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Biophysical characterization of genistein–membrane interaction and its correlation with biological effect on cells — The case of EYPC liposomes and human erythrocyte membranes

Bożena Pawlikowska-Pawlęga [a,b,*], Lucjan E. Misiak [c], Anna Jarosz-Wilkołazka [d], Barbara Zarzyka [e], Roman Paduch [f], Antoni Gawron [a], Wieslaw I. Gruszecki [g]

[a] Department of Comparative Anatomy and Anthropology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[b] Electron Microscopy Laboratory, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[c] Institute of Physics, Maria Curie-Skłodowska University, Pl. M. Curie-Skłodowskiej 1, 20-031 Lublin, Poland
[d] Department of Biochemistry, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[e] Department of Cell Biology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[f] Department of Virology and Immunology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland
[g] Department of Biophysics, Institute of Physics, Maria Curie-Skłodowska University, Pl. M. Curie-Skłodowskiej 1, 20-031 Lublin, Poland

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ABSTRACT

With application of EPR and ^{1}H NMR techniques genistein interaction with liposomes formed with egg yolk lecithin and with erythrocyte membranes was assessed. The present study addressed the problem of genistein localization and its effects on lipid membrane fluidity and protein conformation. The range of microscopic techniques was employed to study genistein effects on HeLa cells and human erythrocytes. Moreover, DPPH bioassay, superoxide anion radical test and enzymatic measurements were performed in HeLa cells subjected to genistein. The gathered results from both EPR and NMR techniques indicated strong ordering effect of genistein on the motional freedom of lipids in the head group region and the adjacent hydrophobic zone in liposomal as well as in red blood cell membranes. EPR study of human ghost showed also the changes in the erythrocyte membrane protein conformation. The membrane effects of genistein were correlated with the changes in internal membranes arrangement of HeLa cells as it was noticed using transmission electron microscopic and fluorescent techniques. Scanning electron and light microscopy methods showed that one of the aftermaths of genistein incorporation into membranes was creation of echinocytic form of the red blood cells with reduced diameter. Genistein improved redox status of HeLa cells treated with H_2O_2 by lowering radicals' level.
In conclusion, the capacity of genistein to incorporate, to affect membrane organization and to change its biophysical properties is correlated with the changes inside the cells.

1. Introduction

Genistein constitutes an important component in the majority of people's daily diet. The common source of it is soybean [1]. This naturally occurring flavonoid has a lot of beneficial properties on human health and because of that it is one of the most studied isoflavones [2]. There is a great interest in flavonoids' potential health benefits that result mainly from their anticancer activity as well as from their ability to reduce heart diseases [3]. Many studies have shown that flavonoids, including genistein, can inhibit the growth of various cancer cell lines including: leukemia, lymphoma, prostate, breast, lung and head and neck cancer cells, both in vitro and in vivo [4,5]. The fact that flavonoids can block or reverse carcinogenesis, makes them promising cancer chemopreventive agents. Genistein is known from its antioxidant, radical scavenging and antimicrobial activities. It has also estrogenic properties and is able to prevent osteoporosis [6–8].

Abbreviations: DPPH• radical, 1,1-diphenyl-2-picrylhydrazyl radical; EPR spectroscopy, electron magnetic resonance spectroscopy; EYPC, 1,2-diacyl-sn-glycero-3-phosphocholine from egg yolk; NMR spectroscopy, nuclear magnetic resonance spectroscopy; 5-SASL, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical; 16-SASL, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical; SAR, superoxide anion radical; Tempo, 2,2,6,6-tetramethyl-1-piperidinyloxy free radical, 4-maleimido Tempo

* Corresponding author at: Department of Comparative Anatomy and Anthropology, Maria Curie-Skłodowska University, Akademicka 19, 20-033 Lublin, Poland. Tel.: +48 81 537 59 28; fax: +48 81 537 59 01.
E-mail addresses: bozka1996@o2.pl (B. Pawlikowska-Pawlęga), lucjan.misiak@umcs.pl (L.E. Misiak), anna.wilkolazka@poczta.umcs.lublin.pl (A. Jarosz-Wilkołazka), basiaza@vp.pl (B. Zarzyka), rpaduch@poczta.umcs.lublin.pl (R. Paduch), agawron@poczta.umcs.lublin.pl (A. Gawron), wieslaw.gruszecki@umcs.pl (W.I. Gruszecki).

