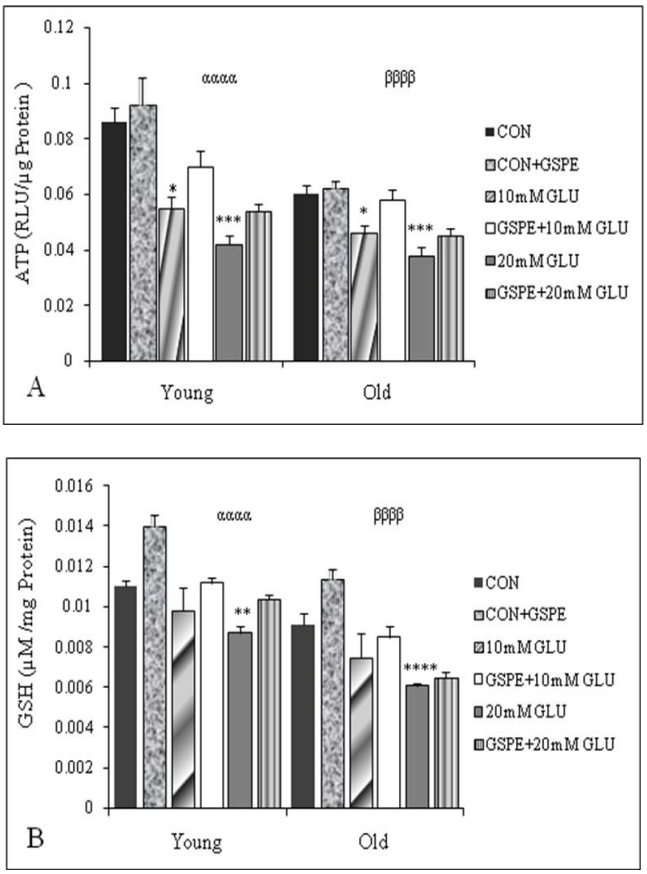


Figure 4: Changes in ATP Levels (A) and GSH (B) in young and old erythrocytes exposed to glucose *in vitro* and effect of grape seed proanthocyanidin extract. Values are mean ± SEM (n=5). GLU, glucose; GSPE; CON, control; RLU, Relative Luminescence unit. Significance was calculated by one-way ANOVA and post-hoc test was in accordance with Tukey's multiple comparison using GraphPad Prism 6 and represented as, ***P<0.001, ** P<0.01, *P<0.05 *v/s* CON; Significance between age groups αααα and ββββ P < 0.0001.

3.6 ATP

Age-dependent and concentration-dependent depletions in the ATP level were observed on glucose exposures. The ATP level in young and old erythrocytes were reduced on exposure to10mM GLU (P<0.05) and further decrease at 20 mM GLU (P<0.001). GSPE was effective in increasing ATP level in young as well old cells though insignificantly (Fig.4A).

3.7 Glutathione

Significant reduction in GSH was noticed in the 20mM GLU-exposed young (p<0.01) and old (P<0.0001) erythrocytes. Improved GSH levels were noticed in the GSPE pre-treated cells prior to GLU exposure. However, this increase did not reach the significance level in the young and old cells (Fig.4B).

Table summarizes the extent of changes with respect to the control in calcium, ROS, MDA, ATP and P-SH levels in young and old erythrocytes subjected to 10mM and 20mM glucose either with or without GSPE pre-treatment.

Table 1: Percentage changes in response to glucose-induced oxidative stress in young and old erythrocytes of male Wistar rats

Treatment	Calcium		ROS		MDA		ATP		P-SH	
	Young	Old	Young	Old	Young	Old	Young	Old	Young	Old
CON+GSPE	- 16	- 19	- 30	- 19	- 34	- 47	+7	+5.0	- 12	+10
10mM GLU	+111	+56	+25	+56	+73	+121	- 36	- 33.3	+37	- 11
GSPE+10mM GLU	+63	+50	+7	+50	+5.6	+61	- 18	- 14.9	+30	0
20mM GLU	+120	+71	+385	+71	+200	+150	- 51	- 35.8	+42	- 15
GSPE+20mM GLU	+89	+52	+7	+52	+128	+94	- 37	- 24.8	+37	- 5

CON, control; GSPE, grape seed proanthocyanidin extract., GLU, glucose. Extent of changes is represented as percentage increase (+) in Ca_i^{+2}, ROS and MDA, and decrease (-) in ATP and P-SH relative to their respective young and old untreated control erythrocytes.

