

Myricetin May Provide Protection against Oxidative Stress in Type 2 Diabetic Erythrocytes

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Oxidative stress is believed to be a major contributing factor in the development of late complications of diabetes. Many *in vitro* and *in vivo* studies have demonstrated that several parameters of red blood cell function and integrity are negatively affected by increased oxidative stress. Plant polyphenols are reported to exert many biological effects due to their antioxidant property. The present study was undertaken to evaluate the antioxidant effect of myricetin on markers of oxidative stress in erythrocytes from type 2 diabetic patients. The study was carried out on blood samples obtained from 23 type 2 diabetic patients and 23 age-matched control subjects. Erythrocytes were subjected to *in vitro* oxidative stress by incubating with 10^{-5} M *tert*-butyl hydroperoxide (t-BHP). Erythrocyte membrane lipid peroxidation and protein oxidation were measured in terms of malondialdehyde (MDA) and protein carbonyl group levels. The results showed an elevated MDA and protein carbonyl content in diabetic erythrocytes which were further increased after incubation with t-BHP. Myricetin at micromolar concentration significantly ($p < 0.01$) protected an t-BHP-induced increase in levels of oxidative stress parameters of diabetic erythrocytes.

Key words: Diabetes mellitus, Erythrocyte, Myricetin, Oxidative Stress

Introduction

Increased oxidative stress is observed for diabetes due to impaired antioxidant defence in the body; this plays an important role in the development of several diabetic complications (Baynes, 1991). Long-term complications of type 2 diabetes include retinopathy, nephropathy, micro- and macrovascular changes including atherosclerosis and shortened life span of erythrocytes (Zelko *et al.*, 2002). Diabetes-induced damage is more prominent in red blood cells compared to other tissues because of their high content of iron, polyunsaturated fatty acids and oxygen (Pasaoglu *et al.*, 2004). In fact, changes in membrane fluidity and ionic parameters, inactivation of membrane-bound receptors and enzymes, increase in lipid peroxidation, oxidation of glutathione and protein sulfhydryl groups, and activation of proteolysis have all been described following the diabetes-induced oxidative stress to erythrocytes (Davies and Goldberg, 1987; Rizvi and Zaid, 2005; Rizvi *et al.*, 2005).

The mechanism(s) involved in the development of oxidative stress for diabetes is not clearly understood. It is thought that glycation of proteins

can lead to oxidative stress by direct release of O_2^- and H_2O_2 and activation of phagocytes through a specialized receptor for advanced glycation end products (Abuja and Albertini, 2001). Many studies have emphasized the importance of antioxidants for diabetes, and low levels of plasma antioxidants have been implicated as a risk factor for the development of the disease (Baynes, 1991; Facchini *et al.*, 2000).

Dietary intervention trials have shown that consumption of flavonoid-rich foods is associated with a significant increase in the plasma antioxidant level of diabetic patients (Hollman and Katan, 1999). Myricetin (3,3',4',5,5',7-hexahydroxyflavone, Fig. 1) is a natural flavonoid ubiquitously

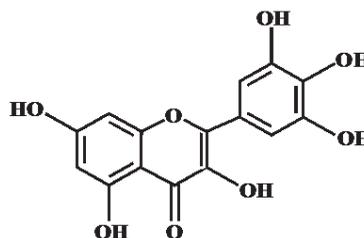


Fig. 1. Chemical structure of myricetin.

present in foods including vegetables, fruits, tea and wine. It is known to possess many beneficial health effects including anticarcinogenic, antimutagenic and anti-inflammatory (Ong and Khoo, 1997). Many of the biological actions of this flavonoid have been attributed to be due to its antioxidant properties (Lee and Choi, 2008). The aim of the present study was to evaluate the antioxidant effect of myricetin on markers of oxidative stress in type 2 diabetic erythrocytes subjected to *in vitro* oxidative stress.