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Ability to interact, permeate and alter biophysical parameters of the membrane is of crucial importance for biological effects of flavonoids. The knowledge concerning drug–membrane interactions is very important since the membrane is the first barrier for the drug on the way into a cell. Moreover, antioxidant activity, transport quality through the cell membrane, and other membrane related-processes such as signal transduction, electron transfer and protein and lipid translocation are coupled with ability of flavonoids to incorporate into membranes [9–14].

In recent years the interactions of flavonoids, including genistein, with the membrane are a frequently appearing subject of articles [15–18] but very few of them applied EPR technique. In our previous work on the saturated DPPC liposomes we have provided evidence of genistein interaction with membrane and revealed molecular mechanism of genistein interaction with lipid membrane [13].

Taken into account the antioxidant activities of genistein, its widespread occurrence in food and its beneficial effects on human health we decided to examine the action of genistein on purified lipids made of EYPC and on biological membranes of human erythrocytes. We decided to examine EYPC liposomes because these model membranes are unsaturated and resemble natural membranes due to high amount of phosphatidylcholine in them. In turn, ghost of human red blood cells are natural membranes that have trilamellar composition that consists of lipid bilayer and underlying protein network. In this regard we wanted to characterize the effects of genistein on the biophysical properties of membranes to extend the knowledge concerning genistein–membrane interactions and to get holistic estimation of such relationship. We also wanted to further correlate these effects with some of its biological properties. Our investigations were performed at physiological pH to maintain the same conditions that are found in vivo.

Thus in the present paper we applied the spin label electron paramagnetic resonance (EPR) in order to examine genistein effect on the lipid fluidity and the conformational changes of membrane proteins. Nuclear magnetic resonance (^{1}H NMR) measurements were also carried out to gain a deeper insight into the genistein–membrane relationship. Transmission, scanning electron and fluorescence microscopy techniques were additionally performed to address the problem of influence of genistein on human cervix carcinoma and red blood cells. Additionally antioxidant tests were performed with usage of DPPH assay, superoxide anion radical examination together with the investigation of chosen enzymes' activities in HeLa cells.

2. Materials and methods

2.1. Chemicals

Genistein (Sigma Chemical Co., USA) dissolved in ethanol (Merck, Germany) was used in the studies. The solution was kept in the dark. Spin labels: 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxy free radical (5-SASL), 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxy free radical (16-SASL), 2,2,6,6-tetramethyl-1-piperidinyloxy (Tempo), 4-maleimido-Tempo, 1,2-diacyl-*sn*-glycero-3-phosphocholine from egg yolk (EYPC) and TRIS were purchased from Sigma Chemical Co. Deuterium oxide (D_2O) was purchased from ARMAR Chemicals Co. (Switzerland). Spin labels were dissolved in absolute ethanol and stored at 4 °C. All other chemicals were of the best quality available.

2.2. Human ghost isolation

Erythrocyte membranes were obtained by hypotonic lysis according to the procedure of Dodge et al. [19] at 4 °C and then suspended in PBS, pH 7.4. Protein concentration was evaluated by the method of Bradford [20] using BSA as a standard.

2.3. Nuclear magnetic resonance (NMR) measurements

For the ^{1}H NMR spectroscopy measurements, mixtures of phospholipids and isoflavonoid were co-dissolved in chloroform/ethanol mixture (55:1 v:v) at the respective concentration [13]. The lipid concentration in the sample was 3.2×10^{-2} M and of the flavonoid was 3.2×10^{-4} M. After evaporating the solvents under a stream of nitrogen and then by vacuum (4 h), the samples were hydrated with D_2O and vigorously shaked (1 h) on a shaker at room temperature. Then the lipid suspension was sonicated (8×3 s) to yield a homogenous lipid dispersion at 4 °C. Shortly before measurements 4 mM praseodymium trichloride ($PrCl_3$) was added. ^{1}H NMR spectra were performed on a Bruker Avance 300 NMR spectrometer using 5-mm probe with pulsed field gradient capabilities. The ^{1}H NMR parameters were as follows: spectral window 3906 Hz, digital resolution 0.238 Hz, pulse width 6.0 ms, acquisition and delay time were 2.09 s and 3 s, respectively, and acquisition temperature 333 K. Relatively high temperature of NMR has been selected in order to compare present data with the previous experiment performed with model membranes formed with DPPC.

