The Effect of Pycnogenol® on the Erythrocyte Membrane Fluidity

M. Sivoňová,[1] I. Waczulíková,[2] E. Kilanczyk,[3] M. Hrnčiarová,[1]
M. Bryszewska,[3] B. Klajnert[3] and Z. Ďuračková[1]

[1] *Department of Medical Chemistry, Biochemistry and Clinical Chemistry, Comenius University, Faculty of Medicine, Bratislava, Slovakia*
[2] *Department of Biophysics and Chemical Physics, Comenius University, Faculty of Mathematics, Physics and Computer Science, Mlynská dolina F1, 842 48 Bratislava 4, Slovakia*
[3] *Department of General Biophysics, University of Lodź, Banacha 12/16, 90-237 Lodź, Poland*

Abstract. In the present study, the *in vitro* effect of polyphenol rich plant extract, flavonoid – Pycnogenol® (Pyc), on erythrocyte membrane fluidity was studied. Membrane fluidity was determined using 1-[4-trimethyl-aminophenyl]-6-phenyl-1, 3,5-hexatriene (TMA-DPH), 1,6-diphenyl-1,3,5-hexatriene (DPH) and 12-(9-anthroyloxy) stearic acid (12-AS) fluorescence anisotropy. After Pyc action (50 μg/ml to 300 μg/ml), we observed decreases in the anisotropy values of TMA-DPH and DPH in a dose-dependent manner compared with the untreated erythrocyte membranes. Pyc significantly increased the membrane fluidity predominantly at the membrane surface. Further, we observed the protective effect of Pyc against lipid peroxidation, TBARP generation and oxidative hemolysis induced by H_2O_2. Pyc can reduce the lipid peroxidation and oxidative hemolysis either by quenching free radicals or by chelating metal ions, or by both. The exact mechanism(s) of the positive effect of Pyc is not known. We assume that Pyc efficacy to modify effectively some membrane dependent processes is related not only to the chemical action of Pyc but also to its ability to interact directly with cell membranes and/or penetrate the membrane thus inducing modification of the lipid bilayer and lipid-protein interactions.

Key words: Flavonoids — Pycnogenol® — Erythrocyte membrane fluidity — Fluorescence anisotropy — Lipid peroxidation — Oxidative hemolysis

Introduction

The balance between oxidants and antioxidants in human organism may be disturbed by depletion of antioxidants. It is known that oxidative stress is associated

Correspondence to: Zdeňka Ďuračková, Comenius University, Faculty of Medicine, Sasinkova 2, 813 72 Bratislava 1, Slovakia. E-mail: zdenka.durackova@fmed.uniba.sk

with diseases such as diabetes mellitus, atherosclerosis, ischemia, cancer, Down syndrom etc. (Ďuračková 1998). Oxidative stress may be assessed in addition to the determination of the products of oxidative damage to important biomolecules, also by analysis of antioxidants profile (Polidori et al. 2001). The antioxidant defence system represents a complex network with interactions, synergism, antagonism and specific tasks of the antioxidants.

For the studies of oxidative damage pathophysiology, erythrocyte membranes are often used (as a model system) because of their simplicity and availability. Oxygen radicals formed during oxidative stress may promote the oxidation of polyunsaturated fatty acids that are present in high concentrations within cell membranes. Lipid peroxidation causes polymerisation of the membrane components, their crosslinking and/or fragmentation (Waczulíková et al. 2000). This damage to the membrane leads to the alterations in the membrane fluidity and cell deformability (Piasecka et al. 2000).

Recently, a growing interest has been seen in the utilisation of polyphenol rich plant extracts as dietary food supplements. Flavonoids are polyphenolic compounds exhibiting antioxidant ability. They scavenge the radicals that initiate lipid peroxidation by binding metal ions and by inhibition of enzymatic systems responsible for free radical generation (such as 5-lipooxygenase, cyclo-oxygenase, mono-oxygenase, or xanthine oxidase) (Laughton et al. 1991; Cotelle et al. 1996). Pycnogenol® (Pyc) is a standardised extract obtained from the bark of the French maritime pine *Pinus pinaster* (Packer et al. 1999; Rohdewald 2002). It is composed of a mixture of flavonoids, mainly procyanidins and phenolic acids. Pyc has been reported to have cardiovascular benefits, such as vasorelaxant activity, angiotensin – converting enzyme inhibiting activity, the ability to enhance the microcirculation by increasing capillary permeability and can be applied in immune, circulatory disorders as well as in neurodegenerative diseases (Packer et al. 1999). Besides these biological properties, Pyc exhibits strong antioxidant properties. These properties include the inactivation of superoxide radical (Blazso et al. 1994; Noda et al. 1997; Wei et al. 1997), hydroxyl radical (Blazso et al. 1994; Noda et al. 1997; Virgili et al. 1998), lipid peroxyl radical (Nelson et al. 1998) and the reactive nitrogen species, such as nitric oxide radical and peroxinitrite (Stadler et al. 1995; Van Acker et al. 1995; Ueda et al. 1996). Pyc can bind to proteins thus altering their structure and modulating the activity of key enzymes (Fitzpatrick et al. 1998).

