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Protective effect of myricetin on osmotic stability of erythrocytes during aging in humans

Kanti Bhooshan Pandey and Syed Ibrahim Rizvi
Department of Biochemistry, University of Allahabad, Allahabad-21102, A.P., India

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Abstract

Enhanced oxidative stress is reported as one of the main determinants of aging and age related events. Several parameters of blood have been demonstrated to be negatively affected by increased oxidative stress including changes in erythrocyte membrane fluidity and integrity, which results into overall malfunctioned cellular physiology. Present study was aimed to determine the erythrocyte osmotic stability during aging in humans and the effect of myricetin treatment. Results obtained from the study show a significant ($p < 0.0001$) age dependent decline in the stability of erythrocyte as evidenced by enhanced osmotic fragility in aged individuals which was significantly correlated with radical scavenging capacity of plasma. Myricetin at 10µM significantly ($p < 0.05$) protected osmotic lysis of erythrocytes obtained from different age subjects.

Key words: Erythrocytes, Aging, Myricetin, ROS, Osmotic fragility

Introduction

Aging is characterized by decline in physiological functions of individuals with accumulation of several adverse changes over time. Among many theories (telomerase, protein error and caloric restriction) which attempt to explain the process of aging , the oxidative stress or free radical theory offers the best mechanistic explanation of aging and age related events (Harman, 2006). Free radicals, commonly known as reactive oxygen species (ROS), are generated during metabolic process as a byproduct of aerobic respiration in the cell. These highly reactive species anonymously react with biomolecules of the cell and damage them in many ways (Halliwell and Gutteridge, 2007). Though the level of ROS is balanced in the cell by inherent antioxidant systems, a condition of oxidative stress occurs when the balance between antioxidant and oxidant is disrupted due to excess generation of ROS or week antioxidant defense. Increased oxidative stress is reported as one of the primary determinants of aging (Droge, 2002 and Pandey and Rizvi, 2010).

Author for correspondence: Professor Syed Ibrahim Rizvi
Department of Biochemistry, University of Allahabad, Allahabad-21102, A.P., India
E-mail: sirizvi@gmail.co
Tel.: +91-9415305910

Red Blood Cells (RBCs) along with its membrane are good model system for oxidative stress related studies because of their susceptibility to oxidative damage in the wake of high cell concentration of oxygen and hemoglobin, a powerful promoter of the oxidative process (Pandey and Rizvi, 2011).

Myricetin (3,3',4',5,5',7-hexahydroxyflavone; Figure 1) is a flavonoid , naturally presents in many vegetables, fruits and wine (Lee and Choi, 2008). Studies have reported that myricetin possesses many health promoting effects, it is reported to be anti-inflammatory, anticarcinogenic and antioxidative (Ong and Khoo, 1997 and Lee and Choi, 2008).

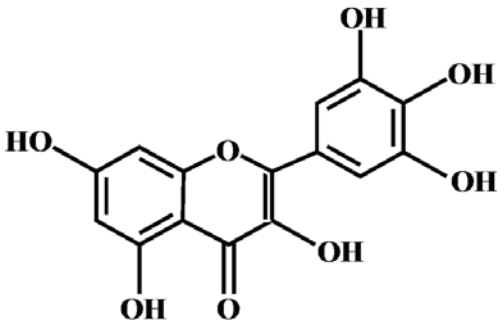

Figure 1: Chemical structure of myricetin

Growing evidence suggest that nutritional sources of antioxidants, such as fruits, vegetables and tea have ability to attenuate tissue damage caused by oxidative stress (Cao *et al*., 1998). Polyphenols present abundantly in these natural sources, could play a major role in protecting cellular molecules from being oxidized as well as in enhancing the antioxidant system of body (Pandey and Rizvi, 2009a; 2012). Keeping this rationale in the mind, the present study was undertaken to determine the erythrocyte stability and its correlation with radical scavenging capacity of plasma during aging in humans. The study was extended to evaluate the effect of myricetin treatment on erythrocytes from persons belonging to different age groups.

