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Short communication

IN VITRO EFFECTS OF PROLONGED EXPOSURE TO QUERCETIN AND EPIGALLOCATECHIN GALLATE ON THE PERIPHERAL BLOOD MONONUCLEAR CELL MEMBRANE

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Abstract: The study aimed to assess biophysical changes that take place in the peripheral blood mononuclear cell (PBMC) membranes when exposed in vitro to 10 μM quercetin or epigallocatechin gallate (EGCG) for 24 and 48 h. PBMCs isolated from hypercholesterolemia patients were compared to those from normocholesterolemia subjects. The membrane fluidity and transmembrane potential were evaluated and the results were correlated with biochemical parameters relevant to oxidative stress, assessed in the patients' plasma. The baseline value of PBMC membrane anisotropy for the hypercholesterolemia patients was lower than that of the control group. These results correlated with the plasma levels of advanced glycation end products, which were significantly higher in the hypercholesterolemia group, and the total plasma antioxidant status, which was significantly higher in normocholesterolemia subjects. In the case of normocholesterolemia cells in vitro, polyphenols induced a decrease in membrane anisotropy (7.25–11.88% at 24 h, 1.82–2.26% at 48 h) and a hyperpolarizing effect (8.30–8.90% at 24 h and 4.58–13.00% at 48 h). The

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Abbreviations used: AGEs – advanced glycation end products; $DiBAC_4(3)$ – bis-(1,3-dibutylbarbituric acid) trimethine oxonol; DPPP – diphenyl-1-pyrenylphosphine; EGCG – epigallocatechin gallate; FCS – fetal calf serum; HC – hypercholesterolemia; NC – normocholesterolemia; PBMCs – peripheral blood mononuclear cells; TAS – total antioxidant status; TMA-DPH – 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluensulfonate

same effect was induced in hypercholesterolemia cells, but only after 48 h exposure to the polyphenols: the decrease in membrane anisotropy was 5.70% for quercetin and 2.33% for EGCG. After 48 h of in vitro incubation with the polyphenols, PBMCs isolated from hypercholesterolemia patients exhibited the effects that had been registered in cells from normocholesterolemia subjects after 24 h exposure. These results outlined the beneficial action of the studied polyphenols, quercetin and EGCG, as dietary supplements in normocholesterolemia and hypercholesterolemia patients.

Keywords: Membrane fluidity, Transmembrane potential, Total antioxidant status, Advanced glycation end products, Hypercholesterolemia, Quercetin, Epigallocatechin gallate

INTRODUCTION

Flavonoids are secondary products that are best known as the yellow, red, blue or purple pigments of various vegetal tissues [1, 2]. Apart from their physiological roles in plants, flavonoids are important components of the human diet. They exhibit multiple biological effects, including antioxidant, antiviral, antibacterial, anti-inflammatory, vasodilatatory, anticancer and hypoglycemic effects, and they reduce platelet aggregation, have anti-ischemic properties and improve capillary permeability and fragility [3–8]. Some flavonoids (e.g., the soy isoflavone subgroup) induce significant effects on erythrocyte membrane fluidity and deformability, affect the cellular mechanics and cause some endocrine perturbations [9, 10]. Epidemiological studies have also revealed that a flavonoid-rich diet correlates with increased longevity and decreased incidence of cardiovascular diseases [3, 11–14].

Polyphenol-rich diets are documented to reduce the incidence of atherosclerotic lesions. They also reduce the severity and progression of already developed lesions and lead to a reduction in dyslipidemia, endothelial dysfunction and hypertension. These benefits occur through several mechanisms, including: inhibition of LDL peroxidation, increase in the HDL level, reduction of platelet activation and thrombosis, inhibition of inflammatory processes, inhibition of MMP-2 expression and activity in vascular tissues, and anti-angiogenic properties [15–18]. The ability of polyphenols to enter membrane lipid bilayers is critical in explaining their antioxidant and cardio-protective effects. For instance, compounds that can locate to the non-polar region of the bilayer inhibit lipid oxidation either by intercepting intra-membrane radicals or by changing membrane fluidity, disorganizing lipid chains and hindering free radical propagation [9, 19–21].

