Protective effect of flavonoids against red blood cell hemolysis by free radicals

S Asgary PhD, GH Naderi PhD, N Askari MS

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BACKGROUND: Flavonoids are polyphenolic substances with antioxidant properties, and they are found in different vegetables and fruits. Epidemiological studies have shown that the consumption of flavonoids reduces the prevalence of cardiovascular diseases. The use of synthetic antioxidants, however, has been limited because of their toxicity. Therefore, medical researchers have intensified their quest to find natural antioxidants.

OBJECTIVES: To investigate the effect of several pure flavonoids, such as kaempferol, quercetin, morin and rutin, on red blood cell hemolysis and evaluate their -SH capacity as an indicator of membrane protection.

METHODS: The rate of hemolysis and cell membrane -SH capacity were determined by spectrophotometry. Red blood cell peroxidation was induced using 2,2′-azo-bis-(2-amidinopropane) dihydrochloride. The effect of each flavonoid on hemolysis was examined at three concentrations (0.5 µg/mL, 5 µg/mL and 10 µg/mL), however, only the greatest concentration (10 µg/mL) of each flavonoid was used to study the effect on -SH groups.

RESULTS: In all cases, the antioxidant activity was dose-dependent. Rutin showed the highest inhibitory effect on hemolysis among flavonoids (42.5%). The protective effect of kaempferol, rutin and morin against -SH group oxidation measured 7.7%, 23.3% and 26.4%, respectively.

CONCLUSIONS: Results showed that flavonoids and flavonoid-containing plants can be used as natural antioxidants for the treatment and prevention of disease conditions, the pathogenesis of which is mediated by lipid peroxidation.

Key Words: *Antioxidant; Kaempferol; Morin; Quercetin; RBC; Rutin; -SH capacity*

Flavonoids are polyphenolic compounds present in all foods of plant origin. They have various effects on mammalian cellular systems and structures (1), and have been shown to protect biological membranes against free radical-induced oxidative damage (2). Additionally, flavonoids have inhibitory effects on the functions of platelets and leukocytes. They also protect endothelial cells, and counterbalance the interactions between the blood stream and vascular wall, which may lead to thrombosis. The latter effect is mediated through the effect of flavonoids on human monocyte tissue factor, which itself may trigger blood coagulation (3).

The strong antioxidant effects of flavonoids have been highlighted by several studies (2-7). Flavonoids may have therapeutic effects on disease conditions caused by oxidative stress, such as coronary atherosclerosis, ischemic damage, diabetes mellitus, aging processes and cancer (5). Flavonoids reduce the risk of developing ischemic heart disease and coronary atherosclerosis by inhibiting low-density lipoprotein oxidation (2,6,7).

Red blood cell (RBC) membranes contain lipids rich in unsaturated fatty acids. RBCs are more frequently exposed to oxygen than other body tissue and, thus, are more susceptible to oxidative damage. Invasion of the RBC membrane by peroxidants may lead to cell hemolysis. Moreover, the hemoglobin in RBCs is a strong catalyst which may initiate lipid peroxidation.

In addition to lipid peroxidation, oxidants affect vital -SH groups of proteins which are highly active and may be targeted during oxidative stress (5). Reduced glutathione levels lead to a decrease in -SH groups (8). Glutathione directly protects membrane proteins and preserves their stability. Decreased levels of glutathione result in oxidization of membrane -SH groups and loss of membrane stability (9).

MATERIALS AND METHODS

Preparation of flavonoid solutions

Stock solutions (1 µg/mL) of flavonoids (ie, rutin, quercetin, kaempferol and morin) were prepared. Rutin, quercetin and kaempferol were purchased from Merck (Germany), and morin from Sigma (Germany). Solutions of each flavonoid were separately prepared in DMSO.

Preparation of globular suspensions

Blood samples from healthy volunteers were obtained in heparinized tubes, and the RBC content was isolated by centrifugation (3000 rpm for 10 min) and, subsequently, washed three times with 0.9% saline. The tubes were then centrifuged at 12,000 g for 10 min to obtain packed cells (10).

