

Protective effect of ellagic acid on erythrocytes subjected to oxidative stress during human ageing

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In the present study, the potential role of ellagic acid on various biomarkers of oxidative stress during human ageing has been studied. The study was carried out on 75 healthy individuals of both sexes. They were divided into three age groups viz., young (<36 years; n=23; male=13, female=10), middle (36-60 years; n=38; male=25, female=13), and old (>60 years; n=14; male=8, female=6). The oxidative stress was induced by using *tert*-butyl hydroperoxide (*t*-BHP). In each age group, erythrocytes were incubated with ellagic acid (final concentration 10^{-5} M). The result demonstrated a significant ($P < 0.01$) decrease in total antioxidant potential in terms of FRAP value during ageing. A significant ($P < 0.05$) *in vitro* protective effect of ellagic acid on malondialdehyde and reduced glutathione levels on erythrocytes against oxidative stress induced by *tert*-butyl hydroperoxide has been reported. The study was performed on erythrocytes of different age groups. The findings suggest a possible role of ellagic acid as an anti-ageing compound.

Keywords: Ageing, Ellagic acid, Glutathione, Malondialdehyde, Polyphenols

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Introduction

Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are the reactive non-radical and radical derived from oxygen and nitrogen. Aerobic cells are responsible for the generation of these reactive species of oxygen and nitrogen and play a crucial role in ageing and age-associated disorders¹. Antioxidants are the molecules that help to prevent, delay or remove oxidative stress from target species². Oxidative stress refers to the disparity between the generation of oxidants and antioxidant defence mechanisms which results in cellular damage to the body³. The free radical theory of ageing was proposed for the first time by Harman in 1956, but in 1990, Sohal highlighted the drawbacks of this theory and gave the concept of oxidative stress. The understanding of human ageing is the origin of the concept of oxidative stress⁴. The oxidative stress theory of the most popular theory given on ageing. According to this theory, ageing and various age-associated disorders are caused by the oxidative damage of macromolecules which includes lipids, carbohydrates, proteins, and nucleic acids⁵. The

process of ageing is characterized by the gradual accumulation of damaged cells, progressive decline in physical activities, and increased susceptibility to the occurrence of various diseases.

Polyphenols are the secondary metabolites produced by plants and are present in a number of fruits and vegetables⁶. These polyphenols possess antioxidant, anti-inflammatory, and antimicrobial activities⁷. Ellagic acid (EA) chemically named as 2,3,7,8-tetrahydroxy-chromeno[5,4,3-cde]-chromene 5, 10-dione is a naturally occurring antioxidant⁸. It is a polyphenolic compound consisting of four hydroxyls and two lactones functional groups⁹. EA is commonly found in berries, pomegranate, and nuts such as almonds and walnuts¹⁰. EA is known to possess anti-inflammatory¹¹, hepatoprotective¹², neuroprotective, and antiviral properties¹³. A study has reported that EA extends the mean lifespan in the case of *Drosophila melanogaster*¹⁴. EA is known to alleviate bleomycin-induced pulmonary functions and also improved the pulmonary functions in the mice model¹⁵. EA has the ability to accept electrons from various substrates and thus participates in redox reactions resulting in efficient scavenging of free radicals^{16,17}. EA was found to decrease the blood glucose level in fasting conditions in diabetic rats by increasing the number and size of the β -cells of the

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pancreas and stimulating the secretion of insulin¹⁸. According to a study, EA is found to significantly increase cell proliferation and decrease the level of ROS, TNF- α , and advanced glycation end products followed by ageing induced by D-galactose¹⁹. EA is known to upregulate the antioxidant enzyme, catalase (CAT) as well²⁰.

Materials and Methods

The present study was carried out on 75 individuals which included both males (61%) and females (39%). The samples selected were healthy normal individuals and were screened for any major health issues. The criteria of selection were based on as mentioned earlier by Maurya & Rizvi²¹. Blood samples for the study were collected from the subjects after their informed consent. The human volunteers were divided into young, middle, and, old age groups. The exclusion criteria for the collection of blood include: i) any kind of drug treatment, ii) The subjects should be non-smokers, and iii) Pregnant women were also excluded from the sampling process. The protocol for blood collection was in accordance with the guidelines provided by the ethical committee of the institute (Ref no. CUH/IHEC/2021/03).

Exactly 3 mL of venous blood was collected from healthy human subjects. Blood was collected in sampling vials containing anticoagulants to prevent coagulation. The centrifugation of blood was done at 4°C at 1800 g for 10 min. The plasma (obtained as supernatant), was stored at -80°C for performing the ferric reducing antioxidant power (FRAP) assay, the buffy coat was removed and packed red blood cells (PRBCs) were obtained as pellets. The PRBCs obtained were further washed 2-3 times using phosphate buffer saline (PBS), pH 7.4. PRBCs were washed by resuspending the pellets following centrifugation 2-3 times. The supernatant was discarded and pellets were used immediately for our study. The malondialdehyde assay and glutathione assay was studied on PRBCs.