The enhanced generation and accumulation of advanced glycation end products (AGEs) is known to contribute to various disorders, increasing the risk for macrovascular and microvascular complications associated with diabetes mellitus, aging and neurodegenerative diseases [22, 23]. Literature data suggests

that AGEs are decreased by some statins, which makes them useful for assessment in correlation with dyslipidemia [22].

We previously studied the effects induced by two polyphenol compounds – quercetin and epigallocatechin gallate (EGCG) – in different models of natural and artificial membranes. We demonstrated that the antioxidant effects of quercetin and EGCG correlate with their ability to modulate membrane anisotropy in a dose-dependent manner [19]. The study was performed on constitutively proliferating human cell lines (Jurkat T lymphoblasts and U937 monocytic cells), corresponding to peripheral leukocytes, in order to further translate the effects to in vivo human studies. We were looking for a reduction in the cardio-vascular risk mediated by the two compounds.

We previously demonstrated that the effect of short exposure (20 min) to the two polyphenols on peripheral blood mononuclear cells (PBMCs) correlates with inflammatory markers indicating endothelial dysfunction (MCP-1) and with the level of insulin resistance [24]. In this study, we used PBMCs isolated from patients with hypercholesterolemia and/or hyperglycemia to demonstrate the ability of the investigated compounds to reduce the cardio-vascular risk associated with insulin resistance and dyslipidemia.

We also evaluated the effect of the two polyphenols on the membrane of small unilamellar vesicles of 2-dimyristoyl-sn-glycero-3-phosphocholine with different amounts of cholesterol [25] and found that polyphenols modulate the transition from the gel phase to the liquid crystalline phase by rendering a more stable membrane in a dose-dependent manner (for quercetin) and independent of concentration (for EGCG).

The aim of this study was to investigate the changes that take place in the PBMC membrane of cells isolated from hypercholesterolemia (HC) patients compared to the changes in those from normocholesterolemia (NC) subjects. PBMCs were selected as the cellular model because they are involved in the initiation and pathological evolution of atherosclerotic lesions. Recent data suggest that the expression of genes such as IL4 in PBMCs could be a useful tool in the diagnosis of femoral artery atherosclerosis in asymptomatic patients [26]. Rentoukas et al. used PBMCs to study endothelial dysfunction associated with metabolic syndrome and proved that in patients with metabolic syndrome characterized by a strong dyslipidemia profile and low diabetes prevalence, significant telomerase activity was detected in circulating PBMCs, along with elevated markers of inflammation and endothelial dysfunction [27].

The results for the PBMC membranes were correlated with the levels of AGEs, the susceptibility to induced lipid peroxidation and the total antioxidant status (TAS) in plasma samples from the patients. We assessed the effects of quercetin and EGCG when cells were exposed in vitro for a prolonged time (24 and 48 h) to the mentioned compounds at a physiologically relevant concentration (10 μM). The exposure time was selected to simulate the chronic use of the tested compounds in the diet or in dietary supplements, when flavonoid blood levels are constantly elevated for a long period of time. However, the period was

limited to 48 h to avoid degradation of the flavonoids and other phenomena linked to the metabolism of cells maintained in culture for a prolonged time.

MATERIALS AND METHODS

Polyphenols

HPLC-grade quercetin dihydrate (code 551600, > 95% purity, Calbiochem – Merck-Millipore) and epigallocatechin gallate (code E4143, purity ≥ 95%, Sigma) were used in the cellular experiments without any prior chemical preparation. Stock solutions (10 mM) of the two polyphenols, respectively prepared in DMSO and water, were kept at –20°C between experiments.

