

Effect of green tea consumption on RBC morphology, membrane properties and antioxidant status in chronic cigarette smokers

Shakeela Begum M¹, Padmavathi P¹, Saradamma B¹, Maturu P², Ananda Vardhan H³,
Varadacharyulu NC¹ & Damodara Reddy V^{1*}

¹Department of Biochemistry, Sri Krishnadevaraya University, Anantapur-515 003, Andhra Pradesh, India

²Department of Pediatrics, Texas children's hospital, Baylor college of medicine, Houston, TX-77 030, United States of America

³Department of Biotechnology, New Horizon College of Engineering, Bangalore-560 103, Karnataka, India

Received 27 March 2018; revised 29 May 2018

Morbidity and mortality attributed to cigarette smoking is a well-known risk factor for diseases and different type of cancers. Free radicals generated by smoking enter into circulation and interacts with blood constituents and RBC membrane thereby causing pathophysiological changes. Herbal extracts have been reported as anti-oxidant and anti-inflammatory supplements, which attenuates free radical induced oxidative stress, among which green tea (*Camellia sinensis*) has been reported as the elixir of life due to its innumerable health benefits. The present study investigates the protective effect of green tea consumption against cigarette smoking-induced oxidative stress on RBC morphology, membrane properties. Blood samples were collected from 120 selected human male volunteers categorized into four groups *viz.*, controls, smokers, control volunteers consuming green tea with no habit of smoking and smokers consuming green tea were analyzed. Results of this study showed that significantly altered membrane lipid peroxidation, protein carbonyls, -SH groups, ATPases, individual phospholipids, morphological changes in RBC structure and membrane proteins as evident by SDS-PAGE in smokers. Besides, smokers showed decreased activities of antioxidant status. The adverse changes observed in the above parameters in smokers were preserved upon supplementation of green tea. In conclusion, the presence of phenols, flavonoids and tannins, in particular, catechins in green tea might be responsible for the observed protection against smoking-induced alterations in blood.

Keywords: Cigarette smoking, Green tea, Oxidative stress, RBC morphology, SDS-PAGE

Cigarette smoking is responsible for more than 6 million deaths worldwide and reported to be the third most common cause of death^{1,2}. Cigarette smoke is a complex mixture of 8700 identified compounds, consists of 8% tar (nicotine, carcinogens, phenol, naphthalene *etc.*) and 92% gaseous constituent components (carbon monoxide/dioxide, ammonia, hydrogen cyanide, *etc.*) which enter blood^{3,4}. The adverse health consequences of smoking have been largely attributed to the abundance of reactive oxygen species (ROS) and reactive nitrogen species (RNS) that readily react with various biomolecules such as DNA, proteins, amino acids and lipids⁵. ROS damage epithelial cells lining the airways by inducing peroxidation of lipids and other cell membrane constituents, activate oxidative-sensitive cellular pathways and induce oxidative damage in the form of

lipid peroxidation^{6,7}. RBCs contain hemoglobin, which is one of the most potent catalysts of lipid peroxidation^{8,9}. Free radicals can directly damage red blood cell (RBC) membranes by peroxidation of membrane polyunsaturated fatty acids^{10,11}.

Cigarette smoking causes marked changes to RBC rheological properties, due to the consequences of inhaled toxins, resulting in increased oxidant levels, followed by impairment in the antioxidant defense mechanisms of the cell. Peroxidation of lipids can disturb the assembly of the membrane, causing changes in fluidity, permeability and alterations in ion transport leading to perturbations in metabolic processes^{12,13}. Moreover, increased level of lipid peroxidation, sulfhydryl group and protein carbonyl formation are also evident in oxidative damages of erythrocyte membrane in cigarette smokers¹⁴.

The first line of antioxidant defense enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GP_X) act against oxidative cell damage preventing lipid peroxidation, protein and

*Correspondence:

Phone: +91-8554-255761; Fax: +91-8554-255244

E-mail: vdp_1975@yahoo.co.in

DNA oxidation^{7,15}, and also to combat the oxidative stress elicited by cigarette smoke exposure^{16,17}. Erythrocyte membrane contains a number of hallmark proteins, namely spectrin, ankyrin, band 3, GAPDH and Glut 1. The former three are the cytoskeleton proteins, which maintain the normal discoid shape of erythrocyte and Glut 1, on the other hand, is the only transporter of glucose and dehydroascorbate (DHA)^{18,19}. According to our knowledge no one has investigated in detail about these proteins in cigarette smokers.

There has been a public demand and also an interest of scientists to develop therapeutic strategies that reduce cigarette smoking-induced damage rather than cessation of smoking²⁰. Natural antioxidants with free radical-scavenging activity such as polyphenols from green tea have received much attention as a potential treatment for oxidative-stress related diseases and damage²¹. Previous reports revealed amelioration of cigarette smoke-induced oxidative damage and lung injury by green tea and its catechins in rats^{17,22}. The multi-beneficiary actions of green tea is a non-oxidized and non-fermented product of leaves of the plant *Camellia sinensis*, belonging to Theaceae family containing high quantities of several polyphenolic components *viz.*, EGCG (Epigallocatechin-3-gallate), EC (Epicatechin), ECG (Epicatechin gallate), EGC (Epigallocatechin) followed by caffeine which have both protective and therapeutic properties²³. EGCG, the most abundant catechin found in green tea, which can make up to 0.05% of the total content of tea by weight¹⁷. Green tea and its catechins are believed to exert protective effects against various diseases such as obesity, diabetes, cardiovascular, respiratory, hepato toxicity, arthritis, neurodegenerative diseases, tooth decay, hepatitis, immune system, allergy, bacterial and viral infection and various types of cancers²⁴⁻²⁶. Here, we report for the first time about the beneficial effects of green tea consumption on smoking-induced alterations such as antioxidant status, membrane properties and RBC morphological changes in human male volunteers.

