After solvent removal under reduced pressure the extract was successively partitioned with hexane, $CHCl_3$, ethyl acetate and butanol to obtain the respective fractions [10].
Since the ethyl acetate fraction exhibited the highest concentration of biflavonoids, it was chosen for further study.

2. Column chromatography

The chromatographic support (CH-Fe) was prepared and characterized according to the literature [7].
The ethyl acetate fraction (150 mg) which contained the biflavonoids, was chromatographed on a chromatographic column (CC) (2.0×30 cm) using 3.5 g of CH-Fe eluted with a $CHCl_3$MeOH gradient and fractions of 5 ml were collected. After being monitored by TLC using silica gel 60 plates eluted with $CHCl_3$MeOH (70 : 30), the similar fractions, which showed positive reaction with $FeCl_3$ (2% in ethanol) solution, were combined and the compounds were identified on the basis of spectral data and Co-TLC with authentic samples previously isolated [8].

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Resorcylidene aminoguanidine improves the pathologically reduced fluidity of erythrocyte membranes in Diabetes mellitus

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Several studies in diabetic patients have reported reduced membrane lipid fluidity of red blood cells of diabetic patients as compared with normal individuals [1–3]. The rigidification of diabetic erythrocyte membranes seems to result from an enhanced cholesterol to phospholipid molar ratio and/or from an increased non-enzymatic glycation of membrane proteins and subsequent formation of advanced glycation end products [4, 5]. The pathologically reduced membrane fluidity occurring in Diabetes results in decreased cell deformability, diminished oxygen releasing capacity, etc. [6, 7]. Therefore, an improvement in the pathological physical state of diabetic membranes may help to prevent late diabetic complications. Aminoguanidine has been found to prevent or ameliorate the Diabetes-induced collagen cross-linking and vascular dysfunction [8, 9]. However, aminoguanidine toxicity limits its application. In search of new suitable non-toxic inhibitors of non-enzymatic glycation, resorcylidene aminoguanidine (RAG) was synthesised [10, 11]. At physiological pH, RAG exists in cationic as well as in neutral forms, with a pK_1 of 7.36 [10] (Scheme). It has been reported that the

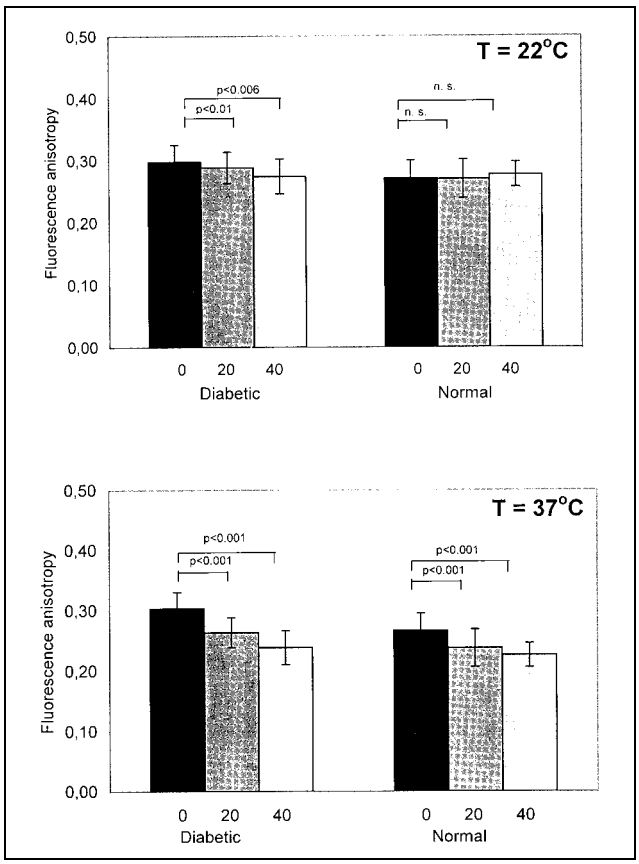
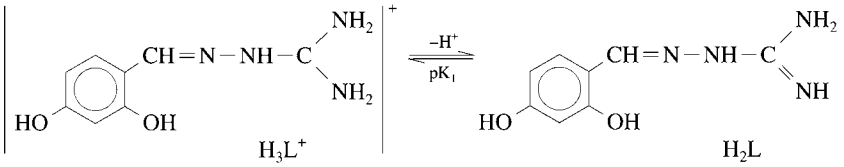


Fig.: Effect of RAG treatment on fluorescence anisotropy of DPH in human erythrocyte membranes (2%). 0 – no treatment, 20 – treatment with 20 μM RAG, 40 – treatment with 40 μM RAG. Data are given as means ± S.D. of 8 independent experiments

Scheme



application of RAG induced a significant diminution of glycation products formation in diabetic hearts and an increase in the fluidity of cardiac sarcolemma in rats with streptozotocin-induced Diabetes mellitus [12]. Further, Hrnčiarová et al. [13] have shown the inhibition of erythrocyte lipid peroxidation in diabetic rats by RAG.