Biological samples

The investigated group consisted of 25 subjects (12 NC subjects and 13 HC patients) that reported for their annual check-up at the Humanitas Medical Biochemical Analysis Laboratory in Bucharest. All of the patients gave informed consent for the study. The experiments were performed in vitro on cells isolated from human blood samples. Patients with severe renal, hepatic or hematological disease, overt cardiovascular disease, or malignancy were excluded from the study. None of the subjects had taken supplements containing known antioxidants (vitamin C, vitamin E, probucol, etc.) in the two weeks prior to the study. The selected subjects were non-smokers and had not consumed green or black tea in the two days prior to the visit. Fasting venous blood samples drawn on heparin as the anticoagulant were used for PBMC isolation, which was performed as previously described [24] using the gradient density centrifugation method on Hystopaque 1077 (Sigma-Aldrich). The resulting platelet-rich plasma was used for the evaluation of AGEs and hydroxiperoxides generation. The study was approved by the Ethics Committee of the Humanitas Medical Center and was carried out according to the principles of the Declaration of Helsinki (2000).

In vitro PBMC treatment

The PBMCs isolated from each patient were washed 3 times in RPMI 1640 culture medium and the cell density was adjusted to 10^5 cells/ml [24]. From each sample, cells were divided into three aliquots that were cultivated for 24 and 48 h respectively in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum (FCS), at 37ºC in a 5% CO_2 atmosphere. One cell aliquot constituted the control, the second was cultivated in presence of 10 μM quercetin, and the third was cultivated in the presence of 10 μM EGCG. Previous experiments showed that the small amount of DMSO in which quercetin was dissolved did not affect any of the investigated parameters [19].

Biochemical evaluation

Plasma samples were used for the metabolic evaluation of the patients. Fasting plasma glucose (Gli), total cholesterol (TC), HDL-cholesterol and triglycerides

(TG) were assayed on an Olympus 400 analyzer with BIORAD and RANDOX commercial kits. The total antioxidant status (TAS) was analyzed using the colorimetric TAS Randox kit (NX 2332, Randox laboratories), based on the inhibition of the production of 2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate] ($ABTS^+$) ion [28], according to the manufacturer's instructions [29].

Fluorescence anisotropy assay

The cell membrane anisotropy measurements were performed using the anisotropy value of 1-(4-trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene p-toluensulfonate (TMA-DPH), which was purchased from Life Technologies Inc. (T204, Molecular Probes). The method was a steady-state fluorescence polarization experiment, as described previously [30, 31]. Briefly, cell suspensions of 10^5 cells/ml were incubated with 2 μM TMA-DPH for 2 minutes at 37ºC in the dark, and then the fluorescence signal was recorded on a LS 50B spectrofluorimeter (Perkin Elmer Ltd.). The fluorescence anisotropy of TMA-DPH-labeled PBMCs was evaluated on the day of blood collection (baseline) and after in vitro incubation for 24 and 48 h with the polyphenols. In the last case, a control sample of cells incubated in the same culture media without polyphenols was also tested. The calculation of the fluorescence anisotropy (r) was performed according to equation (1):

$$r = \frac{I_{vv} - GI_{vh}}{I_{vv} + 2GI_{vh}} \quad (1)$$

where I_{vv}, I_{vh}, I_{hv} and I_{hh} denote the emission intensity (excitation at 340 nm, emission at 425 nm) when the polarizers in the excitation and emission beams were oriented vertical–vertical, vertical–horizontal, horizontal–vertical and horizontal–horizontal, respectively, and $G = I_{hv}/I_{hh}$ is an instrumental factor [32]. Membrane fluidity is proportional to the inverse of the squared anisotropy value (i.e. the higher the anisotropy value, the less fluid the membrane), as stated previously [30, 32].

Transmembrane potential evaluation

Bis-(1,3-dibutylbarbituric acid) trimethine oxonol, $DiBAC_4(3)$ from Life Technologies Inc. (B438, Molecular Probes) was used as a fluorescent probe for the evaluation of the transmembrane potential [31, 33]. PBMCs (10^5 cells/ml) were incubated with 2 μM $DiBAC_4(3)$ for 2 min at 37ºC in the dark. Using a LS 50B spectrofluorimeter (Perkin Elmer Ltd.), $DiBAC_4(3)$-labeled PBMCs were excited at 493 nm and fluorescence emission spectra were collected within the range 500 to 600 nm. Emission was measured at the peak, at 517 nm. Fluorescence intensity was measured at the baseline and after incubation of the cells with quercetin or EGCG. The membrane potential changes were evaluated as the absolute value of the fluorescent signal intensity.