The enzyme activity can be modulated also by changes in membrane fluidity. Planar phenolic units of Pyc components allow it to interact easily with cell membrane thus inducing the membrane rearrangement. Hence, one may expect that mechanism(s) other than the antioxidant protection may be also involved in the positive effect of Pyc.

We were interested: a) in interactions of Pyc with erythrocyte membrane at *in vitro* conditions, b) in its influence on the membrane fluidity, as well as c) in the effect of Pyc on the lipid peroxidation (initiated *in vitro* by H_2O_2) and d) on the oxidative hemolysis.

Materials and Methods

Chemicals

Chemicals of analytical grade were obtained from POCH (Gliwice, Poland); 1-[4-trimethyl–aminophenyl]-6-phenyl-1,3,5-hexatriene (TMA-DPH) from Eugene (Oregon, USA); 1,6-diphenyl-1,3,5-hexatriene (DPH) from Serva (Heidelberg, Germany); 12-(9-anthroyloxy) stearic acid (12-AS) from Sigma (USA); natrium azide (NaN_3) from Merck (Germany); hydrogen peroxide (H_2O_2) from Slavus (Slovakia); thiobarbituric acid (TBA) from Merck (Germany); trichloracetic acid (TCA) from Lancaster (Germany); standard 1,1,3,3-tetraethoxypropane (MDA) from Sigma (USA); Pycnogenol® from Drug Research Institute (Modra, Slovakia).

Isolation of erythrocytes

Blood samples from healthy volunteers obtained from the Central Blood Bank of Lodź, Poland and from University Hospital, Department of Internal Medicine, Comenius University, Bratislava, Slovakia ($n = 12$) were anticoagulated with citrate 3.2%. The erythrocytes were obtained from blood by centrifugation ($3000 \times g$, 10 min, 4℃) and then washed three times with cold (4℃) phosphate buffered saline (PBS) (0.15 mol/l NaCl; 1.9 mmol/l NaH_2PO_4; 8.1 mmol/l Na_2HPO_4; pH 7.4). Erythrocytes were used immediately after isolation.

Measurements of membrane fluidity

Membrane fluidity was measured in the terms of the steady-state fluorescence anisotropy of the fluorescent probes TMA-DPH, DPH and 12-AS incorporated into red blood cell membranes (Fig. 1).

The suspension of erythrocytes was diluted with cold PBS up to hematocrit of 0.05%. The suspension of erythrocytes was then incubated with Pyc at a final concentration from 50 μg/ml to 300 μg/ml, at 37℃ for 2 min in dark. The suspension of erythrocytes was then incubated with TMA-DPH for 5 min, with DPH for 15 min and with 12-AS for 10 min, at room temperature. The probes were used at a final concentration of 1 μmol/l and fluorescence was measured with luminescence spectrometer LS – 50B (Perkin-Elmer, Great Britain). The wavelengths of excitation and emission were equal to 358 and 428 nm, 348 and 426 nm, 360 and 471 nm for TMA-DPH, DPH and 12-AS, respectively.

Measurement of lipid peroxidation and oxidative hemolysis

Sample preparation

The erythrocytes were resuspended at 5% hematocrit with PBS containing 2 mmol/l NaN_3. Natrium azide was necessary for inhibition of catalase, the enzyme involved in hydrogen peroxide decomposition. The erythrocytes were incubated with Pyc at a final concentration from 1 μg/ml to 500 μg/ml at 37℃ for 90 min. Control sample was prepared without Pyc. Then H_2O_2 was added at a final concentration of 10 mmol/l, and the incubation was continued at 37℃ for 120 min.

