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Myricetin can mitigate altered redox status in type 2 diabetic erythrocytes

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ABSTRACT

Several complications of diabetes are known to be associated with increased oxidative stress. Decreased erythrocyte anti-oxidative capacity in type 2 diabetes has been shown to be correlated with diabetic complications. The present study was undertaken to evaluate the protective effects of myricetin on altered cellular redox parameters; reduced glutathione (GSH) and membrane sulphydryl group (-SH) of human erythrocytes in type 2 diabetes. We observe a decreased intracellular GSH (30%) and membrane -SH content (49%) in type 2 diabetic erythrocytes. Incubation with myricetin (0.1μM to 10μM) caused increase in GSH and membrane -SH group content in type 2 diabetic erythrocytes, an effect that was concentration dependent. The effect of myricetin in maintaining the integrity of membrane -SH groups may be due to activation of plasma membrane redox system activity. Since myricetin in present in high concentration in several plant sources, a high intake of such food sources may augment antioxidant defence in type 2 diabetes and may delay the development of diabetic complications.

Keywords: Erythrocyte, diabetes mellitus, oxidative stress, myricetin.

INTRODUCTION

Complications from diabetes, such as coronary artery and peripheral vascular disease, stroke, diabetic neuropathy, amputations, renal failure and blindness, result in increasing disability, reduced life expectancy and enormous health costs (Greene & Lattimer. 1986). Genetic susceptibility to type 2 diabetes coupled with lifestyle changes, is a major factor for the growing incidence of diabetes in Asian countries (Ramachandran *et al.* 1999). Although little can be done to avert genetic susceptibility; efforts can be made to delay/prevent the development of diabetic complications.

Many *in vitro* and *in vivo* studies have demonstrated that several parameters of erythrocyte function and integrity are negatively affected by increased oxidative stress. In fact, changes in membrane fluidity and inactivation of

membrane-bound receptors and enzymes (Halliwell & Gutteridge. 1986), ionic parameters (Rizvi & Zaid. 2005), an increase in lipid peroxidation, oxidation of glutathione and protein sulphydryl group (Rizvi *et al.* 2005) and activation of proteolysis (Davies *et al.* 1987) have all been described following the application of oxidative stress to erythrocytes. Decreased erythrocyte anti-oxidative capacity in non-insulin-dependent diabetes mellitus has been shown to be correlated with several diabetic complications (Baynes 1991).

Several studies have emphasised the importance of anti-oxidants in diabetes and low levels of plasma anti-oxidants have been implicated as a risk factor for the development of the disease (Facchini *et al.* 2000). A rational extension of the proposed role for oxidative stress is the suggestion that the different susceptibility of diabetic patients to micro- and macrovascular complications may be a function of the endogenous antioxidant status (Baynes 1991).

Flavonoids are a group of naturally occurring polyphenolic compounds primarily from fruits and vegetables (Hollman *et al.* 1999). Several small dietary intervention trials have shown that consumption of flavonoid-rich foods is associated with a significant increase in plasma antioxidant level in diabetic patients (Hollman *et al.* 1999-b, Lean *et al.* 1999). Myricetin (3, 3', 4', 5, 5', 7-hexahydroxyflavone) is a natural flavonoid ubiquitously present in foods including vegetables, fruits, tea and wine, myricetin possesses many beneficial health effects including anticarcinogenic, antimutagenic and anti-inflammatory (Ong & Khoo. 1997). Many of the biological actions of this flavonoid have been attributed to be due to its antioxidant properties (Teissedre *et al.*1996, Lee & Choi. 2008).

The present study was undertaken to evaluate the protective effects of myricetin on altered cellular redox parameters; reduced glutathione (GSH) and membrane sulphydryl group (-SH) of human erythrocytes in type 2 diabetes.

