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In vivo influence of extract from *Aronia melanocarpa* on the erythrocyte membranes in patients with hypercholesterolemia

Authors' Contribution:
A Study Design
B Data Collection
C Statistical Analysis
D Data Interpretation
E Manuscript Preparation
F Literature Search
G Funds Collection

Piotr Duchnowicz[1 BCDEF], Agnieszka Nowicka[1 BC], Maria Koter-Michalak[1 ADFG], Marlena Broncel[2 ADFG]

[1] Department of Environment Pollution Biophysics, University of Lodz, Lodz, Poland
[2] Department of Internal Diseases and Clinical Pharmacology, Medical University of Lodz, Lodz, Poland

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Summary

Background: Hypercholesterolemia increases cholesterol concentration in erythrocyte membranes, which results in decrease of membrane fluidity and decreases the deformability of red blood cells. The fruits of *Arona melanocarpa* contains many of polyphenols and other compounds that have beneficial health effects.

Material/Methods: The aim of the study was to estimate the influence of 2-month supplementation of extract from *Aronia melanocarpa* (100 mg Aronox, three times per day) on cholesterol concentration, lipid peroxidation, membrane fluidity, level of thiol groups and activity of ATPase in erythrocytes from patients with hypercholesterolemia. The study involved 25 patients with hypercholesterolemia without pharmacological treatment and 20 healthy individuals as a control group. Blood samples were collected before, and after 1 and 2 months of Aronia administration.

Results: The 2-month Aronia supplementation resulted in a decrease of cholesterol concentration (by 22%) and a decrease of lipid peroxidation (by 40%), and an increase of membrane fluidity. No statistically significant increase of the concentration of thiol groups and of ATPase activity were observed.

Conclusions: Our study shows that supplementation of extract from *Aronia melanocarpa* has a beneficial effect on rheological properties of erythrocytes.

key words: **hypercholesterolemia • *Aronia melanocarpa* • lipids peroxidation • cholesterol • ATPase activity • membrane fluidity**

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Author's address: Piotr Duchnowicz, Department of Environment Pollution Biophysics, University of Lodz 141/143 Pomorska St., 90-236 Lodz, Poland, e-mail: duchno@biol.uni.lodz.pl

Background

Hypercholesterolemia is a major risk factor for atherosclerosis and coronary heart disease [1]. It is a lipid metabolic disorder, not a disease in the strict sense of this word. High concentration of cholesterol in blood is a consequence of changes in human lifestyle. High dietary cholesterol affect lipid metabolism in the liver and other organs. The primary intervention for dyslipidemia is a low-cholesterol diet connected with drug therapy [2,3]. Most current hypolipidemic drugs have potential adverse effects, especially as the patients with hypercholesterolemia have to take many drugs that can interact with each other. Currently, research is focused on natural compounds that show potential effect on blood lipid levels, blood pressure and blood glucose level.

Polyphenols are natural substances of plant origin; they are found in fruits, vegetables, herbal, tea and red wine. Polyphenols are divided into 4 main classes according to the nature of their carbon skeleton [4]. Phenolic acids and flavonoids are abundant in our diets. Stilbenes and lignans are not widespread in food plants and they are less common.

The biological activity of flavonoids is mostly connected with their antioxidant properties. However, flavonoids, especially flavonols, interfere with a large number of biochemical signalling pathways [5]. Five classes of flavonoids have important biological significance: catechins, procyanidins, anthocyanins, flavones, and flavonols.

Several studies have shown that diet rich in flavonoids possesses beneficial effects on our health. Flavonoids reduce the risk of CVD, decrease oxidative stress, reduce blood pressure, and inhibit low-density lipoprotein (LDL) oxidation [6–12]. They may protect against atherosclerosis, and may reduce dyslipidemia, insuline resistance and hypertension [13,14].

Daily intakes of polyphenols have been estimated at between 20 mg to 600 mg, strongly depending on the individual dietary habits and preferences. More important is the proportion of the different polyphenols, especially concentration of flavonoids, in our diet [5,15].

A plant-based dietary supplement can remedy the deficit of polyphenols in our diet. Some supplements contain pure compounds (eg, quercetin), flavonoid mixtures or extracts from plants (fruits). The amount of polyphenols in supplements often far exceeds the dietary intake.