RBC hemolysis

Flavonoid solutions (0.2 mg/mL, 0.1 mg/mL and 0.01 mg/mL) dissolved in DMSO were prepared and the study was conducted on test and control groups. Each flavonoid solution (0.1 mL) was introduced into a separate test tube in the trial group, and 0.8 mL phosphate buffered saline and 0.1 mL of the globular suspension were

Isfahan Cardiovascular Research Center, Isfahan University of Medical Sciences, Isfahan, Iran
Correspondence: Dr S Asgary, Isfahan Cardiovascular Research Center, PO Box 81465—1148, Isfahan, Iran. Telephone 98-311-335-9090 or 98-311-335-9696, fax 98-311-337-3435, e-mail s_asgary@hotmail.com

TABLE 1
Effect of flavonoids on 2,2′-azo-bis-(2-amidinopropane) dihydrochloride-induced red blood cell hemolysis

Flavonoid	Concentration (µg/mL)	Hemolysis inhibition (%)
Kaempferol	10	26.9
	5	11.2
	0.5	0.6
Quercetin	10	35.5
	5	16.7
	0.5	7.1
Morin	10	40.5
	5	28.4
	0.5	21.1
Rutin	10	42.5
	5	34.6
	0.5	23.4

All results were statistically significant (P<0.05)

added to tubes of both groups. After adding 1 mL 2-2′-azo-bis-(2-amidinopropane) dihydrochloride (AAPH) (25 mM), the tubes were incubated for 2 h at 37°C and then centrifuged (3000 rpm for 10 min). The absorption of the supernatant solution at 540 nm was measured using a UV-3100 spectrophotometer (Shimadzu, Japan) (11).

-SH group measurement per milligram of protein

An RBC membrane suspension was prepared and the protective effect of flavonoids on -SH groups was assessed both in the presence and absence of tetrathionate (an -SH group inhibitor).

Flavonoid solution was added to a predetermined volume of membrane suspension, centrifuged at 3000 rpm for 10 min and washed with phosphate buffered saline. Tetrathionate was subsequently added to membrane suspensions. The membrane suspensions were incubated for 30 min at 37°C. To measure the protection of -SH groups by flavonoids, sodium dodecylsulfate (10%) was added to the membranes and 5,5′-dithio-bis-(2-nitrobenzoic acid) (1 mM) was used as an -SH group reagent.

Absorption was measured at 412 nm after incubation for 1 h at 37°C in the presence of reduced glutathione (12).

To measure -SH group content per milligram of protein, protein concentrations were determined using the Lowry method (13).

All tests were repeated four times. Results were calculated and expressed as mean ± SD, and interpreted using Student's *t* test.

RESULTS

The results showed inhibition of hemolysis in the presence of kaempferol, quercetin, morin and rutin. Inhibition of hemolysis was dose dependent. Hemolysis was inhibited by 26.9%, 35.5%, 40.5% and 42.5% at the greatest flavonoid concentrations (10 µg/mL) of kaempferol, quercetin, morin and rutin, respectively. Results were considered statistically significant at P<0.05. Inhibition of hemolysis was also observed at lower concentrations (Table 1). The protective effects of flavonoids against hemolysis decreased in a dose-dependent manner.

As shown in Table 2, flavonoids led to an increased -SH capacity. The effects of morin and rutin were particularly notable.

Morin, rutin and kaempferol protected -SH groups against tetrathionate by 26.4%, 23.3% and 7.7%, respectively.