Materials and Methods

The study was carried out on 91 normal healthy subjects of both genders (59 males and 32 females) between the ages of 18 and 80 years. The criteria of selection were as published earlier (Pandey *et al*., 2010a). The BMI of the subjects ranged from 18.8 to 26.2 Kg/m^2. All volunteers were screened for asthma, tuberculosis, diabetes mellitus or any other major illness. None of the subjects were smokers or were taking any medication. Care was also taken to exclude the volunteers taking/have taken the any nutritional supplements since last three months. The elderly subjects were living at home but functionally independent without any cognitive impairment.

All persons are aware of the study protocol and gave their informed consent for the use of their blood samples for the study. The protocol of study was in conformity with the guidelines of the Allahabad University Ethical Committee.

Isolation of packed red blood cells and plasma

Venous blood was obtained at fasting condition in the morning by venipuncture in sterile polystyrene tubes containing heparin. Plasma was obtained by centrifuging the blood at 800 × g for 10 min at 4^0C and free radical scavenging measurements were performed immediately. Packed Red blood cells (PRBCs) obtained after the removal of plasma, buffy coat and the upper 15% of RBCs, washed twice with cold phosphate buffered saline (PBS) (0.9% NaCl and 10 mM Na_2HPO_4; pH 7.4).

Determination of the osmotic fragility of erythrocytes

The erythrocyte osmotic fragility was determined as described by Sharma *et al*. (2010). Briefly, 0.02 ml of blood was added to tubes containing increasing concentration (0, 0.1, 0.2, 0.3, 0.5, 0.7, 0.8 and 0.9%) of phosphate-buffered sodium chloride (NaCl) solution at pH 7.4. The tubes were gently mixed and incubated at 37^0C for 30 min. The content in each tube was then centrifuged at 1500 × g for 10 min, supernatant was collected and optical density of the supernatant was determined spectrophotometrically at 540nm. Hemolysis in each tube was expressed as a percentage, taking hemolysis in distilled water (0% NaCl) as 100%. The results are expressed in terms of mean erythrocyte fragility (MEF) which was NaCl concentration corresponding to 50% hemolysis of erythrocytes. The experiments were run in replicates of three or more in order to obtain spastically reliable data.

Measurement of radical scavenging capacity of plasma

Radical scavenging capacity of plasma was estimated by DPPH reduction assay as described earlier (Pandey *et al*., 2010b). Briefly 0.1 mL of plasma in phosphate buffered solution (PBS) (10 µM, pH 7.4) was incubated in the methanolic solution of DPPH· (0.1 mM). Absorbance at 517 nm was measured after 30 min of incubation with vigorous shaking. The free radical DPPH· scavenging (*i.e*., reduction) activity was calculated from the equation: Activity [% of DPPH reduction] = [(A"Ax)/A]×100%, where A is the absorbance of DPPH· solution with methanol, Ax is the absorbance of a DPPH· solution with plasma.

Experiments with myricetin

To study effect of myricetin on osmotic fragility of erythrocytes, the volunteers were divided into three age groups; <40 years (n = 31; Young), 40–60 years (n = 30; Middle-aged) and >60 years (n = 30; Old).Washed erythrocytes of all three age groups were suspended in 4 volumes of PBS containing 5 mM glucose (pH 7.4). *In vitro* effects were evaluated by incubating the erythrocytes in the presence of myricetin at 10 µM final concentration at 37ºC for 60 min with mild shaking. After this time, the suspensions were immediately centrifuged at 800 ×g, the RBCs were washed twice with at least 50 volumes of PBS and subjected to assay.

Statistical Analysis

Statistical analyses were performed, using the software PRISM 5 (Graphpad Software Inc., San Diego, CA). Relationships between various parameters were assessed using the Pearson correlation coefficient (*r*) and coefficient of determination (r^2). Effects of myricetin were analyzed by using student's t test with their respective control. Values are expressed as Mean ± SD and were considered to be significant when $p < 0.05$.