Material and Methods

Selection of subjects

The criteria for selection of type 2 diabetic patients were the same as reported earlier (Rizvi *et al.*, 2005). Briefly, blood from 23 diabetic patients (13 men, 10 women) was taken after informed consent has been obtained from all patients. The mean age was (58 ± 7) years, fasting plasma glucose level was (183.5 ± 42.4) mg/dL, body mass index (BMI) was (27 ± 4) kg/m², total plasma cholesterol was (5.4 ± 1.3) mmol/L, and duration of diabetes was (12 ± 5) years. None of the patients had high blood pressure or microalbuminuria. Care was also taken to exclude patients who had a family history of hypertension.

The control group consisted of 23 healthy volunteers, age and sex matched with diabetic subjects. The mean age was (56 ± 8) years, fasting plasma glucose level was (85.2 ± 14.4) mg/dL, BMI was (24.8 ± 3.8) kg/m², and total plasma cholesterol was (5.3 ± 1.3) mmol/L. None of the controls was affected by hypertension. Care was taken to select control subjects with no family history of diabetes mellitus or hypertension (two generations). None of the women studied was receiving any hormonal treatment. All volunteers (diabetic patients and normal subjects) were informed about the nature of the study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Collection of blood, isolation of packed red blood cells and preparation of ghosts

Venous blood was collected from control and type 2 diabetic patients after an overnight fast using ACD (citric acid/sodium citrate/dextrose) as anticoagulant. The blood samples were kept at 37 °C for 3 h prior to experiments for degrada-

tion of endogenous insulin. The blood samples were centrifuged at 4 °C for 10 min at $1000 \times g$ to remove plasma and buffy coat, and the isolated erythrocytes were washed four to five times with 0.154 M NaCl and; finally, packed erythrocytes were obtained. The erythrocyte membrane from leukocyte-free red cells was prepared following the method of Marchesi and Palade (1967), that involves the principle of osmotic shock treatment with hypotonic and hypertonic buffers (pH 7.4). The erythrocyte membrane protein content was determined by the method of Lowry *et al.* (1951), using bovine serum albumin as standard.

Determination of lipid peroxidation

Erythrocyte lipid peroxidation was measured in terms of malondialdehyde (MDA), which was estimated according to the method of Esterbauer and Cheeseman (1990). Packed erythrocytes (0.2 mL) were suspended in 3 mL Krebs-Ringer phosphate buffer (KRPB), pH 7.4. The lysate (1 mL) was added to 1 mL of 10% trichloroacetic acid (TCA) and the mixture was centrifuged for 5 min at $1000 \times g$. The supernatant (1 mL) was added to 1 mL of 0.67% thiobarbituric acid (TBA) in 0.05 M NaOH and boiled for 20 min at a temperature higher than 90 °C. The solution was cooled and read against a complementary blank at 532 nm (OD1) and 600 nm (OD2). The net optical density (OD) was calculated after subtracting the absorbance at OD2 from that at OD1. The concentration of MDA in erythrocytes was determined from a standard plot and expressed as nmol/mL of packed erythrocytes (packed red blood cells, PRBC).

Determination of erythrocyte membrane protein oxidation

Erythrocyte membrane protein oxidation was measured in terms of carbonyls, which was estimated according to the procedure of Levine *et al.* (1990). 0.2 mL of erythrocyte membrane samples in phosphate buffered saline (PBS) were taken in two tubes, as test and control. 4.0 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 M HCl were added to the test sample, and 4.0 mL of 2 M HCl, alone, was added to the control sample. The contents were mixed thoroughly and incubated for 1 h in the dark at 37 °C. The tubes were shaken intermittently every 10 min to facilitate the reaction with proteins. After that, 20% TCA (w/v)

was added to both tubes and the mixtures left on ice for 10 min. The tubes were then centrifuged at $1000 \times g$ for 20 min to obtain the protein pellets. The supernatant was carefully aspirated and discarded. The protein pellets were washed three times with ethanol ethyl acetate (1:1, v/v) solution to remove unreacted DNPH and lipid remnants. Finally, protein pellets were dissolved in 6 M guanidine hydrochloride and incubated for 10 min at 37 °C. The insoluble materials were removed by centrifugation. The carbonyl content was determined by taking the spectra of the supernatant at 370 nm. Each sample was read against the control. The carbonyl content was calculated using an absorption coefficient (ϵ) of $22,000 \text{ M}^{-1} \text{ cm}^{-1}$ and data was expressed in nmol/mg protein.