Materials and Methods

Chemicals

The chemicals used in the present study were of analytical grade (AR) and obtained from Sigma-Aldrich chemical Co. (St. Louis, MO, USA), SRL and HIMEDIA chemicals (Mumbai, India). Green tea (Ripple Premium green tea, Kanan Devan

Hills Plantations Company (P) Limited) was procured from the local market of Anantapuramu, Andhra Pradesh, India.

Preparation of green tea

One gram of green tea leaves in 100 mL of water was brewed in a microwave oven at 60°C for 5 min. The liquid extract was separated by a filter and the filtrate was used for the study.

Study subjects

The study group included 120 human male volunteers aged between 35-55 years, residing in Anantapuramu, India was chosen for the study based on a questionnaire and was categorized into four groups, consists of 30 in each group, *viz.*, controls, smokers, green tea alone and smokers taking green tea. Subjects included were good general health and excluded were coronary artery disease, hypertension, diabetes mellitus and abuse of alcohol. Smokers were smoking at least 15-20 filter cigarettes/day for the past 7 years and above. Green tea bottles were provided along with instructions (tea preparation method, green tea at the rate of 3 cups/day *i.e.*, morning, afternoon and evening for one year, each cup contains 100 mL of green tea) to all smokers and green tea group volunteers. All subjects expressed informed consent in writing prior to study participation. The study was approved by the Institutional Human Ethics Committee. All volunteers participated in the present study were requested to maintain a similar diet and tea drinking patterns throughout the study period. Venous blood samples were collected from fasting subjects and used for analysis.

RBC morphology

Erythrocyte morphology was examined using a microscope (Olympus, lens: 40X with oil immersion) and the changes were analyzed by the method adopted²⁷. Red blood cell morphology was assessed in freshly prepared wet-mount preparations. Blood samples were diluted (1:200 dilutions) in PBS were transferred onto glass slide having silicone grease well of ~1 mm depth and covered with a coverslip and cells were observed under microscope and images were captured at 10X magnification.

Erythrocyte antioxidant status

Erythrocytes were washed thrice with 0.9% NaCl and suspended in 1 volume of 0.9% NaCl. The packed cell volume was adjusted to 5% with PBS pH 7.5

(10 mM phosphate buffer saline). Hemoglobin content in erythrocytes was determined²⁸. Reduced GSH content was estimated and expressed as $\mu\text{mol/g Hb}$ ²⁹. The SOD activity was measured based on the ability of the enzyme to inhibit the autoxidation of adrenaline and activity was expressed as units/mg Hb/min³⁰. The CAT activity in hemolysate was estimated and the activity of the enzyme was calculated using the extinction coefficient of H_2O_2 as $0.071 \text{ cm}^{-1}\text{mol}^{-1}$ and expressed as $\text{IU} \times 10^4/\text{g Hb}$ at 37°C ³¹. The GPx activity was measured and the activity was expressed as $\mu\text{moles of glutathione oxidized}/\text{min}/\text{mg Hb}$ ³².

Erythrocyte membrane studies

Erythrocyte membrane was prepared as described previously³³, and the thiobarbiturate assay was used for the estimation of MDA levels³⁴. The concentration of protein carbonyls was determined using 2,4-dinitrophenylhydrazine (DNPH) assay^{35,36}. Erythrocyte membrane total sulfhydryl groups were determined³⁷. The activity of Na^+/K^+ -ATPase was measured by estimating the phosphorus liberated after the incubation of erythrocyte membrane in a reaction mixture containing the substrate ATP with the co-substrate elements at 37°C for 15 min. The reaction was arrested by adding 1.0 mL of 10% TCA. The phosphorus content from the TCA supernatants was then determined³⁸. Erythrocyte membrane protein concentration was estimated³⁹. The SDS-PAGE technique was used to separate erythrocyte membrane proteins as described previously⁴⁰, and silver staining was carried out to visualize protein bands in gel⁴¹.

Erythrocyte membrane individual phospholipid analysis

Erythrocyte membrane individual phospholipids were separated on silica gel H (Merck) using

two dimensional thin layer chromatography with chloroform:methanol:aqueous ammonia 65:35:5 (v/v) as the first solvent and chloroform:acetone:methanol:acetic acid:water 50:20:10:10:5 (v/v) as the second solvent. The fractions were located with iodine vapors and scraped from the plate and the phospholipids were measured as inorganic phosphorus after digestion with perchloric acid⁴².

Data analysis

Values are subjected to statistical analysis and expressed as mean \pm SD. Duncan's multiple range (DMR) test followed by student "t" test was performed to find out a significant difference between groups. A $P < 0.05$ was considered statistically significant.