TABLE 2
Effect of flavonoids on -SH capacity of red blood cell membranes

	Protein (µmol/mg)	-SH capacity (%)
Control (without tetrathionate)	507±22	100
Tetrathionate	296±15	58.4
Tetrathionate and morin	430±14	84.8
Tetrathionate and kaempferol	235±15	66.1
Tetrathionate and quercetin	295±12	58.2
Tetrathionate and rutin	414±9	81.7

The effect of the flavonoids was tested at a concentration of 10 µg/mL. All tests were repeated four times. All results were statistically significant (P<0.05)

DISCUSSION

In the present study, the antioxidant effect of pure flavonoids in the presence of AAPH was studied. Rutin resulted in 42.5% inhibition of hemolysis at a concentration of 10 µg/mL, demonstrating the highest percentage of hemolysis inhibition among the tested flavonoids. Hemolysis was decreased in a dose-dependent manner in the presence of flavonoids. Rutin attracts superoxide radicals and chelates iron. Studies of RBC-rutin reactions have shown that rutin cannot prevent membrane damage and hemolysis by primaquine (8), but it can significantly impede the oxidation of hemoglobin and its conversion to methemoglobin.

Rutin's exact mechanism of action has yet to be determined. It is still unknown whether rutin can react with intracellular oxidants and prevent hemoglobin oxidation (8). Rutin has also displayed an array of other pharmacological features, such as decreasing capillary permeability and fragility, anti-inflammatory and antitumour properties, and protection against x-rays in mice, all of which are consistent with its antioxidant properties (14). Studies of the effects of flavonoids have shown that quercetin, rutin and kaempferol inhibit glycosylation by 52%, 37% and 15%, respectively. This is an important effect, in view of the results of studies which show that flavonoids are stored in RBCs (15).

In the present study of the antioxidant effect of pure flavonoids, morin inhibited hemolysis by 40.5% at the highest tested concentration (10 µg/mL), and the rate of inhibition decreased at lower concentrations. In another study, it was shown that morin can inhibit hemolysis by as much as 50% at a concentration of 100 µg/mL (5), a result also borne out in the present study.

Pure quercetin inhibited hemolysis by 35.5% at the highest concentration (10 µg/mL), ranking third among the flavonoids tested for hemolysis inhibition. As shown in Table 1, quercetin decreased hemolysis in a dose-dependent manner. This was also shown by Kitagawa et al (2). Additionally, it has been shown that in the absence of AAPH, the relatively hydrophobic flavonoids, quercetin and morin, induce the oxidation of oxyhemoglobin to methemoglobin; however, this oxidation did not induce hemolysis (2). Several studies have referred to quercetin as an antioxidant capable of preventing low-density lipoprotein oxidation and platelet aggregation, crucial factors that prevent the formation of atherosclerotic plaques which predispose individuals to myocardial infarction (5).

Results of the present study reaffirmed the antioxidant properties of quercetin. Kaempferol inhibited lipid peroxidation and hemolysis by 26.9% at the greatest concentration (10 µg/mL). Decreased kaempferol concentrations led to lower rates of hemolysis inhibition.

Other studies have shown oral consumption of bioflavonoid antioxidants in animals to be effective in preventing oxidative stresses which damage RBCs (16). Earlier studies have demonstrated that AAPH-induced hemolysis in RBCs is effectively inhibited by natural antioxidants (17).

Results of the present study showed a direct relationship between the concentration of the tested flavonoid and its antioxidant effect. Given that these effects were also observed at acceptable levels at much lower flavonoid concentrations, it may be possible to use them in the clinical setting with minimal side effects (once in vivo and toxicology tests have been performed). Protein -SH groups play a vital role in preserving cell membrane stability. Under oxidative stress, -SH groups protect cellular structures against free radicals by undergoing oxidization and forming disulfide bonds (7). If antioxidant compounds prove effective in protecting -SH groups against oxidization, they are likely to increase cellular resistance to oxidative stress. The results of the present study showed that morin, kaempferol and quercetin increased -SH groups.

In light of the results of the present and similar studies on flavonoids, these substances may afford beneficial effects in preventing oxidative damage to membranes. They may also be useful in preventing or treating other disease conditions in which lipid peroxidation plays a role. Further studies are required to find new antioxidant substances and assess their effects and mechanisms of actions.

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