MATERIALS AND METHODS

Selection of subjects

The criteria for selection of type 2 diabetic patients were same as reported earlier (Rizvi *et al.* 2005). Blood from 23 diabetic patients (13 men, 10 women) was taken after informed consent has been obtained from all patients, mean age 58 $\pm$ 7 years, fasting plasma glucose level 183.5 $\pm$ 42.4 mg/dL, BMI 27 $\pm$ 4 kg/m^2, total plama cholesterol 5.4 $\pm$ 1.3 mmol/L and duration of diabetes was 12 $\pm$ 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, mean age 56 ± 8 years, fasting plasma glucose level 85.2 ± 14.4 mg/dL, BMI 24.8 ± 3.8 kg/m^2, and total plasma cholesterol 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 generation). None of the women studied was receiving any hormonal treatment. All volunteers (diabetic patients and normal subjects) were informed about the nature of study. The protocol of study was in conformity with the guidelines of the Institutional Ethical Committee.

Collection of blood, isolation of packed RBC 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 sample was kept at 37°C for 3 hours prior to experiments for degradation of endogenous insulin. The blood samples were centrifuged at 4°C for 10 min. at 1000 xg to remove plasma and buffy coat and the isolated erythrocytes were washed 4 to 5 times with 0.154 mol/L NaCl and finally packed erythrocyte was obtained. The erythrocyte membrane from leukocyte free red cells were prepared following the method of Marchesi & 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 BSA as standard.

Determination of erythrocyte GSH and membrane -SH group content:

Erythrocyte GSH was measured following the method of Beutler *et al.* (1963) and membrane-bound -SH group was estimated according to Kitajima's method (1990). Both methods were based on the ability of the SH group to reduce 5, 5'-dithiobis, 2-nitrobenzoic acid (DTNB) and form a yellow colored anionic product whose OD is measured at 412 nm. The concentration of GSH is expressed in mg per mL packed RBCs and was determined from a standard plot. The concentration of the -SH group is expressed as nmol/mg protein.

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 myricetin at different doses (from 0.1M to 10M) at 37°C for 60 min. After this time, the suspensions were immediately centrifuged at 1800 xg, the RBC were washed twice with at least 50 volumes of PBS and then subjected to assay GSH content. For -SH group estimation, erythrocyte ghosts

(0.8-1.5 mg of protein) were incubated with the myricetin at different doses in PBS (pH 7.4) for 1 h at 37°C prior to the estimation of membrane -SH group. Parallel control experiments were also performed in which myricetin was replaced with an equal amount of solvent (DMSO, 0.1%).

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 California USA. Data are expressed in mean $\pm$SD and analysed by student t-test. $P < 0.05$ is considered as significant.

RESULTS AND DISCUSSION

In diabetes mellitus, chronic hyperglycaemia produces multiple biochemical sequelae and diabetes-induced oxidative stress could play a role in the onset and progression of disease. Many of the complications of diabetes, including retinopathy and atherosclerotic vascular disease, the leading cause of mortality in diabetes, have been linked to oxidative stress (Baynes 1991).

We observe a decreased erythrocyte GSH (30%) in type 2 diabetes (figure 1). Reduced glutathione is a non-protein sulphydryl compound and plays an important role in the intracellular defence system of the body. GSH has many biological functions including protection of membrane lipids against peroxidation and maintenance of membrane protein -SH groups in the reduced form, the oxidation of which can otherwise cause altered cellular structure and function and (Meister 1994, Rizvi & Maurya. 2007).