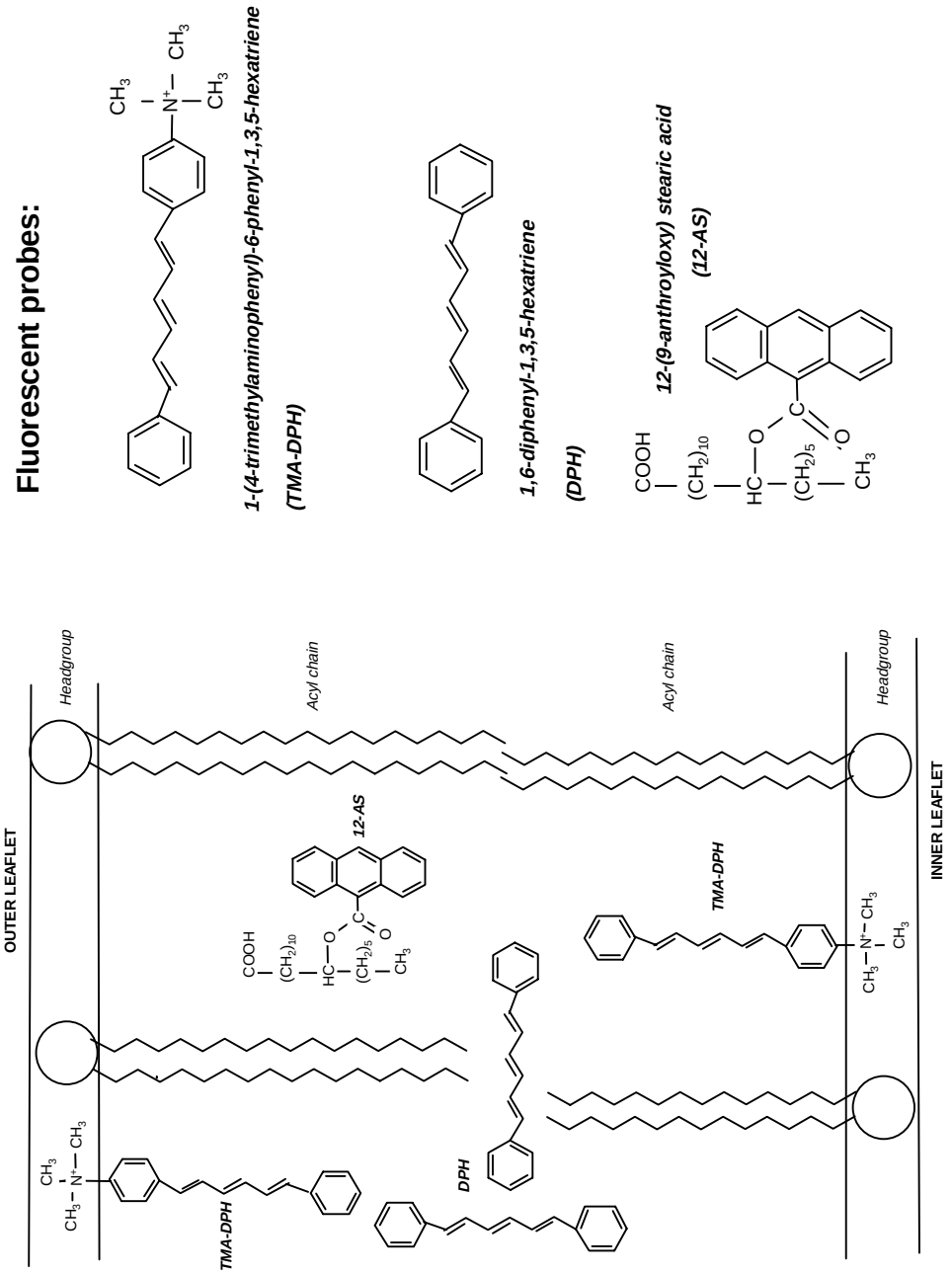

Figure 1. Location of the three fluorescent probes within the lipid bilayer.

The reaction was stopped by adding of 30 % TCA and the reaction mixture was then used for thiobarbituric acid reactive products (TBARP) assessment, as well as for oxidative hemolysis measurements.

Lipid peroxidation assay

MDA and other aldehydes reacted with TBA in acidic medium and their concentration was expressed as nanomoles of TBARP *per* g of hemoglobin (Hb) (Stocks and Dormandy 1971; Bidder and Jaeger 1982). 1,1,3,3-tetraethoxypropane was used as a standard.

Suspension of pre-treated erythrocytes (see Sample preparation) (1 ml) was deproteinised by adding of 30 % TCA (0.5 ml) and subsequently incubated at 0 °C for 120 min. Then it was centrifuged (2500 × *g*, 10 min, 4 °C) and 1 ml of the supernatant was mixed with 0.25 ml of 1 % TBA (dissolved in 0.05 mol/l NaOH) and 0.075 ml of 0.1 mol/l EDTA and heated at 95 °C for 15 min.

The absorbance of the colored product (TBARP) was measured at 535 nm. Standard 1,1,3,3-tetraethoxypropane was dissolved in water and used for construction of standard curve for TBARP (1–10 nmol/ml). All TBARP concentrations were expressed in nmol/g of Hb.

The amount of Hb was determined according to the method of Drabkin and Austin (1936).

Oxidative hemolysis

In vitro oxidative hemolysis can be detected by spectrophotometrical quantitation of the Hb released from erythrocytes. The suspension of pre-treated erythrocytes (see Sample preparation) was centrifuged (2500 × *g*, 10 min, 4 °C). Further, 0.2 ml of supernatant was diluted with 1.4 ml of PBS and the absorbance was immediately measured at 540 nm. The percentage of hemolysis was calculated in relation to the sample without Pyc.

Statistical analysis

Data are expressed as mean ± SEM. Shapiro–Wilk's test was used to verify the normal distribution of the data. Statistical significance was evaluated using one-way ANOVA and Tukey test for multiple comparisons. In order to estimate to which extent the variations in Pyc concentration explained the variability in the lipid fluidity, we employed the simple linear regression analysis.