Induction of oxidative stress

Oxidative stress was induced *in vitro* by incubating washed erythrocytes/erythrocyte ghosts with 10^{-5} M *tert*-butyl hydroperoxide (t-BHP) with or without myricetin. The concentration of t-BHP used in the present study to induce oxidative stress in erythrocytes was in the range of concentrations used in a previously published study (Pandey *et al.*, 2009).

In vitro experiments with myricetin

Washed erythrocytes were suspended in 4 volumes of PBS containing 5 mmol/L glucose (pH 7.4). *In vitro* effects were evaluated by incubating the erythrocytes in the presence of different doses of myricetin at 37 °C for 60 min. After this time, the suspensions were immediately centrifuged at $1800 \times g$, the red blood cells (RBC) were washed twice with at least 50 volumes of PBS and then subjected to assay the MDA content.

For the protein carbonyl group estimation, erythrocyte ghosts (0.8–1.5 mg of protein) were incubated with myricetin at different doses in PBS (pH 7.4) for 1 h at 37 °C prior to the estimation of membrane protein carbonyls. Parallel control experiments were also performed in which myricetin was replaced with an equal amount of solvent.

Blood glucose values were determined by Ames Glucometer GX (Miles, India). Statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA). Data are expressed as mean \pm SD.

Results and Discussion

An increased reactive oxygen species (ROS) production is believed to mediate tissue injury in a wide range of diseases including diabetes. ROS generated during metabolism can enter into reactions that, when uncontrolled, can affect certain processes leading to clinical manifestations. The erythrocyte membrane is prone to lipid peroxidation under oxidative stress that involves cleavage of polyunsaturated fatty acids at their double bonds leading to the formation of MDA, frequently used to determine the oxidant/antioxidant balance in diabetic patients. The attack of ROS against proteins modifies amino acid (lysine, arginine, proline, and histidine) residues generating carbonyl moieties, which has been identified as an early marker for protein oxidation and is used as a measure of protein damage (Levine *et al.*, 1990).

Our results showed that diabetic erythrocytes have higher levels of oxidative stress. These cells have higher MDA and protein carbonyl contents, both are markers of oxidative stress (Figs. 2 and 3). Earlier reports also showed a significantly higher MDA content in diabetic erythrocytes in comparison to the control, explained to be due to increased oxidative stress in diabetic erythrocytes (Rizvi *et al.*, 2005). In another study we have reported that diabetic erythrocytes show greater susceptibility to oxidation when subjected to *in vitro* oxidative stress by incubating with t-BHP (Rizvi and Mishra, 2009). Increased erythrocyte MDA concentrations are known to cause a decrease in the membrane fluidity of the membrane lipid bilayer and increased osmotic stability of cells. It seems likely that oxidation processes and MDA accumulation can contribute directly to changes in the properties of diabetic erythrocytes and may cause the development of long-term complications. Oxidative modification of proteins, considering their multiple functions, can be selective and specific. The use of protein carbonyls as index of oxidative stress has some advantages in comparison with the measurement of other oxidation products because of the relative early formation and the relative stability of carbonylated proteins (Dalle-Donne *et al.*, 2003).

In our experiments, we observed that, upon incubation with t-BHP, the levels of MDA and protein carbonyls increase in both normal and diabetic erythrocytes. Myricetin significantly protected the erythrocyte membrane lipid peroxidation and