Results

Results of our study showed an age dependent decline in erythrocyte stability as evidenced by increased osmotic fragility of mixed erythrocyte populations during aging ($p < 0.0001$, r^2=·0.667, $r = 0.817$, Figure 2). Increase in osmotic fragility correlates significantly ($p < 0.0001$, $r^2 = 0.513$, $r = 0.716$) with decrease in the radical scavenging capacity of the plasma (RSCP) by plotting quotients (MFK/RSCP) as a function of human age (Figure 3). *In vitro* treatment of myricetin at 10µM final concentration positively modulated the erythrocyte osmotic fragility pattern (Figure 4).

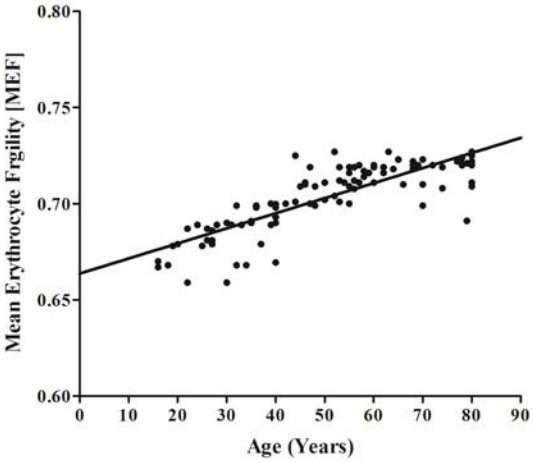

Figure 2: Mean erythrocyte fragility (MEF) plotted as a function of human age. (n = 91); r = 0.817, r^2 = 0. 0.667; p < 0.0001). MEF is NaCl concentration corresponding to 50% hemolysis of erythrocytes at 37^{0}C.

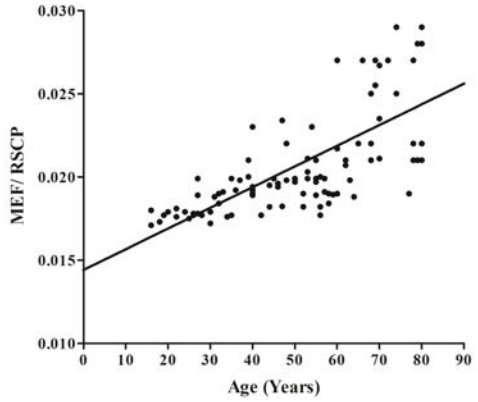

Figure 3: Plot of quotient (mean erythrocyte fragility (MEF)/radical scavenging capacity of plasma (RSCP)) as a function of human age. (n = 91); r = 0.716, r^2 = 0. 0.513; p < 0.0001). RSCP values expressed in the form of % of DPPH· reduced by 0.1 mL of plasma.

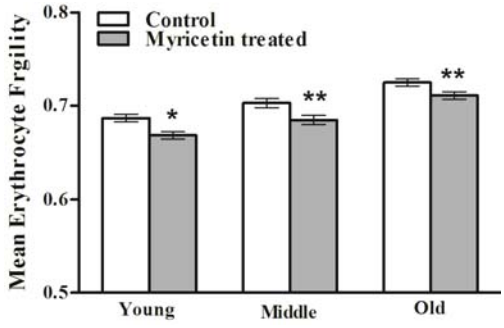

Figure 4: Effects of 10 µM myricetin treatment on mean erythrocyte fragility (NaCl concentration corresponding to 50% hemolysis of erythrocytes at 37^{0}C) with ageing in human erythrocytes. Erythrocytes were obtained from subjects aged <40 years (n = 31; Young), 40–60 years (n = 30; Middle-aged) and >60 years (n = 30; Old). Data are the mean ± SD of 10–12 independent experiments. *p < 0.01, **p < 0.05 compared with respective non-treated (control) group.