Measurement of hydroperoxides generation
We used DPPP (diphenyl-1-pyrenylphosphine) to evaluate the lipid peroxidation processes in vitro. DPPP (code D7894, Life Technologies Inc., Molecular Probes) was dissolved in dimethylsulfoxide (DMSO) at a concentration of 5 mM. Previous experiments showed that DMSO (present at less than 1% in the biological samples) did not influence the measurements [19]. After the addition of DPPP at a final concentration of 5 μM, the biological samples were incubated for 20 min at room temperature, in the dark, then DPPP-labeled samples were treated with 10 μM cumene hydroxiperoxide (code 247502, technical grade, from Aldrich) to induce DPPP-oxide formation. The process was assessed by measuring the fluorescence emission spectra between 360 nm and 410 nm, with excitation at 351 nm, every minute, for 5 min, using a LS 50B spectrofluorimeter (Perkin Elmer Ltd.). The fluorescence intensity maximum was measured at 380 nm [34].

Evaluation of AGEs
The plasma level of fluorescent AGEs was evaluated using a spectrofluorimetric method [35]. Plasma samples were diluted 1:25 with PBS and excited at 350 nm. The emission was registered between 350 nm and 550 nm. Fluorescence of PBS alone was subtracted from each data set.

Fluorescence measurements
All the fluorescence measurements were carried out using a steady state LS 50B Perkin Elmer spectrofluorimeter equipped with an externally thermostated cell holder and a fluorescence polarization accessory.

Statistical analysis
Results were expressed as the mean ± standard deviation (SD). Inter- and intra-group differences were evaluated using the Mann Whitney U test, assuming that the groups included rather few subjects, and probably conform to a non-parametric distribution for the tested parameters. Differences were considered significant for $p < 0.05$.

RESULTS

The cellular parameters investigated in this study were chosen considering that proper anisotropy, transmembrane potential and low levels of lipid peroxides might constitute indicators for membrane integrity and function, which reflect on the overall cell functioning.

General metabolic and redox profile of the selected group
Results were analyzed in correlation with the levels of AGEs, TAS and DPPP-oxide generation in the plasma of the selected subjects. The baseline plasma parameters relevant for our study are presented in Table 1.

Table 1. Relevant plasma biochemical parameters of the investigated NC and HC subjects.

Biochemical parameter	Normocholesterolemia group (NC, n = 12)	Hypercholesterolemia group (HC, n = 13)
Fasting plasma glucose (mg/d)	105.11 ± 9.86	120.27 ± 32.22
Total cholesterol (mg/d)	129.49 ± 13.89	269.30 ± 26.51*
HDL (mg/dl)	43.02 ± 16.38	43.80 ± 0.95
Triglycerides (mg/dl)	124.69 ± 38.81	167.31 ± 67.47
TAS (μM Trolox equivalents/l)	2.71 ± 0.90	1.69 ± 0.51*
AGEs (fluorescence units)	524.85 ± 68.98	624.96 ± 134.59*
DPPP oxide (fluorescence units)	231.71 ± 133.54	299.36 ± 88.39*

The values are expressed as the mean ± SD. *$p < 0.05$ HC vs. NC

The level of total cholesterol was significantly different between the two groups of subjects, but the levels of HDL, glucose blood level and triglycerides were not. The recorded TAS values, which were higher for the NC group, confirmed the generally known fact that hypercholesterolemia is characterized by a lower endogenous antioxidant activity. This point is strengthened by the results obtained for the plasma AGE level, which was significantly higher for the HC group than for the NC one ($p = 0.046$).