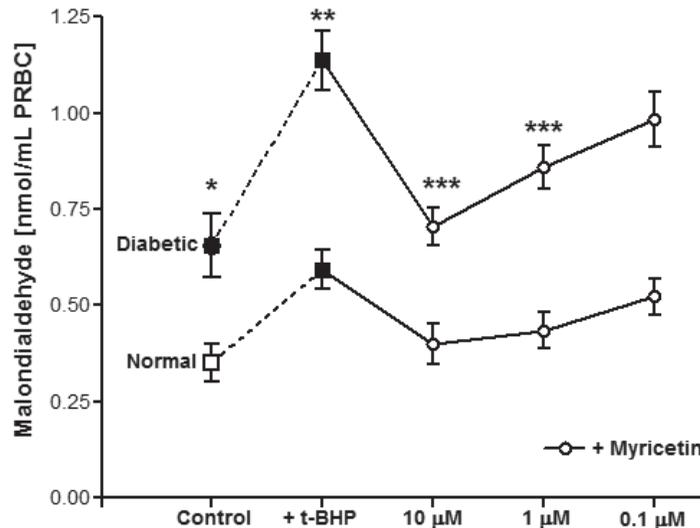


Fig. 2. Dose-dependent effect of myricetin on *tert*-butyl hydroperoxide (t-BHP)-induced changes of the MDA level in type 2 diabetic erythrocytes and control (normal) subjects. There was an increase in the MDA level in diabetic erythrocytes as compared with normal (* $p < 0.005$). Treatment with t-BHP showed an elevated MDA content (** $p < 0.001$, compared with diabetic control). Incubation with myricetin showed significant protection against an increased level of MDA at different concentrations above $0.1 \mu\text{M}$ (***) $p < 0.01$ with respect to t-BHP-treated diabetic erythrocytes). The MDA level is expressed as nmol/mL of packed red blood cells (PRBC).

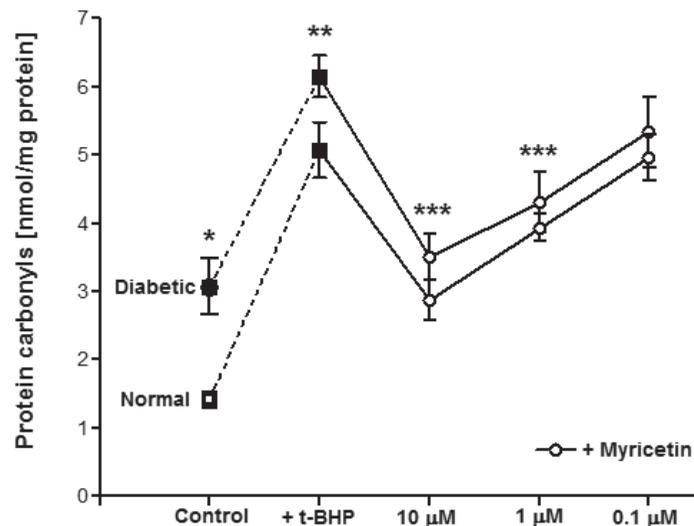


Fig. 3. Dose-dependent effect of myricetin on *tert*-butyl hydroperoxide (t-BHP)-induced changes of the membrane protein carbonyl group content in type 2 diabetic erythrocytes and control (normal) subjects. There was an increase in the protein carbonyl content in diabetic erythrocytes as compared with normal (* $p < 0.001$). Treatment with t-BHP showed an elevated carbonyl content (** $p < 0.0001$, compared with diabetic control). Incubation with myricetin showed significant protection against an increased level of the protein carbonyl group content at different concentrations above $0.1 \mu\text{M}$ (***) $p < 0.01$ with respect to t-BHP-treated diabetic erythrocytes). The carbonyl content is expressed in nmol/mg protein.

protein oxidation subjected to oxidative stress, as evidenced by a decrease in the MDA level and protein carbonyl content (Figs. 2 and 3); the effect of myricetin was higher in diabetic erythrocytes compared to normal. We have also found that the effect of myricetin was dose-dependent; higher protection was observed at increasing concentration. The effect of myricetin at micromolar concentration seems to be relevant because in most of the studies its bioavailability has been reported in the micromolar range (Williamson *et al.*, 2005). Our results are also supported by studies of Knekt *et al.* (2002), in which they have documented that the intake of some specific types of flavonoids including quercetin and myricetin was inversely associated with the risk of incidence of type 2 diabetes. Our present results on the protec-

tive effect of myricetin on diabetic erythrocytes substantiate the antioxidant effect of myricetin on the human cellular system and add another point to the health beneficial effects of myricetin.

In conclusion, we showed that myricetin has a strong antioxidant effect, helpful in alleviating oxidative stress and thereby protecting against several complications of diabetes resulting from increased oxidative stress. Since myricetin naturally occurs in different plant sources, intake of a diet rich in myricetin may provide some protection against the development of long-term diabetic complications.

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