FRAP assay

The FRAP assay was carried out following the methods given by Benzie and Strain²². Acetate buffer (300 mM, pH 3.6), FeCl₃.6H₂O (20 mmol/L), and 2,4,6-tri[2-pyridyl]-s-triazine (10 mM in 40 mM HCl) in the ratio 10:1:1 was mixed together to get a working FRAP solution. For performing the assay, 3 mL of FRAP reagent was added to 100 μ L of the plasma. After vigorously mixing the solution, the absorbance

at 593 nm at 30 s interval for 3 min was read. For calibration purpose, an aqueous ferrous solution having a concentration range of 100–1000 μ mol/L was used. With the help of a regression equation, the FRAP value of the plasma was calculated in μ molFe(II) per litre.

Determination of malondialdehyde in erythrocytes

The malondialdehyde (MDA) content in the erythrocytes is measured following the Esterbauer and Cheeseman²³ method. 200 μ L of PRBCs were suspended in 3 mL Krebs-Ringer phosphate buffer of pH 7.4 (KRP buffer). In 1 mL of 10% trichloroacetic acid (TCA), 1 mL of the lysate was added. The mixture was further centrifuged at 5000 g for 5 min. A 0.67% thiobarbituric acid (TBA) was prepared in 0.05 mol/L NaOH. Exactly 1 mL of the supernatant was added to this solution. This was boiled in a water bath for about 20 min at a temperature of more than 90°C. The mixture was cooled and the absorbance was read at an optical density of 532 nm. A standard plot was used to obtain the total MDA concentration in the erythrocytes. MDA concentration was expressed in nmol/mL of the PRBCs as mentioned by Rizvi and Maurya²⁴.

Determination of glutathione in erythrocytes

Glutathione (GSH) in the erythrocytes was measured following the method given by Fujii, *et al*²⁵. In this method, 5,5-dithio-bis-2-nitrobenzoic acid (DTNB), (Ellman's reagent) gets reduced to form a yellow-coloured product. The absorbance was measured at an optical density of 412 nm. The concentration of GSH in PRBCs was expressed in mg/mL. GSH content was measured using a standard graph as mentioned by Rizvi and Maurya²⁴.

Effect of ellagic acid and *t*-BHP-induced oxidative stress

The effect of EA on the GSH and MDA status in erythrocytes was determined as follows: After washing the blood 2-3 times using PBS, it was then suspended in 4 volumes of KRP, pH 7.4 containing 5 mmol/L of glucose. In the present study, the PRBCs were incubated with 10⁻⁵ mol/L EA and 10⁻⁵ mol/L *t*-BHP together. The effect of 10⁻⁵ mol/L EA alone was studied. Further, the antioxidant property of EA alone and when induced with stress was compared. For the present study, data only for 10⁻⁵ mol/L concentration has been presented as the earlier work reported on other polyphenols has used this concentration²¹. Here, a single concentration has been considered, but further study will be conducted on the concentration

and time-dependent effect. The *in vitro* effect of EA was determined after the treatment of packed erythrocytes with 10^{-5} mol/L of EA and *t*-BHP at 37°C for 60 min. In parallel, control experiments were performed without any treatment of EA or *t*-BHP. *In vitro* oxidative stress was induced in the packed erythrocytes with 10^{-5} mol/L *t*-BHP for 60 min at 37°C . The concentrations of *t*-BHP used for the induction of oxidative stress in the erythrocytes were in the concentration range as reported by Di Simplicio *et al*²⁶.

The statistical analysis was performed with the help of GraphPad Prism version 8.00. The relationship among the various parameters were assessed with the help of Pearson correlation coefficient (*r*). *P*-value <0.05 was considered statistically fit.

Results

Fig. 1 demonstrates the total antioxidant potential of plasma in terms of FRAP value. The present study indicate that there was a significant ($P < 0.01$) decrease in the FRAP values in middle and old age group as compared to the young age group. This suggests that with increasing age the antioxidant status of the body decreases and hence the free radical scavenging property of the antioxidants also decreases.