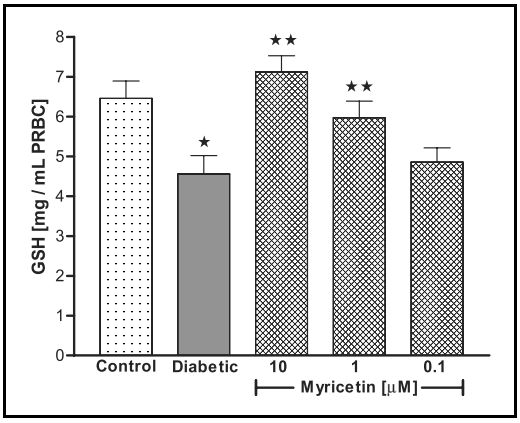

Figure-1: Dose dependent effect of myricetin on GSH content in diabetic erythrocytes. There was a decrease in GSH content in diabetic erythrocytes as compared with control (*$p < 0.005$). Treatment with myricetin showed significant protection against lowered status of GSH at concentration above 0.1M (**$p < 0.05$, with respect to diabetic alone). GSH content is expressed in mg/mL PRBC.

The human erythrocyte is an easily accessible cell type that is rich in sulphydryl functions: the importance of the erythrocyte membrane -SH group to overall cellular redox balance has been emphasised (Reglinski *et al.* 1988). Membrane oxidative damage has a considerable effect upon membrane mechanical properties. Membrane -SH group oxidative damage may be an important molecular mechanism inducing changes in the membrane microelasticity or whole cell deformability of erythrocytes under conditions of physiological and pathological oxidative stress (Wang *et al.* 1999). Previously we have reported the decreased level of both GSH and -SH group in diabetic erythrocytes as compared to normal (Rizvi *et al.* 2005). In the present study we also show a decreased erythrocyte membrane -SH content (49%) in type 2 diabetes (figure 2).

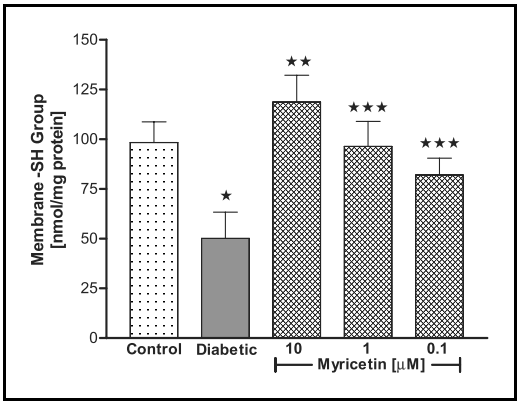

Figure-2: Dose dependent effect of myricetin on -SH group content in diabetic erythrocyte membrane. There was a decrease in membrane -SH content ($*p < 0.01$) in diabetic erythrocytes as compared with control. Treatment with myricetin showed significant protection at different concentrations ($**p < 0.001$, $***p < 0.05$, with respect to diabetic alone).
Membrane -SH group content is expressed in nmol/mg protein.

Decreased levels of GSH in diabetics may be caused by decreased reduction of oxidized glutathione catalysed by glutathione reductase as a consequence of NADPH depletion owing to an up regulated polyol pathway. Decreased GSH content may predispose the cell to a lower defence against the condition of oxidative stress during diabetes. It has also been suggested that diabetic complications may be the result of a long-term effect of small deficiencies in intracellular GSH (Coleman & Rustiom. 1999). In our experiments incubation with myricetin (at micromolar concentrations) significantly recovers the altered intracellular redox status in type 2 diabetic erythrocytes, as evidenced by increased GSH and membrane -SH group contents (figure 1 and 2).

The effect of myricetin was dose dependent; at 10μM final concentration it significantly increased the GSH level (56%) and at 1μM final concentration the increase was 31% when compared with basal diabetic erythrocyte GSH level. No significant effect was observed at 0.1μM final concentration. Myricetin also caused an increase in erythrocyte membrane -SH group content. At myricetin concentrations of 10μM, 1μM and 0.1μM a 136%, 92% and 63% elevation in -SH was observed, as compared to basal diabetic level. Our results assume significance because the bioavailability of flavonoids has been reported in micromolar range (Scalbert & Williamson. 2000, Williamson *et al.* 2005).