The aim of the present study was to investigate the effect of *Aronia melanocarpa* extract on cholesterol concentration, ATPase activity, level of thiol groups, lipid peroxidation and membranes fluidity in erythrocytes during 2-month supplementation.

Material and Methods

Patients

Blood from hypercholesterolemic patients (total cholesterol concentration (TC) >250 mg/dl, LDL cholesterol (LDL-C) >160 mg/dl, triglycerides (TG) < 400 mg/dl) and from healthy donors (TC <200 mg/dl, LDL-C <135 mg/dl, TG <200 mg/dl) were obtained from the Department of Clinical Pharmacology, Medical University of Lodz. Blood was collected with anticoagulant (23 mM citric acid, 45.1 mM trisodium citrate, 45 mM glucose) in a 5: 1 ratio. The study involved 25 patients with hypercholesterolemia (7 males and 18 females, mean age 55.9±7.4). The control group consisted of 20 healthy individuals (7 males and 13 females, mean age 50.3±8.2).

Patients with hypercholesterolemia were treated with 100 mg of Aronia extract (Aronox, Agropharm, Poland) 3 times a day during the 2-month supplementation. Blood samples were collected 3 times: before supplementation, and after 1 and 2 months of supplementation. During the period of suplementation volunteers did not change their individual dietary habits and preferences.

These experiments were carried out in accordance with the ethics standards as formulated in The Helsinki Declaration of 1975 (revised 1983); consent number 241/06/KB of Commission of Medical Research Ethics of Medical University of Lodz, Poland.

Erythrocytes

Erythrocytes were washed 3 times with phosphate-buffered 0.9% NaCl (pH 7.4) and centrifuged at 600× *g*.

Lipids peroxidation

Peroxidation of lipids was estimated as the amount of compounds reacting with 2-thiobarbituric acid (TBA) according to the method of Stock and Dormandy [16]. Absorbance was read at 532 nm. The concentration of TBARS was calculated using a milimolar absorption coefficient for malonyl dialdehyde (MDA), $\varepsilon=1.5\times10^5$ $mol^{-1}\cdot dm^3\cdot cm^{-1}$. The hemoglobin concentration was determined by Drabkin method [17]. Absorbance was read at 540 nm. The results are expressed in μmol TBARs/g Hb.

Cholesterol concentration

The extraction of lipids from erythrocytes was carried out using the Rodriguez-Vico method [18]. Erythrocytes were placed in a flat-bottomed flask and they were shaken with a hexane and 2-propanol mixture (3:2). Everything was centrifuged at 4000× *g* for 15 min. The supernatant was transferred into a dry flask. The flask was connected to a vacuum evaporator in order to evaporate solvents. Dry lipids were dissolved in a mixture of ethanol: chloroform (9:1, v/v).

The concentration of cholesterol was determined with the use of Liebermann-Burchard reagent [19]. Acetic anhydride and concentrated sulfuric acid dehydrate the cholesterol molecule in anhydrous conditions, resulting in setting an additional double bond. Green color products are colorimetric measurement at 660 nm. The concentration of cholesterol in the sample was read from a calibration curve in the range 0.2–1.5 mg/ml. Concentration of cholesterol was expressed as milligrams of cholesterol per milliliter packed cells (mg HC/ml packed cell).

Red cell membrane preparation

The erythrocyte membranes were prepared by the method of Dodge et al. with Tris-HCl buffer [20]. The erythrocytes

Table 1. Mean values of the studied parameters after 1 and 2 months of Aronia supplementation in erythrocytes from patients with hypercholesterolemia.

Parameter	Control group	Before therapy	After 1 month	After 2 month
Cholesterol (mg CH/ml p.c.)	2.26±0.22	4.85$^{###}$±0.65	4.01$^{**,###}$±0.81	3.58$^{***,###}$±0.54
Lipid peroxidation (µmol TBARS/g Hb)	0.172±0.017	0.464$^{###}$±0.019	0.363$^{***,###}$±0.019	0.281$^{***,###}$±0.014
Parameter S	0.742±0.006	0.761$^{###}$±0.008	0.758$^{###}$±0.004	0.749$^{*,##}$±0.005

*** p<0.001; ** p<0.01; * p<0.05 *vs.* before therapy values; $^{###}$ p<0.001; $^{##}$ p<0.01 *vs.* the control group.

Table 2. Mean values of the studied parameters after 1 and 2 months of Aronia supplementation in erythrocyte membranes from patients with hypercholesterolemia.