Results

Information on the effect of green tea consumption on markers of oxidative stress, in particular, membrane LPO, protein carbonyls and $-\text{SH}$ groups are furnished in Fig. 1A-C. The levels of lipid peroxidation product, malonaldehyde (MDA), protein carbonyls and $-\text{SH}$ contents in smokers were significantly ($P < 0.05$) increased when compared to other groups. However, smokers supplemented with GT restored the values close to normal.

Data presented in Fig. 2 reveals the erythrocyte membrane Na^+/K^+ ATPase and Mg^{2+} ATPases activities in controls and other experimental groups. Results showed that increased membrane Na^+/K^+ ATPase and Mg^{2+} ATPases activities in smokers when compared to control and other groups. However, supplementation of green tea restored these enzyme activities to normal level in smokers taking green tea.

Table 1 represents antioxidant machinery of red cells of controls and other experimental groups. The

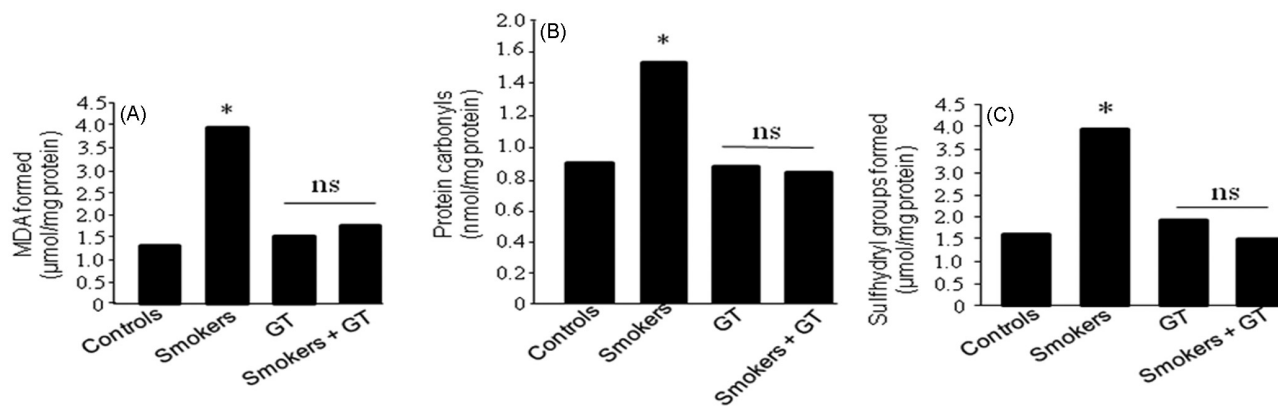


Fig. 1 — Effect of green tea on cigarette smoking induced changes in (A) erythrocyte membrane lipid peroxidation, erythrocyte membrane protein carbonyls (B) & erythrocyte membrane $-\text{SH}$ groups (C). Values are mean \pm SD of each group. *Indicates significant difference from controls. A $P < 0.05$ is statistically significant

GSH and activities of GPx, SOD and catalase decreased in cigarette smokers compared to other groups. Moreover, it is also evident from the results that the regular green tea consumption significantly restored them to normal levels in smokers.

Values representing the contents of individual phospholipid classes phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidyl ethanolamine (PE), phosphatidylserine (PS) and sphingomyelin (SM) from RBCs of controls and other experimental groups of volunteers are presented in Table 2. The levels of PC, PE and SM increased in smokers with a decrease in the contents of PI and PS in comparison with that of controls. These alterations were modulated in smokers taking green tea as moieties of all these phospholipid classes were brought to the normal range. Green tea alone consumed group did not show any significant changes compared to controls.

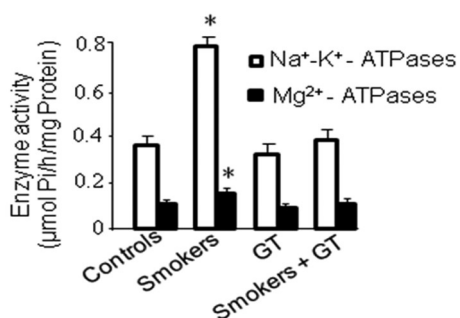


Fig. 2 — Effect of green tea on cigarette smoking induced changes in erythrocyte membrane ATPases. Values are mean ± SD of each group, *Indicates significant difference from controls. A *P* < 0.05 is statistically significant

Red blood cells of different groups *viz.*, controls, smokers, green tea and smokers group consuming green tea were observed under the light microscope and the images were shown in Fig. 3. Microphotographs of red cell morphology revealed changes in the shape of RBCs with spike-like protrusions in smokers when compared to controls. Green tea consuming smokers group did not show any such abnormalities compared to other groups.