Various studies advocate the beneficial health effects of plant polyphenols (Knekt *et al.*2002). Myrecitin is reported to be a strong inhibitor of the human P form phenolsulphotransferase, suggesting its potential use for clinically important drug interactions and as a chemopreventive agent in sulphation-induced carcinogenesis (Eaton *et al.* 1996). Indeed, myricetin has been shown to be a potent anti - mutagen and also has an inhibitory effect on platelet aggregation (Tzeng *et al.* 1991). Possible mechanism by which myricetin exerts these beneficial effects is thought to be its antioxidant activity (Ong & Khoo. 1997, Yang *et al.* 2001). 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 were inversely associated with the risk of incidence of type 2 diabetes.

Dietary antioxidants have attracted wide interest as potent agents that may protect β-cells from free radical-mediated damage, and thus slow progressive β-cell dysfunction. There is evidence to suggest that hyperinsulinemia and postprandial hyperglycemia can elicit oxidative stress by increasing reactive oxygen species (ROS) production and reducing intracellular antioxidant defence (West 2000, Song *et al.* 2005). Further, both *in vitro* and *in vivo* studies show that oxidative stress generation impair pancreatic β-cell insulin secretion and interfere with insulin signalling pathway, thereby accelerating the progression to overt type II diabetes from insulin resistance (Robertson *et al.* 2003, Song *et al.* 2005). Our results substantiate the antioxidant effect of myricetin on human cellular system.

Myricetin has also been shown to penetrate erythrocytes; it has been shown to prevent glutathione depletion induced by dehydroascorbic acid in rabbit red blood cells. While most flavonoids are known to display antioxidant property, only myricetin quercetin and fisetin (flavonoids in which the B ring is combined with 2, 3 double bond and 4-oxo function of the C ring) have the ability to act as intracellular substrates for the plasma membrane redox system (PMRS) activity. The PMRS in erythrocytes represents a mechanism for cell-dependent reduction of extracellular oxidants and is an important process used by the erythrocytes to

maintain the redox status of the plasma (Fiorani *et al.* 2005). One of the important functions of human erythrocyte PMRS is to maintain membrane -SH groups in reduced form (VanDuijn *et al.* 1998), we hypothesize that myricetin protects membrane -SH groups through a mechanism which involves activation of erythrocyte PMRS activity. The involvement of erythrocyte PMRS activity in modulating redox balance of the plasma has already been shown (Rizvi *et al.* 2006). Since myricetin in present in high concentration in several plant sources, a high intake of such food sources may augment antioxidant defence in type 2 diabetes and may delay the development of diabetic complications.

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ميريستن قادر على تخفيف التغير في حالة الأكسدة والاختزال في كريات الدم الحمراء في مرض السكر من النوع الثاني

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خلاصة

من المعروف أن مضاعفات مرض السكر ترتبط بزيادة الإجهاد المؤكسد. إن النقصان في سعة erythrocyte المضاد للأكسدة في النوع الثاني من السكر قد أظهر مضاهاه مع التعقيدات السكرية. لقد أجريت الدراسة الحالية لتقييم تأثير الـ myricetin على عناصر خلايا الاختزال – المؤكسدة، وتخفيض glutathione (GSH) وغشاء مجموعة sulphydral (-SH) لإنسان erythrocyte في النوع الثاني من السكر. لقد تمت ملاحظة نقصان 30٪ من الوسط الخلوي (GSH) و49٪ من محتوى أنسجة (-SH) في النوع الثاني لمرض السكر.

erythrocyte وبإحتضان myricetin بتركيز (0.1 μM to 10 μM) أدى إلى الزيادة في محتوى GSH ومجموعة أنسجة (-SH) في النوع الثاني من السكر Erythrocyte كتأثير يعتمد على التركيز. وبما أن myricetin يوجد بتركيز عالي في مصادر عدة نباتات، فإن استخدام كمية عالية من مصادر الغذاء هذه قد تدمج دفاع المضاد للتأكسد في مرض السكر النوع الثاني ومن الممكن تأخير تطوير التعقيدات السكرية.