Parameter	Control group	Before therapy	After 1 month	After 2 month
Thiol groups (µmole –SH/mg proteins)	0.364±0.019	0.250$^{##}$±0.024	0.209$^{##}$±0.014	0.263$^{##}$±0.021
Total ATPase activity (nmol Pi/mg protein × h)	366.47±26.72	265.14$^{###}$±49.95	285.90$^{###}$±75.27	297.06$^{###}$±63.08
Na^+K^+ ATPase activity (nmol Pi/mg protein × h)	144.78±23.01	116.55$^{###}$±26.08	115.66$^{###}$±33.11	117.45$^{###}$±28.93

$^{###}$ p<0.001; $^{##}$ p<0.01 *vs.* the control group.

were hemolyzed with 20 mM Tris-HCl buffer, pH 7.4, supplemented with 1 mM EDTA and 0.01% PMSF on ice for 15 min. The erythrocyte membranes were centrifuged at 20000× *g* for 5 min. The membranes were washed several times with the above-mentioned buffer until "white ghost" (hemoglobin-free) state. Used buffer was chilled down to 5°C and the whole preparation procedure was performed in the ice-bath conditions.

The protein concentration was estimated according to the Lowry methods [21]. Absorbance was read at 715 nm. The concentration of protein in the sample was read from a calibration curve in the range 30–300 µg proteins/ml using albumin from bovine serum as the standard.

Activity of ATPase

Activity of ATPase was measured by means of Bartosz's method based on the measurement of released orthophosphate from ATP during the incubation of erythrocyte membranes with medium (1 mmol/dm^3 ATP, 10 mmol/dm^3 $MgCl_2$, 100 mmol/dm^3 buffer Tris-HCl, pH 7.4, 0.1 mmol/dm^3 ouabain) [22]. Concentration of orthophosphate released from ATP was determined in supernatant by the method of van Veldhoven and Mannaerts [23]. Absorbance was read at 610 nm for membranes incubated in the absence (total ATPase activity) and presence (minus Na^+K^+ ATPase activity) of ouabain in the incubation medium. The concentration of orthophosphate in the sample was read from a calibration curve in the range 2–20 µM using KH_2PO_4 as the standard. The results are expressed in nmol orthophosphate/mg proteins × h. Na^+K^+ ATPase activity was calculated as the difference between activity of ATPase without and with ouabain in incubation medium.

Level of thiol groups

Level of thiol groups in erythrocyte membranes was estimated according to the Ellman's methods with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The absorbance was read at 412. The concentration of – SH groups was calculated using a milimolar absorption coefficient for 2-nitro-5-thiobenzoate (NTB^-), ε_{412}=14.15 mmol^{-1}·dm^3·cm^{-1} [24]. The results are expressed in mmol – SH/mg proteins.

Membrane fluidity

Fluidity of erythrocyte membranes was determined by means of electron paramagnetic resonance (EPR) spectroscopy with using 5-doxylstearic acid (5-DSA) spin label. EPR measurements were performed in a Brucker 300 Spectrometer (Germany). The order parameter is a degree of the distribution of molecular orientations with respect to a reference axis, chosen in this study to be normal to the membrane surface. An increase in order parameter reflects a decrease in the segmental flexibility of the spin sample. Order parameter S was calculated as described in Koter et al. [25].

Chemicals and reagents

Tris, TBA, ATP, DTNB, 5-DSA, albumin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used were of high-purity grade.

Statistical analysis

Results are reported as mean ± SD to show variations in a group. Statistical analyses were performed by the one-way ANOVA test with *post-hoc* Tukey test using the statistical software STATISTICA 9.0 (StatSoft Inc., Tulsa, OK, USA). Differences were considered significant at $p<0.05$.