2.4. Electron paramagnetic resonance (EPR) measurements of liposomes and human ghosts

In our work we examined the effect of genistein on egg yolk lecithin liposomes representing unsaturated type of membranes. The concentration of the lipid (EYPC) in phosphate buffer was 10^{-5} M. The concentration of genistein was 5 mol% and of the spin label 1 mol% with respect to the lipid. Dispersion of EYPC (100 mg/ml of chloroform) was prepared by mixing solutions of respective compounds and evaporating solvent, first in a stream of nitrogen and subsequently by vacuum (3 h). EYPC samples were hydrated with the phosphate buffer (100 mM, pH 7.4) then manually agitated at room temperature and the lipid suspension was sonicated in buffer to yield homogenous lipid dispersion. The samples, to be measured, were placed in a 1.3-mm diameter capillary (Hyland Lab. Inc.) and sealed with miniseal wax. EPR spectra were recorded with a SE/X-2547 (Radiopan, Poznań) spectrometer working at the X band and equipped with variable temperature-stabilizing unit under the following conditions: modulation amplitude 5 G in the case of spectra scanning and 10 G for determining an accurate position of the maxima time constant 0.3 s, scan time 2 min, and scan range 3200–3300 G. The n-SASL spin labels applied in the study are commonly used to monitor the fluidity of model membranes [13,21]. In the spectra, the maximum splitting value $2T'_{||}$, an empirical parameter related to an order parameter of the alkyl chain and the rate of the alkyl motion of the spin label in a lipid core, reflecting the fluidity of a membrane, was analyzed.

In ghosts of human red blood cells we have applied Tempo and maleimido Tempo spin labels. Before measurements the ghosts were incubated with genistein solutions (0 and 50 μg/ml) in isotonic PBS solution. The suspensions of erythrocytes membranes in a PBS (pH 7.4) was vigorously vortexed in a glass tube with a film of Tempo spin label. The concentration of the label was 2.5×10^{-4} mol per 1 mg of total protein in the erythrocyte membranes. Maleimido spin label examines the changes in protein conformation of the membranes [12]. The membranes were incubated with various concentrations of genistein (10, 25, 50 μg/ml) in the dark for 1 h at 37 °C. After incubation, the membranes were washed three times with PBS and then labeled with maleimido Tempo spin label for 12 h at 4 °C, in a ratio of 1 mg per 25 mg of membrane protein. We have examined the changes of partition coefficient B/A using polar spin label Tempo. High field spectra of Tempo spin label show two peaks: one correspondent to a relatively mobile fraction of spin label in the water phase (A) and the fraction of spin label immobilized within the membrane (B). This parameter is also connected with the membrane fluidity [12,22].

In the studies the changes of W/S ratio have been monitored to examine the changes in protein conformation. Maleimido tempo binds

covalently to membrane proteins and (W) represents weakly and (S) strongly immobilized state of the labeled sites in the membrane proteins.

EPR spectra were finally measured using typical instrument parameters: modulation amplitude 2 G, time constant 1 s, scan time 4 min, and scan range 3300–3400 G.

All data are expressed as means ± SD (n = 3). Statistical comparison was performed by Student's t-test and values of $p \leq 0.05$ were considered significant.

2.5. Cell culture

In experiments, human cervix carcinoma cell line (HeLa B, ECACC No. 85060701) cultured in RPMI 1640 medium (GIBCO BRL) supplemented with 5% fetal bovine serum (FBS) (GIBCO BRL) (v/v) and antibiotics (penicillin 100 units/ml, streptomycin 100 μg/ml, amphotericin B 0.25 μg/ml) (Sigma) was used. Cells at density of 1×10^6 cells/ml were seeded on a cover slide in Leighton dishes (2 ml) and in Falcon vessels for electron microscopy observation (5 ml). To dissolve genistein in the growth medium a stock solution (15 mg/ml) in dimethyl sulfoxide (DMSO) was applied. Then the cells were incubated at 37 °C in humidified atmosphere of 5% CO_2/95% in an incubator. The cultures were incubated with 15 μg/ml of genistein. The final concentration of DMSO in culture medium did not exceed 0.25%.