According to the literature, hydroperoxides exogenously added to biological samples that were treated with DPPP induced an increase in fluorescence. The effect was very small if the peroxidation was induced with H_2O_2, but was significant and very rapid in the case of organic hydroperoxides. We observed that, in the case of HC patients, the generation of hydroperoxides in the plasma under the effect of cumene peroxide was significantly more intense than in samples from NC subjects ($p = 0.008$; Table 1).

Our results showed that there was an inverse correlation of the values obtained for DPPP-oxide and TAS in the plasma samples of the selected patients. The HC group, characterized by a reduced TAS value, generated an increased level of DPPP-oxide fluorescence (299.36 ± 88.39 relative fluorescence units). The NC group was characterized by significantly higher values of TAS ($p = 0.019$) and a lower DPPP-oxide generation (231.71 ± 133.54 relative fluorescence units).

Effects of the tested polyphenols on the membrane anisotropy of PBMCs

PBMC membrane anisotropy for HC patients was lower compared to the value corresponding to the control group (Table 2). The membrane anisotropy of untreated cells significantly increased after 24 and 48 h of cultivation, compared to baseline values. The increase was stronger in the case of HC patients (88.98% increase at 24 h and 111.54% at 48 h) compared to the NC subjects (63.1% increase at 24 h and 73.96% at 48 h; variations are calculated using the mean value for each group).

Table 2. Anisotropy values (*r*) for PBMCs after 24 and 48 h of incubation with quercetin and EGCG.

Group	Untreated cells	+10 μM quercetin	+10 μM EGCG
0 h incubation (harvesting day)			
NC	0.0888 ± 0.0248	-	-
HC	0.0771 ± 0.0131†*	-	-
24 h incubation			
NC	0.1448 ± 0.0541	0.1343 ± 0.0475	0.1276 ± 0.0521
% change	↑63.10$^{\#}$*	↓7.25§*	↓11.88§*
HC	0.1457 ± 0.0567	0.1557 ± 0.0560	0.1523 ± 0.0556
% change	↑88.98$^{\#}$*	↑6.86§	↑4.53§
48 h incubation			
NC	0.1545 ± 0.0270	0.1517 ± 0.0388	0.1510 ± 0.0263
% change	↑73.96$^{\#}$*	↓1.82§	↓2.26§
HC	0.1631 ± 0.0495	0.1538 ± 0.0383	0.1593 ± 0.0380
% change	↑111.54$^{\#}$*	↓5.70§	↓2.33§

The values are expressed as the mean ± SD, *$p < 0.05$. Statistical significance was analyzed on harvesting day for HC compared to NC (†). Values at 24 and 48 h were compared to the corresponding ones obtained on harvesting day ($^{\#}$). For 24 and 48 h exposure to polyphenols, the results were analyzed for treated cells compared to untreated ones (§).

After 24 and 48 h of incubation with the tested polyphenols, the membrane anisotropy of PBMCs isolated from NC subjects decreased compared to the control group. After 24 h the changes were significant (7.25% under the effect of quercetin and 11.88% under the effect of EGCG), but the effect following 48 h exposure was negligible in the NC cells.

For PBMCs from HC patients, following 24 h exposure to polyphenols, a further increase in the membrane anisotropy was registered. The anisotropy decrease (as observed in normal cells) was obtained only after 48 h of incubation with the tested polyphenols (5.70% under the effect of quercetin and 2.33% under the effect of EGCG). Our results showed that the general trend for the anisotropy response of PBMCs to flavonoid exposure (as indicated by the results obtained for NC) was a decrease, thus reflecting an increase in membrane fluidity (membrane "softening").

Effects of the tested polyphenols on the transmembrane potential of PBMCs

$DiBAC_4(3)$ is a slow-response potential-sensitive probe that can enter depolarized cells. It binds to membrane proteins, exhibiting enhanced fluorescence. An increase in depolarization results in additional influx of the anionic dye, leading to increased fluorescence. Conversely, hyperpolarization (increase in the absolute value of the membrane potential) is indicated by a decrease in fluorescence.