Discussion

Erythrocyte membrane is prone to oxidative stress induced damages due to high polyunsaturated fatty acid (PUFA) content. Attack of ROS causes cleavage of PUFA at their double bonds leading to formation of secondary lipid peroxidation products and thus reduces the deformability of membrane (Pandey and Rizvi, 2011). Plasma membrane regulates numerous accepts of cell physiology and plays fundamental roles in regulating cellular homeostasis, signaling, transport and cell adhesion (Mattson, 1998). It has been reported that membrane proteins and lipids are susceptible to oxidative stress modifications (Niki, 2009 and Pandey and Rizvi, 2009b). Damage to these vital biomolecules of the membrane may contribute cellular dysfunction, degeneration of neurons and other age-related pathological events (Mattson, 1998 and Pandey and Rizvi, 2010).

In blood, the normal function of erythrocytes depends upon the integrity of membrane. The normal red cell membrane is characterized its durability; however, its capacity to resist stress gets compromised under certain pathological conditions (Sharma *et al*., 2010). Determination of erythrocyte fragility pattern provides an index of membrane's integrity; therefore, erythrocyte fragility parameter is most frequently used to measure erythrocyte tensile strength (Uzum *et al*., 2006 and Rizvi and Srivastava, 1999). Our results on age dependent increase in osmotic fragility of erythrocytes gives an indication that the vulnerability of the erythrocytes in circulation increases with age. Results are also supported by our previous study, in which we have shown that sulfhydryl (-SH) group concentration in erythrocyte membrane get deceased as we age (Rizvi and Maurya, 2009). The -SH group in erythrocyte membrane plays a very crucial role in maintenance of overall cellular redox balance and provides mechanical strength to the membrane. Reduced concentration of membrane -SH groups due to oxidation in elderly people influences changes of membrane microelasticities of erythrocytes (Reglinski *et al*., 1988) and may be one of the reasons for increased fragility of erythrocytes during aging.

Significant correlation is observed between the RSCP measured through DPPH· assay and pattern of osmotic fragility of erythrocytes during aging, strength the oxidative stress theory of aging; increased oxidative stress is responsible for malfunctioned cellular physiology during aging. We have already reported an age dependent decline in radical scavenging capacity of plasma (Murtaza and Rizvi, 2012). Age dependent increase in osmotic fragility of red blood cells seems to be closely correlated with increasing extracellular oxidative stress measured in terms of RSCP.

Plant polyphenols have attracted wide interest as potent scavengers of ROS. Flavonoids are largest class among six classes of the polyphenols. In myricetin, the B ring is combined

with a 2,3 double bond with a 4-oxo function of the C ring, this chemical structure makes it one of the most biologically active flavonoid. Studies have reported that myricetin has the ability to enter inside cell and to activate plasma membrane redox system, a complimentary tool of all eukaryotic cells to neutralize oxidative stressors and to maintain the redox balance in plasma (Fiorani and Accorsi, 2005). In our earlier study, we have reported that myricetin provides protection to lipids, proteins and inherent antioxidant molecules in the erythrocytes against oxidation (Pandey *et al*., 2009). We hypothesize that decline in osmotic fragility of erythrocytes after treatment of myricetin may be due to its antioxidant property. To the best of our knowledge, no such report is yet available on the effect of myricetin on age dependent changes in erythrocytes.

Conclusion

Based on the results obtained from our study, we conclusively report significant decline in the stability of erythrocyte membrane during aging in humans. Susceptibility of erythrocytes of elderly subjects towards membrane damage may be correlated with the reduced plasma radical scavenging capacity. Myricetin elicited protective effect against erythrocyte osmotic fragility; the mechanism may be due to strong antioxidative property present in this flavonoid. Since myricetin is naturally present in many fruits and vegetables, consumption of myricetin rich diet may be beneficial in age related disorders.

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