Fig. 2 showed the *in vitro* effect of EA on MDA content. Results demonstrated that there is significant ($P < 0.001$) increase in MDA content in age groups when oxidative stress was induced by *t*-BHP (final concentration 10^{-5}M) as compared to respective

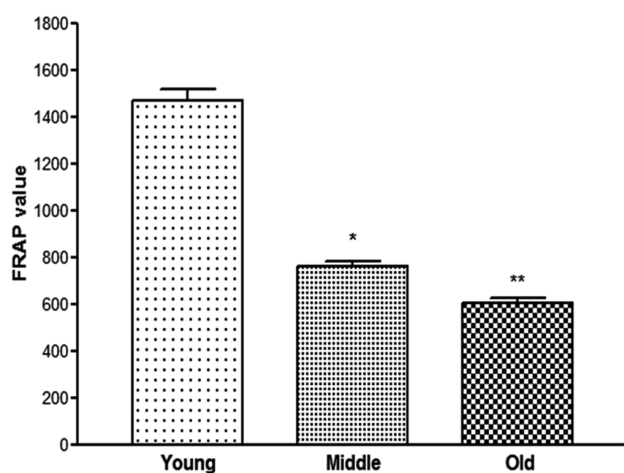


Fig. 1 — Total antioxidant potential of plasma in term of FRAP value in different age group viz. young (<36 years; $n=23$), middle ($36-60$ years; $n=38$), and old (> 60 years; $n=14$). *, $P < 0.001$; **, $P < 0.0001$ compared with young age group. FRAP values expressed as $\mu\text{mol Fe (II)}$ per litre of plasma. FRAP, ferric reducing ability of plasma.

control. Incubation of erythrocytes with EA (final concentration 10^{-5}M) caused significant ($P < 0.001$) decrease in MDA content in all age groups.

GSH is a primary antioxidant and an important biomarker of oxidative stress. Fig. 3 represents the *in*

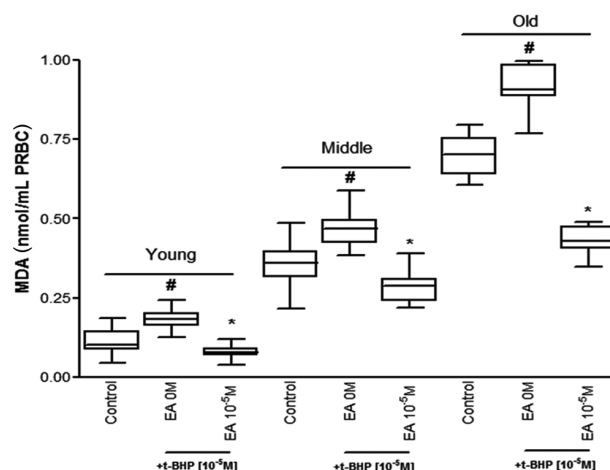


Fig. 2 — Effect of ellagic acid administration (10^{-5}M final concentration) on red blood cell malondialdehyde content in oxidative stressed red blood cell in young (<36 years; $n=23$), middle ($36-60$ years; $n=38$) and old (> 60 years; $n=14$) group. MDA level were expressed as nmol mL^{-1} Packed Red Blood Cells (PRBC). Data are expressed as mean $\pm$ SD. #, $P < 0.001$ compared with respective control and *, $P < 0.001$ compared with the respective oxidative stress induced group. MDA, malondialdehyde; EA, ellagic acid, *t*-BHP, *tert*-butyl hydroperoxide, M, molar.

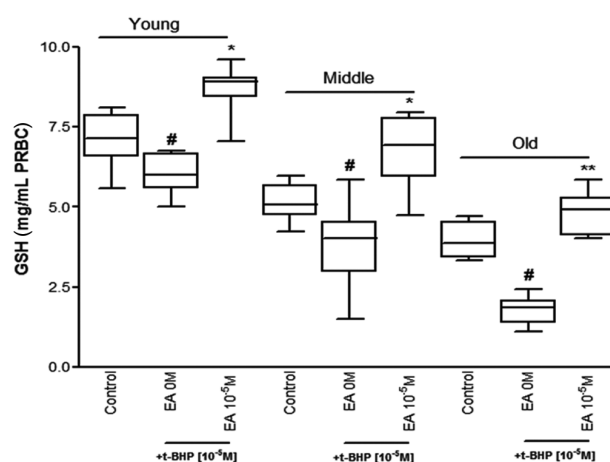


Fig. 3 — Effect of ellagic acid administration (10^{-5}M final concentration) on red blood cell reduced glutathione content (GSH) level in oxidative injured red blood cell in young (<36 years; $n=23$), middle ($36-60$ years; $n=38$) and old (> 60 years; $n=14$) group. GSH level were expressed as mg mL^{-1} Packed Red Blood Cells (PRBC). Data are expressed as mean $\pm$ SD. #, $P < 0.001$ compared with respective control and *, $P < 0.001$; **, $P < 0.0001$ compared with the respective oxidative stress induced group. GSH, reduced glutathione; EA, ellagic acid, *t*-BHP, *tert*-butyl hydroperoxide, M, molar.