Results

Erythrocyte membrane fluidity at different depths of the lipid bilayer was estimated by measuring the fluorescence anisotropy values (r) for TMA-DPH, DPH, and 12-AS. The anisotropy values were decreasing in Pyc-treated, thus pointing to increasing membrane fluidity (Shinitzky and Barenholz 1978). The alterations in TMA-DPH anisotropy values (Fig. 2) exceeded those in DPH (Fig. 3), which indicates that the Pyc made the erythrocyte membrane more fluid in the regions of

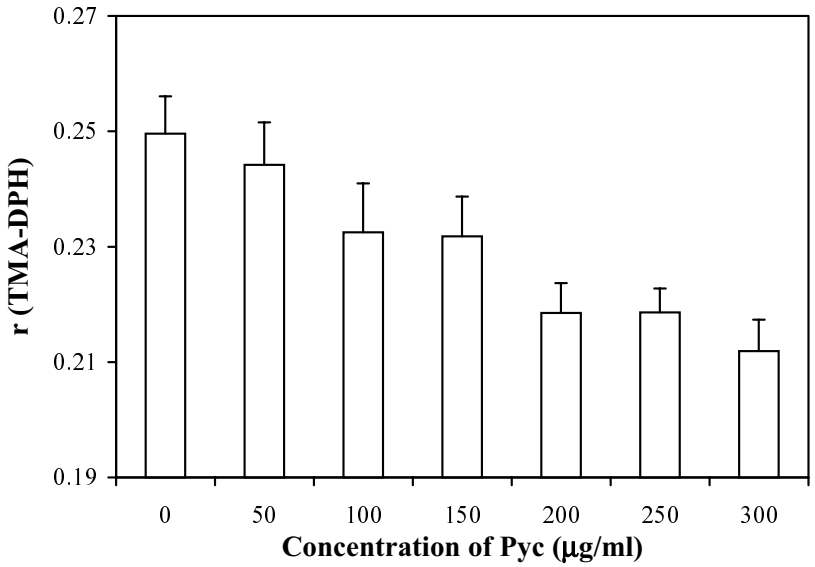

Figure 2. Fluorescence anisotropy (r) of TMA-DPH in the erythrocyte membranes treated with Pyc. The suspension of the erythrocytes (hematocrit of 0.05 %) was incubated with Pyc at 37 °C for 2 min then incubated with TMA-DPH (1 μmol/l) for 5 min at room temperature. Results are presented as mean ± SEM for $n = 7$. Significance estimated by means of simple linear regression was $p < 0.0001$.

membrane surface ($p < 0.001$) than in the centre of the lipid bilayer ($p > 0.05$). The phenomenon of the increased fluidity of the treated erythrocyte membranes was significantly pronounced at the higher concentrations of Pyc. Although alterations in the DPH values were not significant, we observed a reciprocal linear dependence of the anisotropy on the Pyc concentration suggesting a clear dose-dependent effect of the antioxidant on the erythrocyte membrane fluidity (Fig. 3). The relevant correlation coefficient was significantly different from zero ($p < 0.01$) as followed from the calculus of simple linear regression. For TMA-DPH, the correlation coefficient and related coefficient of determination were (-0.64) and 0.41, respectively ($p < 0.0001$). That means, 41 % of the total variability in TMA anisotropy values could be attributed to the Pyc concentration.

The results obtained with a fatty acid analogue 12-AS, which is known to bind to acyl chains of phospholipids at a position of C-12, showed a different behaviour of the related dynamic properties. In our study, the anisotropy values for 12-AS did not apparently change with increasing Pyc concentration ($p = 0.9$). Similarly, no trendline in the functional dependence $r_{\text{12-AS}} = f(c_{\text{Pyc}})$, where r is fluorescence anisotropy and c is concentration, was observed: Pyc had no effect on the dynamic parameter reflected by 12-AS in the erythrocyte membranes, regardless of its concentration.