The baseline transmembrane potential of PBMCs was higher for NC subjects, compared to the HC group (Table 3). Incubation of PBMCs with RPMI and

FCS, in the absence of polyphenols, induced cellular depolarization, which was stronger in the case of HC cells (86.90% at 24 h and 122.09% at 48 h), compared to NC ones (82.31% at 24 h and 95.88% at 48 h).
The tested polyphenols induced the decrease of the fluorescence signal, thus showing a hyperpolarizing action. The effect was higher in the case of HC cells at 24 h (3.26% fluorescence decrease under the effect of quercetin and 6.92% decrease under EGCG effect for NC group; 8.90% fluorescence decrease under the effect of quercetin and 8.34% decrease under the effect of EGCG for HC cells). At 48 h, the same hyperpolarizing tendency was observed for both HC and NC cells. As such, the response of HC and NC cells to flavonoids in terms of transmembrane potential changes was a tendency to lower the depolarization normally observed at 24 and 48 h of cell cultivation (Table 3).

Table 3. The transmembrane potential (expressed as $DiBAC_4(3)$ absolute value of fluorescence intensity) for PBMCs after 24 and 48 h of incubation with quercetin and EGCG**.**

Group	Untreated cells	+10 μM quercetin	+10 μM EGCG
0 h incubation (harvesting day)			
NC	177.90 ± 44.05	-	-
HC	161.18 ± 30.27†*	-	-
24 h incubation			
NC	324.34 ± 46.09	313.76 ± 55.76	301.88 ± 32.76
% change	↑82.31$^{\#}$*	↓3.26§	↓6.92§
HC	301.24 ± 92.35	274.44 ± 178.55	276.12 ± 149.50
% change	↑86.90$^{\#}$*	↓8.90§*	↓8.34§*
48 h incubation			
NC	348.47 ± 55.98	307.84 ± 38.74	325.70 ± 72.33
% change	↑95.88$^{\#}$*	↓11.66§*	↓6.53§
HC	357.97 ± 58.28	341.59 ± 68.77	311.45 ± 66.31
% change	↑122.09$^{\#}$*	↓4.58§	↓13.00§*

The values are expressed as the mean ± SD, *$p < 0.05$. Statistical significance was analyzed on harvesting day for HC compared to NC (†). Values at 24 and 48 h were compared to the corresponding ones obtained on harvesting day ($^{\#}$). For 24 and 48 h exposure to polyphenols, the results were analyzed for treated cells compared to untreated ones (§).

DISCUSSION

Long-term consumption of a polyphenol-rich diet is associated with a reduction of cardio-vascular (CVD) risk, according to the literature [11, 12]. This study aimed to assess the effects of prolonged in vitro exposure to quercetin and EGCG (exposure time of 24 and 48 h, respectively) on the membrane anisotropy and transmembrane potential of PBMCs isolated from NC subjects and HC patients. We performed the study on PBMCs because these cells are directly involved in the development of CVD generally associated with hypercholesterolemia. The aim of the study was to investigate whether the ability of polyphenols to reduce

CVD incidence might be correlated to their effect on cell membranes. Biophysical membrane parameters (membrane anisotropy and transmembrane potential) were analyzed in correlation with redox status markers in the plasma (AGE level, total antioxidant status and susceptibility to induced lipoperoxidation via the measurement of hydroxiperoxide generation).

The consumption of antioxidant supplements was limited for the selected patients for two weeks prior to the study in order to allow the evaluation of their endogenous antioxidant status and the total hydroperoxide level in the plasma. It was assumed that these parameters are influenced by the fat-soluble molecules, which are characterized by their tendency to remain in the system for prolonged periods of time.

This study was a preliminary one, with a quite small number of subjects included in the experiments, so there is an inherent limited statistical power of the study.