Results

Tables 1 and 2 present the characteristics of the erythrocytes from patients with hypercholesterolemia and the control group at baseline. Cholesterol concentration, lipid

peroxidation and parameter S were significantly higher in the patients with hypercholesterolemia than in the control group. Concentration of thiol groups, total ATPase activity and Na^+K^+ ATPase activity were significantly lower in the patients with hypercholesterolemia than in the control group.

Tables 1 and 2 illustrated the effect of Aronia extract 2-month therapy on investigated parameters. After 1 month of therapy a significant decrease of cholesterol concentration and level of lipid peroxidation were observed, and these effects were intensified after 2 months. Although the parameters decreased, they were still higher in patients with hypercholesterolemia than in the control group. Order parameter S decreased significantly after 2-month therapy, but it was still higher in patients with hypercholesterolemia than in the control group. Increase of membrane fluidity (decrease of parameter S) was a consequence of the decrease of cholesterol concentration.

One- and 2-month-long Aronia extract administration had no statistically significant influence on thiol groups' level, total ATPase activity and Na^+K^+ ATPase activity. In spite of a tendency to increase, those parameters were still lower in patients with hypercholesterolemia than in the control group.

Discussion

Aronia berries contain 7849.21 mg phenolic compounds/100 g dry weight, including 5181 mg polymeric procyanidins, 1958.76 mg anthocyanins, 101.15 mg flavonols (quercetin derivatives), 15.04 mg (–)epicatechin, 301.85 mg chlorogenic acid and 290.81 mg neochlorogenic acid [26]. Chokeberry contain 4 major anthocyanins: cyanidin 3-galactoside (66.9%), cyanidin 3-arabinoside (27%), cyanidin 3-xyloside (3.5%) and cyanidin 3-glucoside (2.5%) [27]. Other biologically important compounds were found in *Aronia* berries: vitamin C, niacin, panthothenic acid, carotenoids, citric acid, malic acid and minerals [28]. According to data from the producer, 100 mg of Aronox (extract from fruits of *Aronia melanocarpa*) contains about 50 mg of polyphenols, and no less than 20 mg of anthocyanins.

In the USA, the daily intake of anthocyanins is estimated at 180 mg during the winter and 215 mg during the summer [29]. In our study the daily intake of anthocyanins was about 60 mg.

Patients with hypercholesterolemia have higher level of total cholesterol, LDL-cholesterol and triglycerides, decreased level of HDL-cholesterol, and reduced activity of antioxidant enzymes [30]. Hypercholesterolemic subjects have higher level of lipid peroxidation in serum, which can be connected with lower activity of antioxidant enzymes [25]. In hypercholesterolemic rats, a higher level of lipid peroxidation in serum and liver was observed [31,32].

In vivo studies described an antioxidant effect of Aronia extract in an animal model, where extract reduced lipid peroxidation and enhanced the activity of antioxidant enzymes [33]. In our study, we noticed higher lipid peroxidation in erythrocytes from patients with hypercholesterolemia. Significant decrease in level of lipid peroxidation after 1 (by 20%) and 2 months (by 40%) of Aronia administration was observed. This decrease may be connected with increase of antioxidant enzymes activity, which was noticed after 2-month Aronia supplementation [30]. A similar effect was observed in hyperlipidemic rats after 4 weeks of administration of mulberry extract [32].

Although a direct antioxidant effect of dietary polyphenols *in vivo* has always been doubtful, indirect effects of dietary polyphenols on the antioxidant system are possible. Additional antioxidants as carotenoids, tocopherols and ascorbate are present in fresh Aronia fruits and in extracts. Especially ascorbate is a much more effective plasma antioxidant than any dietary polyphenols. These antioxidants can play a significant role in decrease in lipid peroxidation [34,35].

Cholesterol is an essential structural and functional component of the cell membranes, but abnormal level of cholesterol causes a disturbance of structure and function of cell membranes [25,39]. Human and animal studies have shown that the accumulation of cholesterol in cell membranes is a result of the higher concentration of cholesterol in plasma [25,31,37]. In the present study, the content of cholesterol in erythrocytes from patients with hypercholesterolemia was over 2-fold higher than that in the control group. Two-month Aronia administration reduced the cholesterol concentration in erythrocytes (by 22%), but it was still higher than in the control group.