2.6. Cell viability analysis by neutral red (NR) uptake assay

NR cytotoxicity assay was based on the uptake and lysosomal accumulation of the supravital dye, neutral red. Dead or damaged cells do not take up the dye [23]. Cells were grown in 96-well multiplates (HeLa for 4 and 24 h) in 100 μl of culture medium with genistein at the doses of 5, 10 and 15 μg/ml. Subsequently, the medium was discarded and 0.4% NR (Sigma) solution medium was added to each well. The plate was being incubated for 3 h at 37 °C in a humidified 5% CO_2/95% air incubator. After incubation, the dye-containing medium was removed, cells fixed with 1% calcium chloride ($CaCl_2$) in 4% paraformaldehyde, and thereafter the incorporated dye was solubilized using 1% acetic acetate in 50% ethanol solution (100 μl). The plates were being gently shaken for 20 min at room temperature and the extracted dye absorbance was measured spectrophotometrically at 550 nm using a microplate reader (Emax; Molecular Devices Corp., Menlo Park, CA). Three independent experiments were performed. The results were calculated as a percentage of control, arbitrarily set to 100%.

2.7. Fluorescence of endoplasmic reticulum under the influence of genistein

HeLa cells were incubated with genistein (15 μg/ml) for 4 h in the dark at 37 °C. Then the cells were stained by 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (Vybrant DIL) (Lonza) dissolved in growth medium, incubated at 37 °C for 15 min [13]. Fluorescence of endoplasmic reticulum in the cells on cover glass was analyzed under a fluorescence microscope NIKON E-800 (Japan). At least 100 cells in randomly selected microscopic fields were observed under a microscope. The data were registered in the fluorescence channel (λ = 488 nm). The relative level of pixel fluorescence was measured along a chosen line passing through the cytoplasm and nucleus using LSM5 Image Examiner software (Zeiss, Germany).

2.8. Transmission electron microscopy of HeLa cells

The cells were being treated with genistein at the concentration of 15 μg/ml for 24 h. HeLa cells were gently scraped off flasks using a cell scraper. Then they were fixed in 4% glutaraldehyde in 100 mM cacodylate buffer for 2 h and in 1% osmium tetroxide for the next 2 h, all at 4 °C. The cells were dehydrated in series of alcohol and acetone and embedded in LR White resin. Ultrathin sections were cut with a diamond knife on microtome RMC MT-XL (Tucson, AZ, USA), collected on copper grids and contrasted with the use of uranyl acetate and Reynold's liquid. For each experimental variant at least 100 cells were examined. The samples were observed under a LEO-Zeiss 912 AB electron microscope (Oberkochen, Germany).

2.9. Measurements of erythrocyte diameter and their shapes

The cells were incubated with genistein solutions (10, 25, 50 μg/ml) in PBS buffer (37 °C, 1 h) in the dark. Then the cells were fixed with 4% glutaraldehyde (30 min) and suspended in isotonic saline buffer. The diameter of 200 cells was measured in a microscope with micrometric ocular. Only non-deformed cells were measured. For the quantitative analysis of the erythrocytes' shapes, the cells were analyzed under a light microscope. In each samples 1000 cells were observed. All data are expressed as means ± SD (n = 3). Statistical comparison was performed by Student's t-test and values of $p \leq 0.05$ were considered significant.

2.10. Electron microscopy of red blood cells

2.10.1. Negative staining of red blood cells in TEM

Erythrocytes were suspended in PBS (pH 7.4) with genistein solution 0 (control) and 10, 25, and 50 μg/ml. Then 1% of ammonium molybdate was added for 10 min and the suspension of the cells was deposited on formvar coated grids and dried at room temperature for 10 min. The samples were observed under a LEO-Zeiss 912 AB electron microscope (Oberkochen, Germany).

2.10.2. Scanning electron microscopy (SEM)

Red blood cells were fixed with 4% glutaraldehyde and 1% osmium tetroxide in 100 mM cacodylate buffer for 2 h and in 1% osmium tetroxide for the next 2 h, all at 4 °C. Then the cells were dehydrated in a graded series of acetone, dried in critical point and coated with gold in Emitech K550X Sputter Coater. The samples were observed with a TESCAN vega 3 LMU microscope (Czechy).