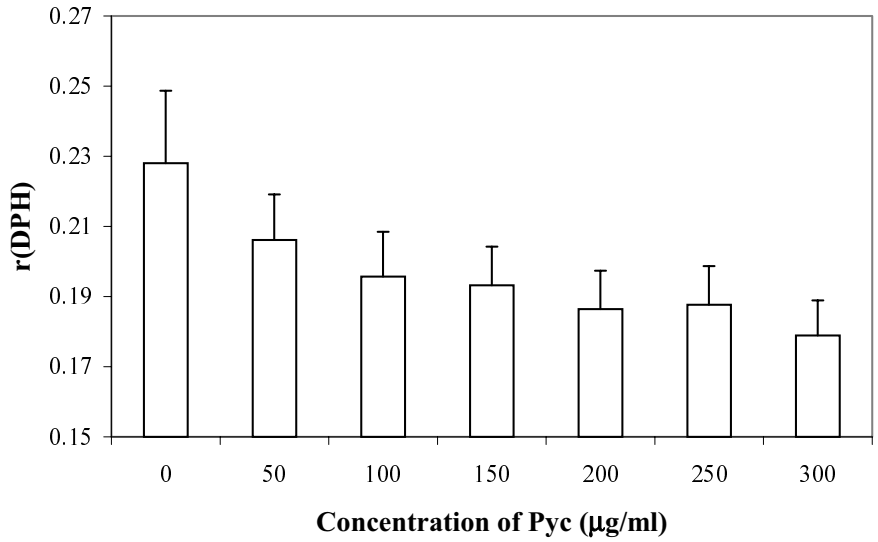

Figure 3. Fluorescence anisotropy (r) of DPH in the erythrocyte membranes treated with Pyc. The suspension of the erythrocytes (hematocrit of 0.05%) was incubated with Pyc at 37°C for 2 min then incubated with DPH (1 μmol/l) for 15 min at room temperature. Results are presented as mean ± SEM for $n = 7$. Significance estimated by means of simple linear regression was $p < 0.01$.

We investigated also the antioxidant effect of Pyc on lipid peroxidation and oxidative hemolysis at *in vitro* conditions (concentrations of Pyc: 1–500 μg/ml). Oxidative damage was induced with H_2O_2 (10 mmol/l).

The rate and degree of the oxidation of the erythrocyte membranes was measured spectrophotometrically with the TBA assay which measures the amount of malondialdehyde and/or malondialdehyde like products (TBARP) formed by the lipid peroxidation processes. As shown in Fig. 4, with increasing Pyc concentrations, the lipid peroxidation and the production of TBARP were significantly inhibited in a dose-dependent manner ($r = 0.96,\ p < 0.001$, for all concentrations of Pyc). Pyc exhibited strong antioxidant activity even at the lowest concentrations used (1–50 μg/ml). At these concentrations, Pyc caused an inhibition in lipid peroxidation of about 10–60% in comparison with controls without Pyc. However, at higher concentrations (200–500 μg/ml), the antioxidant activity of Pyc did not rise further (Fig. 4). That result has suggested that amount of TBARP reached saturated level at the chosen experimental conditions.

The effect of Pyc on the oxidative hemolysis, expressed as an amount of the Hb released, was investigated at *in vitro* conditions. We observed a concentration-dependent decrease in the hemolysis ($r = 0.97,\ p < 0.001$, for all concentrations of Pyc). The dependence was non-linear with apparent saturation at the concentration of Pyc about 200 μg/ml (Fig. 5).

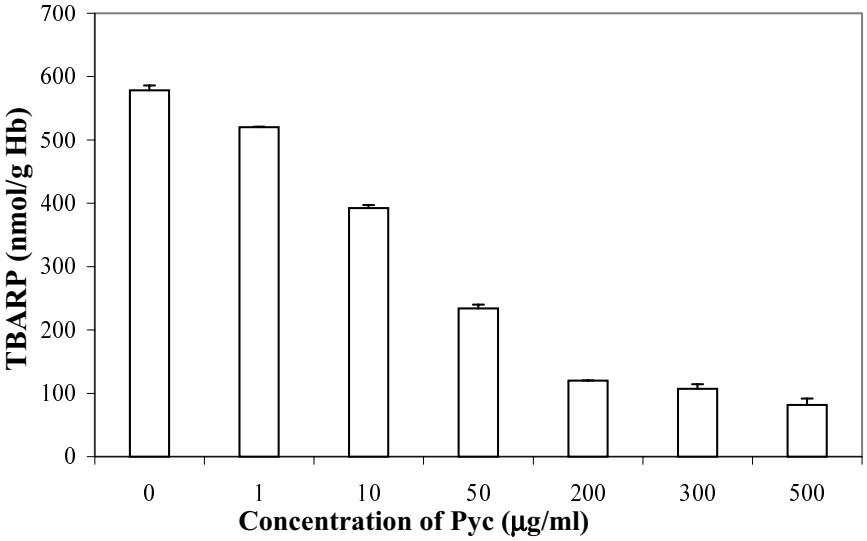

Figure 4. Influence of various Pyc concentrations on TBARP formation in the H_2O_2-treated erythrocytes. The suspension of erythrocytes (hematocrit of 5%) was incubated with Pyc at 37°C for 90 min then incubated with H_2O_2 (10 mmol/l) at 37°C for 120 min. Results are presented as mean ± SEM for $n = 6$. Significance estimated by means of simple linear regression was $p < 0.001$.