The mechanism of reduction of lipid (cholesterol) level in serum and cell membranes is probably a sum of several effects. Flavonoids from green tea can inhibit the intestinal absorption of dietary lipids [38]. Quercetin, one of the most abundant flavonoids in human diet, can inhibit fatty acid and triacylglycerol synthesis in liver cells [39]. Fruit juice from *Aronia melanocarpa* showed hypolipidemic effects in an experimental model of hyperlipidemia in rats [40]. Decrease in serum total cholesterol level was observed in men with mild hypercholesterolemia after 6 weeks of regular chokeberry juice drinking [41].

Other compounds also show hypolipidemic effects. Niacin decreases the secretion of cholesterol by the liver and panthothenic acid improves the lipid profile in the blood and liver [42,43].

Decrease in erythrocyte membrane fluidity is a result of the increased cholesterol concentration in plasma, and, in consequence, in erythrocyte membranes [25,36]. Higher fluorescence polarization (lower fluidity) in glands membranes was observed in rats fed a cholesterol-rich diet after 5 weeks [36]. In erythrocyte membranes from rats with hypercholesterolemia induced by feeding a cholesterol-rich diet, increase of fluorescence anisotropy (decrease of membrane fluidity) was reported [44].

In this study, the erythrocyte membrane fluidity at the depth of the fifth carbon atom in the fatty acid chains of phospholipids was examined. Higher value of order parameter S in erythrocyte membranes from patients with hypercholesterolemia is evidence of decreased membrane fluidity (parameter S is inversely proportional to membrane fluidity). After 2-month Aronia supplementation, an increase of membrane fluidity was observed. Statin and hypolipidemic drugs showed similar effect. Atorvastatin, simvastatin and pravastatin decreased cholesterol concentration in the plasma

membranes of erythrocytes and improved the fluidity of erythrocyte membranes after 4 and 12 weeks of therapy [45].

Thiol groups play important roles in transport processes through membranes [46]. Changes in content of thiol groups in erythrocytes membranes from hypercholesterolemic patients was reported earlier [25]. In the present study, a 32% decrease of the concentration of thiol groups in hypercholesterolemic patients was also noticed. The Aronia supplementation did not cause any significant changes in level of thiol groups. Decrease of thiol groups' level is a result of the oxidative damage to membrane proteins or changes in structure of proteins [47].

The membrane lipid composition has a strong influence on membrane enzyme activity and ion transport. Higher cholesterol concentration in membranes decreases activities of Ca^{2+},Mg^{2+} ATPase and Na^{+},K^{+} ATPase, and disturbs ion transport [39,48]. Inhibition of the activity of Na^{+},K^{+} ATPase could be caused by lipid peroxidation products [49,50]. In the present study,a tendency to increase the total ATPase activity and the activity of Na^{+},K^{+} ATPase was observed after 2-month Aronia administration (changes were no statistically significant). The positive changes of the short-term supplementation suggests that this may require a longer period of Aronia administration.

Na^{+},K^{+} ATPase is responsible for sodium and potassium transport across the membrane, as well as for the maintenance of cell shape. Increase of the echinocyte population in the erythrocytes from hypercholesterolemic rats was reported [44].

Cyanidin 3-glycosides are metabolized in the human body. Glucuronides and methylglucuronides are the major metabolites found in human serum and urine after oral administration of cyanidin 3-glycosides. Only 32% of total anthocyanins detected in the serum and urine were intact [51]. Also, quercetin glycosides are metabolized. Several metabolites appeared in human plasma and urine after consumption of a rich source of quercetin derivatives, including glucuronides, sulfates and methyl conjugates of quercetin and isorhamnetin. Polymeric procyanidins are not absorbed by humans and they are metabolized by colon microflora to phenolic acids [52]. Glucuronides, O-methylated and sulfated metabolites of flavonoids have limited antioxidant activity and other biological properties [53].

Conclusions

Our data show that hypercholesterolemia causes some essential changes in erythrocyte membranes. Two-month supplementation with extract from *Aronia melanocarpa* has beneficial influence on rheological properties of erythrocytes – decrease of cholesterol concentration, decrease of lipid peroxidation, and increase of membrane fluidity. These beneficial effects can be connected with anthocyanidins and flavonols, as well as with other compounds present in Aronia extract.

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