Erythrocyte membrane protein profile was analyzed by SDS-PAGE and the data was presented in Fig. 4. SDS-PAGE analysis of erythrocyte

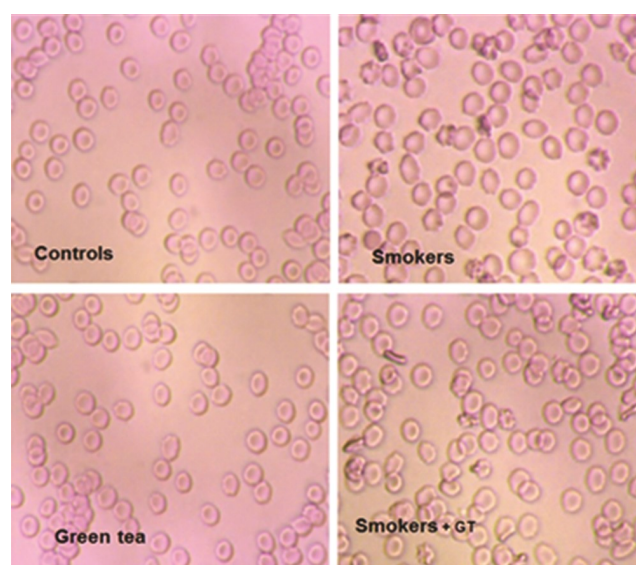


Fig. 3—Effect of green tea on cigarette smoking induced changes in red cell morphology. Cells were observed under microscope and images were captured at 10X magnification

Table 1 — Effect of green tea on red cell antioxidant status in smokers

[Values are mean ± SD of 30 human volunteers in each group. ns: not significant. *Indicates A *P* < 0.05 is statistically significant between groups]

Parameter	Control	Smokers	GT	Smokers + GT
GSH (µmol/gHb)	6.38 ± 0.03	4.12 ± 0.07*	5.52 ± 0.05	6.18 ± 0.02 ^{ns}
GPx (µmole of GSH oxidized/min/mg Hb)	18.2 ± 0.69	13.3 ± 1.12*	17.5 ± 0.55	18.7 ± 1.17 ^{ns}
SOD (U/mg Hb/min)	6.60 ± 0.19	4.73 ± 0.25*	6.35 ± 0.17	6.95 ± 0.14 ^{ns}
Catalase (IU × 10 ⁴ /g Hb)	12.9 ± 0.34	9.8 ± 0.30*	13.1 ± 0.28	13.7 ± 0.32 ^{ns}

Table 2 — Effect of green tea on RBC membrane individual phospholipids in smokers

[All the values are expressed as µg/mg protein. Values are mean ± SD of 30 human volunteers in each group. #Indicates significantly different from all other groups; ns, not significant; *Indicates A *P* < 0.05 is statistically significant between groups]

Parameter	Control	Smokers	GT	Smokers + GT
Phosphatidylcholine	22.9 ± 1.1	24.3 ± 0.5*	21.9 ± 0.9 [#]	22.2 ± 0.7 ^{ns}
Phosphatidylinositol	5.8 ± 0.5	4.2 ± 0.4*	5.6 ± 0.3 [#]	5.2 ± 0.4 ^{ns}
Phosphatidylethanolamine	33.6 ± 1.12	43.9 ± 2.64*	32.2 ± 1.10	39.9 ± 2.19 ^{ns}
Phosphatidylserine	16.9 ± 0.5	13.9 ± 0.2*	15.3 ± 0.7	16.2 ± 0.3 [#]
Sphingomyelin	7.94 ± 0.9	8.29 ± 1.3*	7.83 ± 0.7	7.25 ± 0.4 ^{ns}

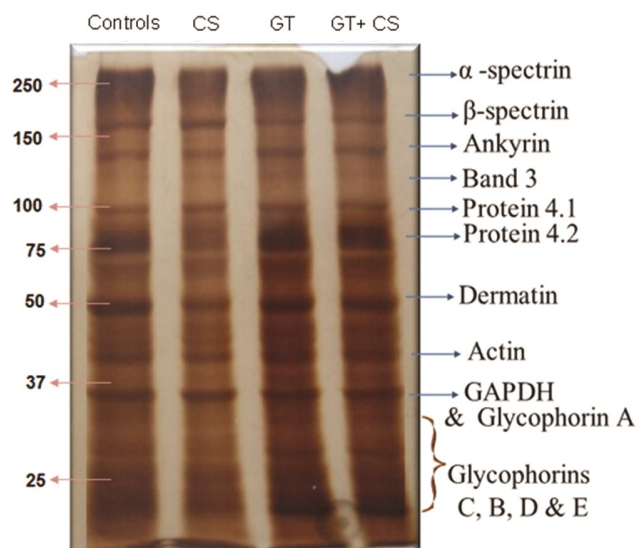


Fig. 4 — SDS-PAGE analysis of cigarette smoking-induced alterations in erythrocyte membrane proteins prepared from control and other subjects. Erythrocyte membrane proteins were resolved in 12% polyacrylamide gel and protein bands were visualised by using silver staining as described in materials and methods

membrane proteins revealed an increase in band densities of band 3, protein 4.2 and glycophorins followed by less dense or decrease in intensities of dematin, actin and ankyrin with no change in bands of spectrin and protein 4.1 of corresponding membrane proteins in smokers reflecting qualitative and quantitative changes in comparison with other groups. Bands that appeared in electropherogram from smokers using green tea matched with that of control.