AGEs result from the non-enzymatic glycation and oxidation of proteins and lipids. Under normal physiological conditions, AGEs are formed at a very slow rate. Their production is accelerated in ageing, inflammatory conditions, and in cases of hyperglycemia and oxidative stress [34–39]. Various studies showed that AGEs impaired vascular function by accumulating in the vessel wall and by quenching the nitric oxide released as an endogenous vasodilatatory and antithrombotic molecule, thereby potentially impacting on vascular relaxation and function. AGEs bound to their specific receptors (RAGE) in the endothelium triggered the production of reactive oxygen intermediates [39–42]. Therefore, AGEs induce vascular pathological changes through direct and also through an indirect (redox stress) mechanism.

TAS evaluation is generally used to assess the overall capacity of the body to fight against reactive oxygen species. TAS evaluation is also used in research settings for the in silico evaluation of a biological sample's ability to neutralize reactive oxygen species. The lower the TAS value, the lower the antioxidant capacity of the sample.

DPPP added to the plasma samples reacted with lipids (including lipoprotein particles), so the fluorescence increase registered after cumene peroxide addition reflected the peroxidation of lipids. In order to confirm the results obtained by this method, we correlated them with TAS values. Our results showed that, for HC patients, the plasma lipid peroxidation susceptibility was higher than in the case of NC subjects. In the case of HC patients, the level of TAS was significantly reduced compared to NC ones, allowing a more intense oxidation of lipids and lipoproteins in plasma. The same kind of difference was obtained for the AGE levels.

Our study aimed to correlate the cell membrane properties with the parameters that characterize the extracellular environment (plasma). This is a limitation of our approach due to the lack of data regarding the intracellular antioxidant systems. Regarding the correlation with the glutathione system, Pytel et al. performed a study on red blood cells isolated from patients with previous myocardial infarction and proved that no differences in glutathione peroxidase

activity or in the level of SH groups were observed between patients with CVD in comparison to healthy controls [43], even if there was an increase in the level of lipid peroxidation (13%) and total cholesterol (19%), and a decrease in membrane fluidity (14%) in the sub-surface layers and in the deeper layers of erythrocyte membrane (7%). Ziobro et al. observed that the concentration of thiol groups in erythrocyte membranes from patients with metabolic syndrome was significantly lower (about 16%) compared to the control group. The results were correlated with a substantially lower membrane fluidity (metabolic syndrome vs. control) [44].

The biochemical profile of the investigated patients indicated the increase in the CVD risk for HC patients compared to NC subjects. This type of risk is associated with changes in the membrane function of PBMCs, which have an increased tendency to infiltrate under the endothelium. In order to support this theory, the membrane anisotropy of PBMCs isolated from the selected patients and the transmembrane potential were evaluated at baseline and after prolonged in vitro exposure (24 and 48 h) to two polyphenols (Q and EGCG) at physiological exposure levels (10 μM) [15].

Membrane microviscosity and transmembrane potential are among the most important biophysical properties of cell membranes. There are several instances in the literature that argue that the microviscosity of cell membranes is directly influenced by the effect of toxic stimuli. Sergent stated that some xenobiotics (such as tacrine, ximelagatran or ethanol) that induce liver injury increase the hepatocytes membrane fluidity, thus leading to disturbances in the cell function [40]. A role for membrane fluidity in red blood cell deformability and stability was recently suggested [45, 46]. It was proven that at lower alcohol concentrations (methanol, ethanol, propanol and butanol), fluidity gradually increased and the effect was reversed at higher concentrations.

Many studies were devoted to the effect of flavonoids on the cell membrane, using different methods to assess membrane fluidity [10, 21, 47, 48]. Steady-state fluorescence polarization of TMA-DPH bound to the cell membrane was used in this study, as the dye displays in isotropic media-specific fluorescence anisotropy properties, which are changed if the free orientation of the probe is restricted by the local microenvironment: membrane fluidity is proportional to the inverse of squared anisotropy [19, 30, 31].

Polyphenols could affect cell function by modifying plasma membrane structure and physical characteristics, such as fluidity and electrical properties [19, 24, 25, 43, 47–52]. EGCG for example, interacts with the lipid rafts, thus disturbing the downstream activation of signals involved in the allergic response and in cell proliferation [53]. Most flavonoids, mainly due to their lipophilicity, have the tendency to accumulate in biological membranes, particularly in lipid rafts, where they can interact with different receptors and signal transducers and influence their function through the modulation of the lipid-phase behavior [54]. In vitro studies demonstrated that compounds differing in the number of hydroxyl groups showed a broad distribution along the membrane [55].