3.8. Correlation between age and glucose-induced oxidative stress-related parameters

Figure 5A-I show correlative analysis (r) between age and oxidative stress-related parameters in erythrocytes exposed to10mM and 20mM glucose and those pre-treated with GSPE as well.

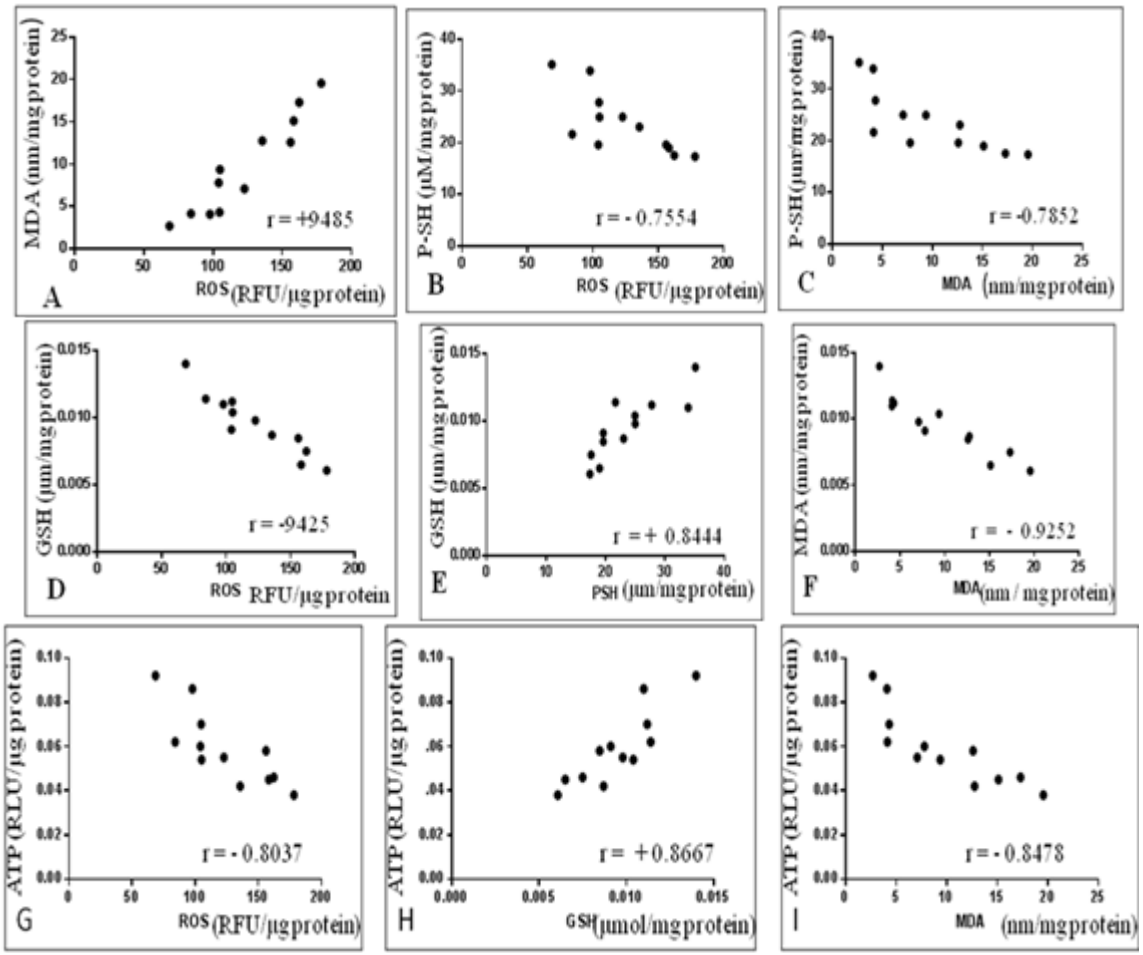


Figure 5: Correlation (r) between age and parameters of oxidative stress-related parameters in hyperglycemic and GSPE-treated erythrocytes was calculated using GraphPad Prism 9