Discussion

Cigarette smoking is a human habit of global scale and the leading cause of preventable death⁴³. The changes in erythrocyte shape and rheological properties are proven to play a major role in the disease pathology and progress which may affect the signal transduction pathway and eventually cause defects in signaling and block the lung capillaries thus propagating the disease⁴⁴. Free radicals are highly reactive atoms, which damages the biological membrane as well as interact with biomolecules, in particular, fatty acids which in turn generate many free radicals causing increased lipid peroxidation. Lipid peroxides are by-products of lipid oxidation and are also formed enzymatically through the action of lipooxygenases⁴⁵. The heavy metal ions lead and cadmium present in smoke are also capable of inducing lipid peroxidation and hence provoke

damage of organs propagating lipid peroxidation^{7,46}. In the present study, the contents of MDA and carbonyls in RBCs from smokers were significantly increased when compared to controls indicating enhanced lipid peroxidation and protein oxidation leading to oxidative stress resulting in damage to tissues^{47,48}. It is evident from the studies that smoking creates a significant oxidant load in the erythrocytes, as a result, toxic free radicals and other oxidant substances in cigarette smoke damage unsaturated fatty acids in the erythrocytes leading to increase in MDA level⁴⁹. However, green tea supplementation reduced lipid peroxidation and protein carbonyls in smokers. The phenolic hydroxyl groups present in green tea catechins act as electron donors and also efficient scavengers of superoxides, peroxy, nitric oxide radicals, hydroxyl and peroxynitrite radicals leading to decreased LPO, protein carbonyl and with increased sulfhydryl group levels⁵⁰.

Microphotographs of red cells from smokers revealed some abnormalities in shape, showing spikes and protrusions. Such changes were not seen in smokers consuming green tea. In all, the study demonstrated that cigarette smoking-induced significant changes in chemical composition and organization of lipids as well as proteins, properties such as fluidity and also external morphology. Constituents of cigarette smoke interact with biomolecules and electrolytes of the red cell bringing about changes in the structure and functions of the red cell. In addition, it is evident from this study that regular green tea intake alleviates/protects the red cell morphology and chemistry⁵¹.

Antioxidant defense enzymes such as GPx, SOD and CAT reduce oxidative stress and play an important role in determining individual risk of developing diseases, such as cancer and atherosclerosis⁵². The major antioxidant in erythrocytes is GSH, which protects important proteins such as spectrin, the oxidation of which can lead to increased membrane stiffness. GSH not only supports antioxidant defense but also an important sulfhydryl buffer, maintaining -SH groups in hemoglobin and enzymes in the reduced state¹⁶. In the present study, depletion in the activities of GPx, SOD, CAT and the content of GSH was observed in cigarette smokers in comparison with other groups may be due to the increased utilization of these antioxidants to counteract free radicals. The superoxide, peroxy radicals and lipid hydroperoxides overload might inhibit these antioxidant enzymes^{53,54}.

Green tea supplementation increased the activities of GSH, GPx, SOD, CAT and these findings are consistent with a recent report that EGCG can induce antioxidant enzymes⁵⁵. Perhaps, EGCG may have scavenged superoxide, H₂O₂ and hydroxyl radicals and restored the levels of antioxidant enzymes⁵⁰.

Phospholipids play a significant role in the maintenance of membrane morphology and properties. In the present study, decreased PS and PI followed by an increase in PC, PE and SM were observed in smokers when compared to other groups. The values were brought normal in smokers taking green tea. In general, asymmetric distribution of phospholipids in the membrane bilayer is maintained by using Mg²⁺-ATPase through the entire process of lysing red cells and of washing and resealing membranes. Though phospholipids slowly but continuously move to outer leaflet from inner leaflet, the amino phospholipids PS and PE are rapidly shuttled back to the inner leaflet by amino phospholipid translocase enabling the maintenances of asymmetric phospholipid distribution^{56,57}. Probably, in addition to ATPase, translocases might have become targets for the action of selected smoke constituents in the present study affecting the insertion of the phospholipid⁵⁸, which needs further confirmation. It is well known that catechins, due to their lipophilic nature, can be inserted in the hydrophobic core exerting a beneficiary and modulatory role. Also interactions of catechins with hydrophilic surface groups were also reported. Therefore, green tea causes improvement in the antioxidant system and partially prevents cell membrane disorganization⁵⁰.

Increased Na⁺-K⁺ ATPase and Mg²⁺ ATPase in smokers in the present study revealed the interaction of smoke constituents at the membrane level. The damage to RBC membrane by oxidative agents was reported to alter the activities of membrane-bound enzymes⁵⁶. The membrane LPO and protein degradation and free radical generation by cigarette smoke might contribute to the increase in activities of these membranes bound enzymes. At the same time, green tea consumption in smokers caused significant restoration of these enzyme activities. Possibly direct actions between cigarette smoke constituents and GT as well as the incorporation of green tea catechins in the hydrophobic core of membranes might have played a role in the observed effect⁵⁹. EGCG administration also resulted in an increase in the activities of membrane-bound ATPases in animals⁶⁰.

Electropherogram of erythrocyte membrane protein profile of smokers revealed increased band densities of band 3, protein 4.2, dematin, actin and glycophorins followed by less dense or decrease in intensities of ankyrin, spectrin and protein 4.1 of corresponding membrane proteins in smokers reflecting qualitative and quantitative changes in comparison with other groups⁵⁶. As spectrin, ankyrin and actin are the cytoskeletal proteins responsible for the biconcavity of the red cell, the observed change in band 3 and as well in G3PDH indicated cigarette smoke-induced degradation of respective proteins affecting the structural organization as well functions of the red cell. G3PDH is a glycolytic enzyme and band 3 protein an antiporter that plays an important role in anion transportation especially affecting HCO₃⁻ and Cl⁻ exchange through membrane⁶¹. However, green tea prevented these alterations in the proteins caused by cigarette smoke exposure in erythrocyte ghosts. Tea catechins efficiently prevent degradation of proteins associated with erythrocyte membrane⁶². The preventive effect on the alterations in protein bands indicates the protective role offered by green tea on cigarette smoke-induced molecular damage.