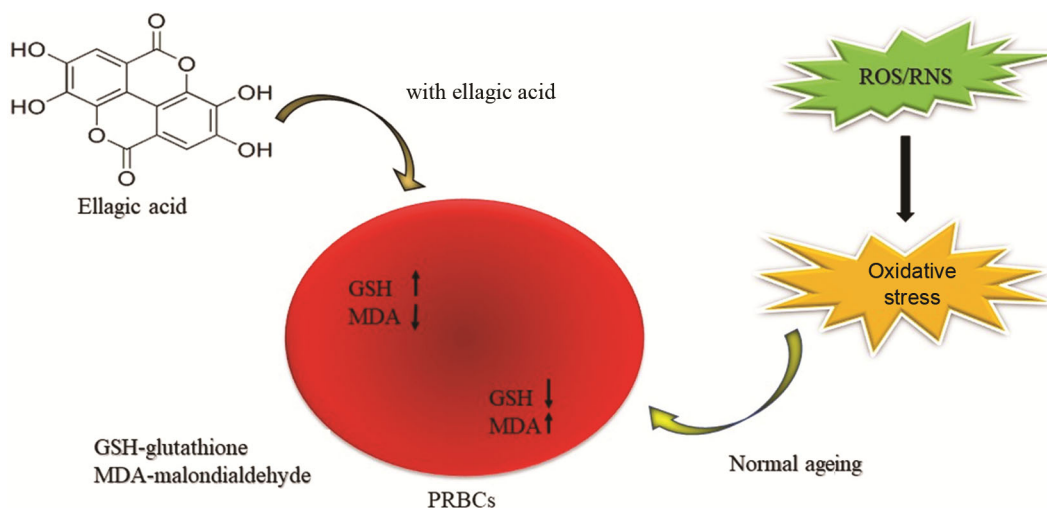


Fig. 4 — Role of ellagic acid in red blood cell metabolism.

vitro effect of EA on *t*-BHP-induced oxidative stress in erythrocytes GSH level. Results showed that there is significant ($P < 0.01$) decrease in GSH level in all age groups when oxidative stress was induced by *t*-BHP (final concentration 10^{-5} M) as compared to their respective controls. Incubation of erythrocytes with EA (final concentration 10^{-5} M) caused significant ($P < 0.001$) increase in GSH level in different age groups.

Discussion

The erythrocytic membrane is well equipped with various types of mechanisms to protect itself from the effect of oxidative stress. MDA is a byproduct of peroxidation of polyunsaturated fatty acids (PUFA). An increase in the level of MDA is indicator for the peroxidation of lipid²⁷. In the present study, it was hypothesized that on induction of the cells with *t*-BHP, stress is generated. The PUFA present in the walls of membrane undergoes peroxidation and thereby, the MDA content of the cells increases. The biomarkers of the oxidative stress are present on the membrane of the PRBCs. The non-enzymatic antioxidant system such as GSH is present in the RBCs. The -SH group of cysteine present in GSH helps to keep the thiol group of haemoglobin, membrane proteins and enzymes in reduced state²⁸. As the cells are stressed, the level of GSH also decreases. EA behaves as an antioxidant and treating the cells with EA scavenges the free radicals inside the cell and hence reduces the level of MDA and increases the level of GSH. This change in the biomarkers was found to be significant when the *t*-BHP induced cells were compared with the control of each age group.

Based on the present study, it can be concluded that EA acts as an impressive antioxidant and the polyphenol exhibits age-related changes in the biomarkers of oxidative stress i.e., MDA and GSH during ageing. The statistical analyses show a significant P value after the treatment of EA in the stress induced PRBCs. Based on the data generated and after subsequent analyses it can be hypothesized that there is a correlation between the biomarkers of oxidative stress, MDA and GSH, and the polyphenol EA. EA decreases the level of MDA and increases the GSH level as we proceed towards aged PRBCs. Further findings are required to state that EA acts as an anti-ageing compound. Fig. 4 shows the role of EA in the metabolism of red blood cells.

Conclusion

Plant products have been used as therapeutics since ancient times. Several studies have reported the anti-ageing property of other polyphenols such as tea-catechins and resveratrol. EA is a potent antioxidant compound but its effect on ageing has not yet been elucidated in human erythrocytes. Here the effect of EA on human erythrocytes of different age groups, which has not been reported yet has been studied. The data reported the anti-ageing property of EA when studied for a single concentration. The authors' aim to work on different concentrations of EA to confirm it as an anti-ageing molecule. Based on results, it can be concluded that ellagic acid has a significant antioxidant potential and may be used to delay ageing to some extent. Further studies are required to confirm whether ellagic acid acts as anti-ageing compound or not.

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Conflict of interest

The authors declare that they have no conflict of interest in the publication.

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