4. Discussion

Damages caused by ROS are implicated in the development of diabetes-related pathologies [18]. Our observation on a higher fragility at 20mM glucose compared to the 10mM glucose *in vitro* is suggestive of a higher extent of glycated Hb (HbA1c)-related hemolysis in the young and old erythrocytes [19]. HbA1c is also a reliable marker of erythrocyte membrane degeneration resulting in its loss of function [20].

In the present study, in addition to high ROS, glucose above the normal concentration i. e. at 10 mM and 20 mM also lead to higher levels of intracellular calcium in the old erythrocytes compared to the young. Aside from increases in ROS and calcium, reduction in the high energy molecule, ATP compared with the control cells was observed. Hyperglycemia affects the membrane Ca^{+2} ATPase activity in expelling excess of accumulated intracellular Ca^{2+} due to lesser availability of intracellular ATP as a consequence of inefficient glycolytic function [21]. Our observations on low ATP levels in old control erythrocytes suggest low glucose transport across the membrane. Studies indicate that in contrast to the old erythrocytes, the young have 2.5 times more glucose metabolism [22]. Further, the lowered levels of ATP in the old as well as young erythrocytes incubated at 10 and 20mM glucose can be identified with a report on a 30-40% drop in the cellular ATP resulting from down-regulation of Embden-Meyerhof pathway [23].

In addition, our observations on reduced glutathione in young and old erythrocytes at 20 mM glucose exposure may be based on the fact that GSH synthesis is exclusively by *de novo* synthesis in the erythrocytes. Also, lowered protein thiol levels are identified as early markers of hyperglycemia in human erythrocytes [24]. The purpose of this study was also to compare the observed increase in osmotic fragility of young and old erythrocytes to the plasma membrane protein SH concentration during the induction of hyperglycemia. Our findings on higher ROS and MDA leading to increased membrane fragility in the young erythrocytes was further exacerbated with decreased protein thiol level compared to controls, a situation resembling old erythrocyte membrane [25]. The insignificant improvement in GSH and protein thiol levels in response to GSPE-pretreatment perhaps is suggestive of the need for a higher concentration of GSPE to improve their synthesis. . Findings from the present study on increased MDA in the glucose incubated young and old erythrocytes along with a strong and positive correlation with ROS is in accordance with the findings on human erythrocytes [26]. GSPE pre-treatment was beneficial in alleviating MDA level at 10mM glucose and 20mM as well in the young and old erythrocytes.

5. Conclusions

Enhanced intracellular oxidative stress associated with hyperglycemia could be a determining signal of a curtailed

lifespan in erythrocytes, more so, in the young than old since sensitivities to stress correlate to their longevity *in vivo* [27]. Further, our findings suggest that GSPE, an antioxidant could be a natural polyphenol for promoting healthy and normal lifespan of young and old erythrocytes in circulation under glucose concentrations above normal level. Further studies are underway in analyzing the specific constituents of GSPE that are anti-hyperglycemic.

6. Acknowledgements

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7. Disclosure

The authors have no conflict of interest related to the manuscript.

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Dr. S. Asha Devi is a biogerontologist researching in the field of aging at the Department of Zoology, Bangalore University, Bangalore, India. She earned her B. Sc and M. Sc degrees from Bangakore University and was awarded Ph. D in 1984. She has been awarded post-doctoral and visiting fellowships to collaborate with scientists from Japan, USA and U. K. Further, her concept of synthetic and natural neutraceuticals for improving functions of the aging brain, red blood cells and myocytes have gained recognition of biologists and clinicians. Asha has published several International and National research and review articles. She has delivered several talks in understanding and modulating ageing.