Conclusion

In conclusion, the present study revealed that cigarette smoking induces oxidative stress, which in turn causes morphological changes as evident from altered protein and lipid profile in RBC, diminishing the activities of membrane-bound ATPases and enzymatic antioxidants. Green tea supplementation restored the above changes induced by cigarette smoking. Therefore, this study suggests that green tea catechins possess antioxidant properties and fight against cigarette smoke-induced toxicity in erythrocytes, cells highly prone to oxidative stress.

Acknowledgment

The authors thank DST-SERB, New Delhi, India for providing a research grant to Dr. Vaddi Damodara Reddy (SB/S2/RJN-043/2014 dated 17-12-2015).

References:

- 1 Goodchild M, Nargis N & Tursan d'Espaignet E, Global economic cost of smoking-attributable diseases. *Tob Control*, 27 (2018) 58.
- 2 Kar D, Gillies C, Zaccardi F, Webb D, Seidu S, Tesfaye S, Davies M & Khunti K, Relationship of cardio metabolic parameters in non-smokers, current smokers, and quitters in diabetes: a systematic review and meta-analysis. *Cardiovasc Diabetol*, 15 (2016) 158.

- 3 Gambardella J, Sardu C, Sacra C, Del Giudice C & Santulli G, Quit smoking to outsmart atherogenesis: Molecular mechanisms underlying clinical evidence. *Atherosclerosis*, 257 (2017) 242.
- 4 Stabbert R, Dempsey R, Diekmann J, Euchenhofer C, Hagemeister T, Haussmann HJ, Knorr A, Mueller BP, Pospisil P, Reininghaus W, Roemer E, Tewes FJ & Veltel DJ, Studies on the contributions of smoke constituents, individually and in mixtures, in a range of *in vitro* bioactivity assays. *Toxicol In Vitro*, 42 (2017) 222.
- 5 Padmavathi P, Reddy VD, Bulle S, Marthadu S, Maturu P & Varadacharyulu NC, Chronic cigarette smoking-induced oxidative/nitrosative stress in human erythrocytes and platelets. *Mol Cell Toxicol*, 14 (2018) 27.
- 6 Boukhenouna S, Wilson MA & Bahmed K, Kosmider B, Reactive oxygen species in chronic obstructive pulmonary disease. *Oxid Med Cell Longev*, (2018) 5730395.
- 7 Kamceva G, Arsova-Sarafinovska Z, Ruskovska T, Zdravkovska M, Kamceva-Panova L & Stikova E, Cigarette smoking and oxidative stress in patients with coronary artery disease. *Maced J Med Sci*, 4 (2016) 636.
- 8 Pandey KB & Rizvi SI, Markers of oxidative stress in erythrocytes and plasma during aging in humans. *Oxid Med Cell Longev*, 3 (2010) 2.
- 9 Ansari FA & Mahmood R, Sodium nitrate induces reactive oxygen species that Lower the antioxidant power, damage the membrane, and alter pathways of glucose metabolism in human erythrocytes. *J Agric Food Chem*, 63 (2015) 10372.
- 10 Diederich L, Suvorava T, Sansone R, Keller TCS, Barbarino F, Sutton TR, Kramer CM, Luckstadt W, Isakson BE, Gohlke H, Feelisch M, Kelm M & Cortese-Krott MM, On the effects of reactive oxygen species and nitric oxide on red blood cell deformability. *Front Physiol*, 9 (2018) 332.
- 11 Maheshwari N, Khan FH & Mahmood R, Sodium meta-arsenite induced reactive oxygen species in human red blood cells: impaired antioxidant and membrane redox systems, haemoglobin oxidation, and morphological changes. *Free Radic Res*, 51 (2017) 483.
- 12 Roy A, Sikdar J, Seal P & Haldar R, Cigarette smokers develop structurally modified hemoglobin: a possible way of increasing oxidative stress. *Inhal Toxicol*, 27 (2015) 300.
- 13 Sparrow RL, Red blood cell components: time to revisit the sources of variability. *Blood Transfus*, 15 (2017) 116.
- 14 Dalle-Donne I, Colombo G, Gornati R, Garavaglia ML, Portinaro N, Giustarini D, Bernardini G, Rossi R & Milzani A, Protein carbonylation in human smokers and mammalian models of exposure to cigarette smoke: focus on redox proteomic studies. *Antioxid Redox Signal*, 26 (2017) 406.