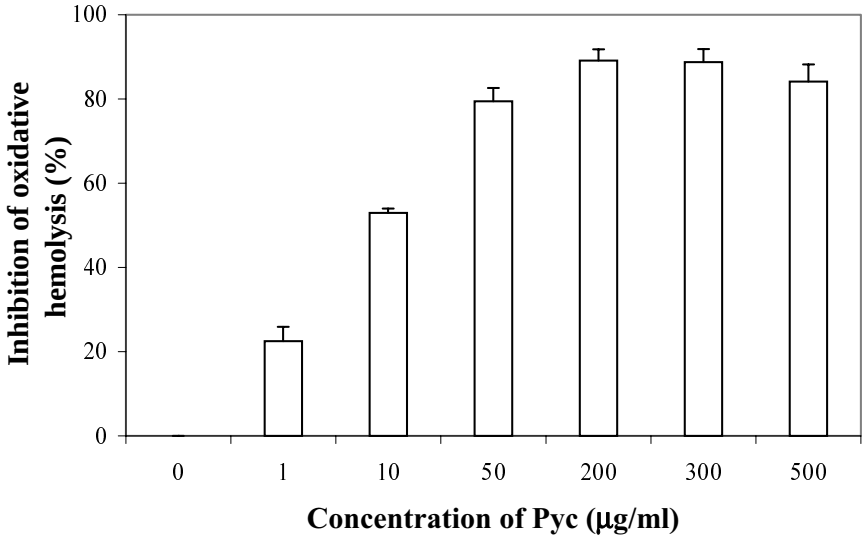

Figure 5. Effect of Pyc on oxidative hemolysis in the H_2O_2-treated erythrocytes. The suspension of erythrocytes (hematocrit of 5%) was incubated with Pyc at 37°C for 90 min then incubated with H_2O_2 (10 mmol/l) at 37°C for 120 min. Results are presented as mean ± SEM for $n = 6$. Significance estimated by means of simple linear regression was $p < 0.001$.

Discussion

In healthy tissue, cell membrane fluidity is at an optimum state, which is necessary for the right function of the membrane. Membrane fluidity is influenced in particular by cholesterol content, lipid composition, and protein-lipid interactions. It plays a role in the control of a number of physiological processes, though the mechanism on molecular scale is not exactly known (Gennis 1989). If abnormal, it may be involved in some pathological states especially those accompanied with oxidative stress. Free radical attack decreases membrane fluidity by modifying lipids *via* lipid peroxidation, which can significantly alter membrane properties and possibly disrupt the function of membrane-associated proteins (Beckman and Ames 1998).

The polyphenolic structure of flavonoids confers them the ability to interact with biological membranes and scavenge free radicals, as well as chelate transition metals directly in the membranes. This implies that such an interaction might result in the altered membrane lipid arrangement (reciprocal to lipid fluidity) at different depths of the lipid bilayer.

Localisation of flavonoids within the artificial and biological membranes has already been documented (Saija et al. 1995; Arora et al. 2000). Some studies on the membrane fluidity changes upon action of flavonoids report decreases in this parameter (Arora et al. 2000), although, some researchers suggest that the fluidity is increased (Joseph et al. 1998). However, the effect of Pyc on cell membrane fluidity has not been studied yet.

The membrane structure and fluidity can be investigated using fluorescence polarisation technique employing appropriate fluorophores.

In the present study, three probes were used to monitor the fluidity changes in isolated erythrocyte membranes treated with Pyc at various concentrations: TMA-DPH, DPH and 12-AS. All these probes exhibit strong fluorescence enhancement when incorporated into membranes, with practically negligible fluorescence in water. Their fluorescence anisotropy values respond to lipid arrangement in various regions of membranes (Fig. 1). TMA-DPH, a cationic derivative of DPH, incorporates into the hydrocarbon region near the membrane surface anchoring by its charged group at the surface. DPH is located preferentially in the hydrocarbon core of lipid bilayer, and the position of the anthroyl moiety is on the C-12 of the membrane phospholipids. The present study shows that Pyc had a pronounced effect on the physico-chemical state of the erythrocyte membranes. The observed anisotropy reduction can be attributed to lower organisation of phospholipid molecules in the hydrocarbon region of the membranes, i.e. to increased membrane fluidity especially near the membrane surface. However, our observations are not in agreement with previous study by Arora et al. (2000) who found that flavonoids and isoflavonoids cause a decrease in liposomal membrane fluidity. They suggest that localisation of flavonoids and isoflavonoids in the membrane interiors and their resulting restrictions on fluidity of membrane components could sterically hinder diffusion of free radicals and thereby decrease the kinetics of free radical reactions. Anyway, another explanation may be given: the interaction of Pyc with membrane (or membrane

surface) can cause membrane expansion and consequently the rise of free acyl-chain volume. Therefore, we assumed that the action of Pyc could be more pronounced at the interfacial region than in the lipid core. From this, it can be inferred that the acyl-chain dynamic parameters are more influenced at the membrane surface as reflected by monitoring TMA-DPH fluorescence anisotropy. Further, some small compounds of the Pyc mixture may easily penetrate the membrane when they are in uncharged form. Such an interaction may also lead to the fluidisation of lipid bilayer similarly to the interaction of uncharged phenols with model membranes as was shown by Van Dael and Ceuterickx (1984). The negatively charged portion of phenol was bound to the polar water molecules. The authors also found that the vesicle diameter did not play a significant role in the interaction. So, one can anticipate that the interaction of polyphenolic structures with lipid bilayer and their effect on the monitored parameters might exert similar effects.