In the present work, we examine whether RAG is able to improve the pathological physical state of human diabetic erythrocyte membranes, namely the membrane lipid fluidity. Changes in fluidity are quantitatively assessed in terms of diphenylhexatriene (DPH) fluorescence anisotropy.

Our data show that the values of fluorescence anisotropy of DPH in the isolated human diabetic erythrocyte membranes were significantly higher as compared to erythrocytes from normal (healthy) donors at both temperatures examined: by 9.96% and by 13.86% at 22 °C and at 37 °C, respectively (Fig.). As shown by Shinitzky and Barenholtz [14], DPH fluorescence anisotropy positively correlates with ordering of phospholipid molecules in the hydrocarbon region of membranes, i.e. with membrane lipid packing. Moreover, packing density inversely corresponds to membrane fluidity [14]. Thus, the presented data on the increase in DPH anisotropy values in diabetic membranes can be interpreted as a decrease of erythrocyte membrane fluidity evoked by Diabetes. These results are in agreement with the well-known rigidification effect of Diabetes mellitus on human erythrocyte membranes [1–3].

Application of RAG on isolated erythrocyte membranes of both diabetic and normal donors induced changes in the values of DPH fluorescence anisotropy. The observed changes were less pronounced at the laboratory temperature (22 °C) than at the physiological temperature (37 °C). Incubation of RAG with isolated erythrocyte membranes of diabetic donors at 22 °C resulted in a statistically significant reduction of anisotropy values by 3.14% and 6.71% at RAG concentrations of 20 μM and 40 μM, respectively. On the other hand, treatment of erythrocyte membranes isolated from normal donors with RAG at 22 °C induced only very slight non-significant changes for both RAG concentrations used. At 37 °C, RAG treatment resulted in statistically significant changes in both diabetic and normal (healthy) groups. In the diabetic group, RAG at concentrations of 20 μM and 40 μM reduced the values of DPH anisotropy by 13.14% and 19.98%, respectively. In the normal group, the reduction of anisotropy values was only 10.79% and 13.78%, respectively.

These studies show that RAG acts as a modulator increasing erythrocyte membrane fluidity and its activity is more pronounced in diabetic membranes than in normal ones, and is also more pronounced at the physiological temperature than at the laboratory temperature. At 37 °C, the concentration of RAG 20 μM induced an increase of the pathologically decreased fluidity in diabetic erythrocyte membranes up to the level almost identical to the fluidity in normal erythrocyte membranes. However, the RAG concentration of 40 μM lifts the membrane fluidity up to a level even higher than the fluidity of healthy donors. This excessive fluidization effect at high RAG concentrations must be controlled by an appropriate dosage.

Experimental

Erythrocyte membranes were isolated from human blood of diabetic (type 1) or healthy (control) adult donors according to the standard method of Hanahan and Ekholm [15]. Resorcylidene aminoguanidine (RAG, synthesized by J. Carsky et al. [10]) at 20 μM, 40 μM was incubated with ghosts (2% in 20 imOsM TRIS buffer, pH = 7.4) for 30 min at 22 °C and 37 °C, respectively. Erythrocyte membrane fluidity was assessed using the fluorescence polarization technique [14]. For this purpose, a suspension of erythrocyte membranes were stained with the DPH (1,6-diphenyl−1,3,5-hexatriene; Koch − Light Co.) probe yielding the final concentration of DPH 1 μM, the final concentration of membranes was 1% and the final concentrations of RAG were 10 and 20 μM, respectively. The measurements were carried out at room temperature (22 °C). Statistically significant differences among the diabetic and normal groups were tested by Student's t-test and Student's paired t-test was applied for anisotropy changes induced by RAG treatment. The criterion of statistical significance was a p-value less than 0.01.

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