There are also studies pointing out that the effects of genistein [21], which is a well-known isoflavone, in combination with cholesterol-lowering therapies inhibited prostate cancer cells by downregulation of the androgen receptor in the lipid raft microdomain, and influenced signal transduction pathways [56].

The results obtained in this study showed that baseline membrane anisotropy of PBMCs was lower for HC patients compared to the NC group. Incubation of PBMCs with RPMI culture media supplemented with 10% FCS led to a significant increase of membrane anisotropy (compared to the baseline value), both at 24 and at 48 h. This is probably due to the ageing process of the cells. The increase was significantly higher in the case of HC cells compared to NC cells, so cell membranes from control subjects proved to be less susceptible to become more rigid in vitro.

Cell membranes have a complex composition, where lipids can be continuously added to or removed from microdomains, depending on the culture media and conditions, thus influencing the physical properties, including fluidity [57–59]. When dealing with cultured mammalian cells, these changes can be sufficient to alter membrane functions. Spector and Yorek believed that the best approach in the study of such alteration is to make functional observations with cultured cells and then explore the mechanism producing these effects with either a reconstituted system or isolated membranes modified by incubation with liposomes [60]. However, this approach needs not only a detailed analysis of the linkages between theoretical and experimental studies, but also different methods to evaluate cell membrane properties [61–63].

Ageing can also influence the cell membrane composition. Naudi et al. stated that during ageing the membrane fatty acid profile changes with increased peroxidizable PUFAs. Consequently, a relative increase in the PUFA content of a membrane would be expected to render the membrane more fluid. Paradoxically, the opposite is systematically reported: membrane fluidity decreases with age, probably due to a change in the cholesterol content of the membrane and the oxidation of membrane components [64].

The polyphenols tested in this study, quercetin and EGCG, induced a decrease in membrane anisotropy (compared to control cells grown in polyphenol free media) after 24 and 48 h of exposure, in the case of NC cells. The same effect was induced in the case of HC cells, but only after 48 h, indicating that a longer time is necessary in order to obtain this effect. Accordingly, there is a tendency for the polyphenols to reduce the changes in the cell membranes and to diminish the rigidity that they exhibit in vitro.

The hyperpolarizing action of the tested polyphenols was induced at 24 and 48 h incubation in both the NC and HC group. At all incubation periods, the level of variation registered for NC cells was significantly lower than the effect obtained for HC cells. Therefore, we can assume that PBMCs isolated from normocholesterolemia environments have an increased stability to outside stimuli, compared to PBMCs isolated from hypercholesterolemia patients.

Still, we need to point out that not all studies support the beneficial effects of flavonoids. For example, van der Heide et al. suggested that several flavonoids could interfere with thyroid hormone synthesis, inhibiting the organification in thyroid cells, displacing T4 from transthyretin, and leading to disturbances in thyroid hormone availability in the tissues [65]. Also, some flavones and isoflavones had the ability to interfere with the activity of the estrogen receptor α, to disrupt estrogen pathways and alter the steroid hormone synthesis [66–68].
Flavonoids, like many other antioxidants, can act as pro-oxidants in particular circumstances. Some authors stated the possibility that flavonoid pro-oxidant function could be related to their beneficial actions. One example is EGCG, which reduces O_2 to yield H_2O_2, thus promoting apoptosis and having bactericidal activity [3].
According to our in vitro results, polyphenols like quercetin and EGCG could be administered as chronic treatment, especially in conditions associated with impaired lipid metabolism, in order to induce the expected effects on PBMC cell membranes. Their effect on the PBMC membrane could represent one of the mechanisms leading to the overall reduction of atherosclerosis and the incidence of cardiovascular events induced by polyphenol-rich diets.

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