- 15 Kuhn V, Diederich L, Keller TCS, Kramer CM, Luckstadt W, Panknin C, Suvorava T, Isakson BE, Kelm M & Cortese-Krott MM, Red blood cell function and dysfunction: Redox regulation, nitric oxide metabolism, anemia. *Antioxid Redox Signal*, 26 (2017) 718.
- 16 Colombo G, Rossi R, Gagliano N, Portinaro N, Clerici M, Anniba A, Giustarini D, Colombo R, Milzani A & Dalle-Donne I, Red blood cells protect albumin from cigarette smoke-induced oxidation. *PLoS One*, 7 (2012) e29930.
- 17 Metta S, Basalingappa DR, Uppala S & Mitta G, Erythrocyte antioxidant defenses against cigarette smoking in ischemic heart disease. *J Clin Diagn Res*, 9 (2015) BC08.
- 18 Montel-Hagen A, Kinet S, Manel N, Mongellaz C, Prohaska R, Battini JL, Delaunay J, Sitbon M & Taylor N, Erythrocyte Glut1 triggers dehydroascorbic acid uptake in mammals unable to synthesize vitamin C. *Cell*, 132 (2008) 1039.
- 19 Sikdar J, Seal P, Roy A & Haldar R, Cigarette smokers develop altered erythrocyte membrane composition: An investigation unmasking the role of membrane bound integral protein GLUT-1. *Free Radic Res*, 51 (2017) 375.
- 20 Brawley OW, The role of government and regulation in cancer prevention. *Lancet Oncol*, 18 (2017) e483.
- 21 Mosbah R, Yousef MI & Mantovani A, Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: the protective effects of green tea extract. *Exp Toxicol Pathol*, 67 (2015) 253.
- 22 Chen L, Mo H, Zhao L, Gao W, Wang S, Cromie MM, Lu C, Wang JS & Shen CL, Therapeutic properties of green tea against environmental insults. *J Nutr Biochem*, 40 (2017) 1.
- 23 Pastoriza S, Mesias M, Cabrera C & Rufian-Henares JA, Healthy properties of green and white teas: an update. *Food Funct*, 8 (2017) 2650.
- 24 Mazzanti G, Di Sotto A & Vitalone A, Hepatotoxicity of green tea: an update. *Arch Toxicol*, 89 (2015) 1175.
- 25 Hayakawa S, Saito K, Miyoshi N, Ohishi T, Oishi Y, Miyoshi M & Nakamura Y, Anti-cancer effects of green tea by either anti- or pro-oxidative mechanisms. *Asian Pac J Cancer Prev*, 17 (2016) 1649.
- 26 Saeed M, Naveed M, Arif M, Kakar MU, Manzoor R, Abd El-Hack ME, Alagawany M, Tiwari R, Khandia R, Munjal A, Karthik K, Dhama K, Iqbal HMN, Dadar M & Sun C, Green tea (*Camellia sinensis*) and l-theanine: Medicinal values and beneficial applications in humans-A comprehensive review. *Biomed Pharmacother*, 95 (2017) 1260.
- 27 Alimi H, Hfaeidh N, Bouoni Z, Sakly M & Rhouma KB, Protective effects of *Opuntia ficus indica* f. inermis prickly pear juice upon ethanol-induced damages in rat erythrocytes. *Alcohol*, 46 (2012) 235.
- 28 Samuel KM, Fundamentals of clinical chemistry (Philadelphia, PA: WB Saunders and Company) 1989, 602.
- 29 Beutler E, Dixon O & Kelly BM, Improved method for the determination of blood glutathione. *J Lab Clin Med*, 61 (1963) 882.
- 30 Mishra PH & Fridovich I, The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J Biol Chem*, 247 (1972) 3170.
- 31 Chance B, Catalase and peroxidases. Part II. Special methods. *Meth Biochem Anal*, 1 (1954) 408.
- 32 Rotruck JT, Pope AL, Ganther HE & Swanson AB, Selenium: biochemical role as a component of glutathione peroxidase. *Science*, 179 (1973) 588.
- 33 Dodge JT, Mitchell C & Hanahan DJ, The preparation and chemical characteristics of hemoglobin free ghosts of human erythrocytes. *Arch Biochem Biophys*, 100 (1963) 119.
- 34 Ohkawa H, Ohishi N & Yagi K, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem*, 95 (1979) 351.
- 35 Reznick AZ & Packer L, Oxidative damage to proteins: spectroscopic method for carbonyl assay. *Methods Enzymol*, 233 (1994) 357.