As to the antioxidant properties of Pyc, the results, obtained in experiments performed at *in vitro* conditions, have shown the protective effect of Pyc in hydrogen peroxide-induced processes. Although H_2O_2 is not very reactive oxidising agent, it easily penetrates cellular membranes. If the metal ions are present, H_2O_2 can trigger hydroxyl radical formation in the membrane. This process leads to the lipid peroxidation (Edwards and Fuller 1996; Sokolowska et al. 1999). Endogenous generation of MDA (product of lipid peroxidation) after exposure of the erythrocytes to H_2O_2 as well as to exogenous MDA resulted in destabilisation erythrocyte membrane lipid asymmetry (Jain 1984).

Various studies have shown, that phenolic acids, polyphenols and in particular flavonoids composed of one (or more) aromatic ring bearing one or more hydroxyl groups are potentially able to quench free radicals by forming resonance-stabilised phenoxyl radicals (Rice-Evans et al. 1996; Packer et al. 1999; Rohdewald 2002). Many authors reported that Pyc has a strong antioxidant activity and participates in the cellular antioxidant network (Virgili et al. 2000; Rohdewald 2002).

It is known that Pyc can protect the chains of lipid bilayer against oxidative damage. Rong et al. (1994-95) have reported that preincubation of cultured normal endothelial cells with Pyc at the concentrations from 20 μg/ml to 80 μg/ml is associated with significant protection of these cells against lipid peroxidation and cell damage induced with tert-butyl hydroperoxide. The generation of TBARP is also significantly decreased, indicating that the protective effect of Pyc is due to its antioxidant activity activity (Rong et al. 1994-95).

We observed the protective effect of Pyc on lipid peroxidation, TBARP generation and oxidative hemolysis induced by H_2O_2 (Figs. 4, 5). We suppose that Pyc through chelating of metal ions was able to eliminate hydrogen peroxide from its participation in the production of a very reactive hydroxyl radical. Hydroxyl radicals can initiate the peroxidation of polyunsaturated fatty acids in membranes of erythrocytes, which results in generation of lipoperoxyl radicals.

Phenolic molecules may behave as metal chelators due to their catechol structure (Packer et al. 1999). It is not clear whether Pyc reduces lipid peroxidation and oxidative hemolysis either by quenching free radicals or by chelating metal

ions, or by both. Pyc capacity to modify membrane dependent processes, such as free-radical-induced membrane lipoperoxidation, is related not only to structural characteristics but also to its ability to interact with and/or penetrate cell membrane.

Conclusion

We found that Pyc was able to interact directly with erythrocyte membrane in a concentration dependent manner, which increased membrane fluidity near the surface. Pyc prevents the possible damage to erythrocyte membrane as a consequence of H_2O_2 exposure. From this it can be inferred that such an interaction of Pyc with the membrane may induce modification of the lipid bilayer and lipid-protein interactions thus preventing at least to some extent the lipid peroxidation.

Acknowledgements. This study was supported partly by VEGA grants of Ministry of Education of Slovakia, No. 1/8303/01 and 1/9243/02, by grant APVT No. 51-013802 and by Drug Research Institute, Modra, Slovakia.

References

Arora A., Byrem T. M., Nair M. G., Strasburg G. M. (2000): Modulation of liposomal membrane fluidity by flavonoids and isoflavonoids. Arch. Biochem. Biophys. **373**, 102—109

Beckman K. B., Ames B. N. (1998): The free radical theory of aging matures. Physiol. Rev. **78**, 547—581

Bidder T. G., Jaeger P. D. (1982): Malondialdehyde production by erythrocytes from alcoholic and non-alcoholic subjects. Life Sci. **30**, 1021—1027

Blazso G., Gabor M., Sibbel R., Rohdewald P. (1994): Antiinflammatory and superoxide radical scavenging activities of procyanidins containing extract from the bark of pinus pinaster sol. and its fractions. Pharm. Pharmacol. Lett. **3**, 217—220

Cotelle N., Bernier J. L., Catteau J. P., Pommery J., Wallet J. C., Gaydou E. M. (1996): Antioxidant properties of hydroxy-flavones. Free Radic. Biol. Med. **20**, 35—45

Drabkin D. L., Austin J. K. (1936): A technique for the analysis of undiluted blood and concentrated hemoglobin solutions. J. Biol. Chem. **112**, 105—115

Ďuračková Z. (1998): Free Radicals and Antioxidants in Medicine (I), Slovak Academic Press, Bratislava (in Slovak)

Edwards C. J., Fuller J. (1996): Oxidative stress in erythrocytes. Comp. Haematol. Int. **6**, 24—31

Fitzpatrick D. F., Bing B., Rohdewald P. (1998): Endothelium-dependent vascular effects of Pycnogenol. J. Cardiovasc. Pharmacol. **32**, 509—515

Gennis R. B. (1989): Biomembranes. Molecular Structure and Function. Springer-Verlag, New York

Jain S. K. (1984): The accumulation of malonyldialdehyde, a product of fatty acid peroxidation, can disturb aminophospholipid organization in the membrane bilayer of human erythrocytes. J. Biol. Chem. **259**, 3391—3394

Joseph J. A., Denisova N., Fisher D., Shukitt-Hale B., Prior R., Cao G. (1998): Membrane and receptor modifications of oxidative stress vulnerability in aging. Nutritional considerations. Ann. N.Y. Acad. Sci. **854**, 268—276

Laughton M. J., Evans P. J., Moroney M. A., Hoult J. R. C., Halliwell B. (1991): Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. Biochem. Pharmacol. **42**, 1673—1681

Nelson A. B., Lau B. H. S., Ide N., Rong Y. Q. (1998): Pycnogenol inhibits macrophage oxidative burst, lipoprotein oxidation and hydroxyl radical-induced DNA damage. Drug Dev. Ind. Pharm. **24**, 139—144

Noda Y., Anzai K., Mori A., Kohno M., Shinmei M., Packer L. (1997): Hydroxyl and superoxide anion radical scavenging activities of natural source antioxidants using the computerized JES-FR 30 ESR spectrometer system. Biochem. Mol. Biol. Int. **42**, 35—44

Packer L., Rimbach G., Virgili F. (1999): Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (Pinus maritima) bark, pycnogenol. Free Radic. Biol. Med. **27**, 704—724

Piasecka A., Leyko W., Krajewska E., Bryszewska M. (2000): Effect of combined treatment with perindoprilat and low-power red light laser irradiation on human erythrocyte membrane fluidity, membrane potential and acetylcholinesterase activity. Scand. J. Clin. Lab. Invest. **60**, 395—402

Polidori M. C., Stahl W., Eichler O., Niestroj I., Sies H. (2001): Profiles of antioxidants in human plasma. Free Radic. Biol. Med. **30**, 456—462

Rice-Evans C. A., Miller N. J., Paganga G. (1996): Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. **20**, 933—956

Rohdewald P. (2002): A review of the French maritime pine bark extract (Pycnogenol®), a herbal medication with a diverse clinical pharmacology. Int. J. Clin. Pharmacol. Ther. **40**, 158—168

Rong Y., Li L., Shah V., Lau B. H. (1994-95): Pycnogenol protects vascular endothelial cells from t-butyl hydroperoxide induced oxidant injury. Biotechnol. Ther. **5**, 117—126

Saija A., Scalese M., Lanza M., Marzullo D., Bonina F., Castelli F. (1995): Flavonoids as antioxidant agents: importance of their interaction with biomembranes. Free Radic. Biol. Med. **19**, 481—486

Shinitzky M., Barenholz Y. (1978): Fluidity parameters of lipid regions determined by fluorescence polarization. Biochim. Biophys. Acta **515**, 367—394

Sokolowska M., Oleszek A., Wlodek L. (1999): Protective effect of alpha-keto acids on the oxidative hemolysis. Pol. J. Pharmacol. **51**, 429—434

Stadler J., Schmalix W. A., Doehmer J. (1995): Inhibition of biotransformation by nitric oxide (NO) overproduction and toxic consequences. Toxicol. Lett. **82**, 215—219

Stocks J., Dormandy T. L. (1971): The autoxidation of human red cell lipids induced by hydrogen peroxide. Br. J. Haematol. **20**, 95—111

Ueda T., Ueda T., Armstrong D. (1996): Preventive effect of natural and synthetic antioxidants on lipid peroxidation in the mammalian eye. Ophthalmic Res. **28**, 184—192

Van Acker S. A. B., Tromp M. N. J., Haenen G. R. M., van der Vijgh W. J. F., Bast A. (1995): Flavonoids as scavengers of nitric oxide radical. Biochem. Biophys. Res. Commun. **214**, 755—759

Van Dael H., Ceuterickx P. (1984): The interaction of phenol with lipid bilayers. Chem. Phys. Lipids **35**, 171—181

Virgili F., Kobuchi H., Packer L. (1998): Procyanidins extracted from Pinus maritima (Pycnogenol®): scavengers of free radical species and modulators of nitrogen monoxide metabolism in activated murine RAW 264.7 macrophages. Free Radic. Biol. Med. **24**, 1120—1129

Virgili F., Pagana G., Bourne L., Rimbach G., Natella F., Rice-Evans C., Packer L. (2000): Ferulic acid excretion as a marker of consumption of a French maritime pine (Pinus maritima) bark extract. Free Radic. Biol. Med. **28**, 1249—1256

Waczulíková I., Šikurová L., Čársky J., Štrbová L., Krahulec B. (2000): Decreased fluidity of isolated erythrocyte membranes in type 1 and type 2 diabetes. The effect of resorcylidene aminoguanidine. Gen. Physiol. Biophys. **19**, 381—392

Wei Z. H., Peng Q. L., Leu B. H. S. (1997): Pycnogenol enhances endothelial cell antioxidant defenses. Redox Rep. **3**, 219—224 and by Drug Research Institute, Modra, Slovak Republic.

Final version accepted: January 26, 2004