- 36 Bailey SM, Patel VB, Young TA, Asayama K & Cunningham CC, Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1system and protein oxidation status in rat liver. *Alcohol Clin Exp Res*, 25 (2001) 726.
- 37 Sedlak J & Lindsay RH, Estimation of total protein bound and non-protein sulphhydryl groups in tissues with Ellman's reagent. *Anal Biochem*, 25 (1968) 192.
- 38 Ismail B & Edelman B, Effects of ethanol administration on rat liver plasma membrane-bound enzymes. *Biochem Pharmacol*, 34 (1985) 2685.
- 39 Lowry OH, Roseberough NJ, Farr AL & Ramdall R, Protein measurement with the Folin-phenol reagent. *J Biol Chem*, 193 (1951) 263.
- 40 Laemmli UK, Cleavage of structural proteins during the assembly of the head of bacteriophage T₄. *Nature*, 227 (1970) 680.
- 41 Oakley BR, Kirsch Dr & Morris NR, A simplified ultra-sensitive silver stain for detecting proteins in polyacrylamide gels. *Anal Biochem*, 105 (1980) 361.
- 42 Fiske CH & Subbarow Y, The colorimetric determination of inorganic phosphorous. *J Biol Chem*, 66 (1925) 375.
- 43 Park JM, Chang KH, Park KH, Choi SJ, Lee K, Lee JY, Satoh M, Song SY & Lee MY, Differential effects between cigarette total particulate matter and cigarette smoke extract on blood and blood vessel. *Toxicol Res*, 32 (2016) 353.
- 44 Gangopadhyay S, Vijayan VK & Bansal SK, Lipids of erythrocyte membranes of COPD patients: a quantitative and qualitative study. *COPD*, 9 (2012) 322.
- 45 Shah AA, Khand F & Khand TU, Effect of smoking on serum xanthine oxidase, malondialdehyde, ascorbic acid and α -tocopherol levels in healthy male subjects. *Pak J Med Sci*, 31 (2015) 146.
- 46 Aboulmaouahib S, Madkour A, Kaarouch I, Sefrioui O, Saadani B, Copin H, Benkhalifa M, Louanjli N & Cadi R, Impact of alcohol and cigarette smoking consumption in male fertility potential: Looks at lipid peroxidation, enzymatic antioxidant activities and sperm DNA damage. *Andrologia*, 50 (2018).
- 47 Fabrizio G, Arcaro A, Pizzimenti S, Daga M, Cetrangolo GP, Dianzani C, Lepore A, Graf M, Paul R, Ames J & Barrera G, DNA damage by lipid peroxidation products: implications in cancer, inflammation and autoimmunity. *AIMS Genetics*, 4 (2017) 103.
- 48 Khoubnasab Jafari M, Ansarin K & Jouyban A, Use of malondialdehyde as a biomarker for assessing oxidative stress in different disease pathologies: a review. *Iran J Public Health*, 44 (2015) 714.
- 49 Kurku H, Kacmaz M, Kisa U, Dogan O & Caglayan O, Acute and chronic impact of smoking on salivary and serum total antioxidant capacity. *J Pak Med Assoc*, 65 (2015) 164.
- 50 Gokulakrishnan A & Ali AR, Cigarette smoke-induced biochemical perturbations in human erythrocytes and attenuation by epigallocatechin-3-gallate-tea catechin. *Pharmacol Rep*, 62 (2010) 891.
- 51 Bulle S, Reddy VD, Padmavathi P, Maturu P, Puvvada PK & Nallanchakravarthula V, Association between alcohol-induced erythrocyte membrane alterations and hemolysis in chronic alcoholics. *J Clin Biochem Nutr*, 60 (2017) 63.
- 52 Pestic G, Jeremic J, Nikolic T, Zivkovic V, Srejovic I, Vranic A, Bradic J, Ristic B, Matic A, Prodanovic N & Jakovljevic V, Interleukin-6 as possible early marker of stress response after femoral fracture. *Mol Cell Biochem*, 430 (2017) 191.
- 53 Sharma S, Kaur M, Suhalka ML & Shrivastav C, Correlation of α -lipoic acid and S. glutathione level with free radical excess in tobacco consumers. *J Clin Diagn Res*, 10 (2016) CC01.
- 54 Villagran M, Munoz M, Del Pozo M, Maldonado M & Mardones L, Catalase is more sensible to the inhibitory effect of soluble component of tobacco smoke than Glutathione peroxidase and superoxide dismutase. *Rev Toxicol*, 34 (2017) 148.
- 55 Ramlagan P, Rondeau P, Planesse C, Neergheen-Bhujun VS, Bourdon E & Bahorun T, Comparative suppressing effects of black and green teas on the formation of advanced glycation end products (AGEs) and AGE-induced oxidative stress. *Food Funct*, 8 (2017) 4194.
- 56 Padmavathi P, Reddy VD, Kavitha G, Paramahansa M & Varadacharyulu N, Chronic cigarette smoking alters erythrocyte membrane lipid composition and properties in male human volunteers. *Nitric Oxide*, 23 (2010) 181.
- 57 Fajardo VA, McMeekin L & LeBlanc PJ, Influence of phospholipid species on membrane fluidity: a meta-analysis for a novel phospholipid fluidity index. *J Membr Biol*, 244 (2011) 97.
- 58 Klem S, Klingler M, Demmelair H & Koletzko B, Efficient and specific analysis of red blood cell glycerophospholipid fatty acid composition. *Plos One*, 7 (2012) e33874.
- 59 Babu PV, Sabitha KE & Shymaladevi CS, Green tea impedes dyslipidemia, lipid peroxidation, protein glycation and ameliorates Ca²⁺ ATPase and Na⁺/K⁺ ATPase activity in the heart of streptozotocin-diabetic rats. *Chem Biol Interact*, 162 (2006) 157.
- 60 Cyboran S, Strugała P, Włoch A, Oszmianski J & Kleszczynska H, Concentrated green tea supplement: Biological activity and molecular mechanisms. *Life Sci*, 126 (2015) 1.
- 61 Assuncao M & Andrade JP, Protective action of green tea catechins in neuronal mitochondria during aging. *Front Biosci (Landmark Ed)*, 20 (2015) 247.
- 62 Chu C, Deng J, Man Y & Qu Y, Green tea extracts epigallocatechin-3-gallate for different treatments. *Biomed Res Int*, (2017) 5615647.