2.11. Enzymatic measurements

For enzymatic measurements the cells were incubated with 5, 10, and 15 μg/ml of genistein. The final concentration of DMSO in culture medium did not exceed 0.25%. To evoke oxidative shock (Os), cells were being incubated with hydrogen peroxide (2 mM) for 1 h. Then the cells were rinsed with phosphate buffer (pH 7.4) and incubated at 37 °C for 18 h. In other experimental variant the cells were incubated with genistein and hydrogen peroxide for 2.5 h at 37 °C. Genistein was added 1.5 h before the oxidative shock and was present during oxidative shock. Medium was removed from flasks, and monolayer cells were rinsed with PBS, scraped using cell scrapers, and resuspended in cold PBS. Following centrifugation for 10 min 8000 ×g, at 4 °C, the supernatant was discarded, cells were resuspended in cold PBS and homogenized in an ice-chilled motor-driven Potter S homogenizer (Sartorius, Germany). The homogenates were then centrifuged for 10 min at 10,000 ×g, at 4 °C, and an aliquot of the supernatant was used for the respective enzyme assay. Catalase (CAT) activity was determined by following decrease of absorbance during the decomposition of H_2O_2 [24]. Superoxide dismutase (SOD) activity was assayed by its ability to inhibit the auto-oxidation of adrenaline determined by the increase in the absorbance at 480 nm at 30 °C, as previously described [25] and expressed as U/mg of proteins. The measurement of the level of superoxide anion radicals (SAR) was done according to the method previously described [26].

2.12. DPPH• free radical scavenging test

Free radical scavenging activity of genistein was measured by the 1,1-diphenyl-2-picrylhydrazyl (DPPH•) assay [14]. This method is based on the ability of antioxidants to reduce the stable dark violet radical DPPH• (Sigma) to the yellow colored diphenyl-picrylhydrazine. Briefly, 100 μl of DPPH• solution (0.2 mg/ml in methanol) was added to 100 μl of genistein at concentrations 5, 10 and 15 μg/ml. Trolox (Sigma) at increasing concentrations (1–50 μg/ml) was used as a reference for free radical scavenging activity. After 10 min of incubation at room temperature, the absorbance of the solution was measured at 515 nm. The lower the absorbance, the higher is the free radical scavenging activity of genistein. The activity of genistein was determined by comparing its absorbance with that of a blank solution (reagents without genistein) and standard. The capability to scavenge DPPH• radical was calculated by the following formula:

$$\text{DPPH}\bullet\text{ scavenging effect }(\%) = [(\text{Xcontrol}-\text{Xgenistein}/\text{Xcontrol}) \times 100]$$

where: Xcontrol is the absorbance of the control and Xgenistein is the absorbance in the presence of genistein.

3. Results

3.1. ^{1}H NMR investigation of EYPC liposomes

Fig. 1 presents ^{1}H NMR spectrum of EYPC liposomes and EYPC liposomes with addition of 1 mol% genistein and Table 1 shows parameters that were measured in our investigations. Starting from right-hand side several bands are visible in the graph. The first and second are the bands corresponding to CH_3 and CH_2 groups of the hydrophobic regions of the membrane. The third and fourth are the bands of choline groups from polar head region of the membrane. The liposome suspension was supplemented with $PrCl_3$. Addition of praseodymium ions effects in the split of the ^{1}H NMR band corresponding to the $-N^+(CH_3)_3$ group owing to the pseudocontact shifts produced by shift reagents from the group of lanthanides (e.g. Pr^{3+}) [27]. The resonance maximum that shifted towards higher ppm values corresponds therefore to the lipid molecules forming the outer leaflet of the liposome membranes, whereas the one

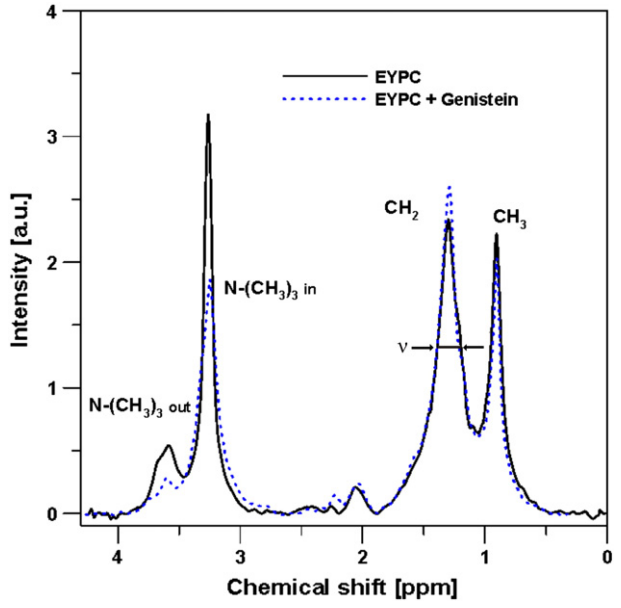

Fig. 1. ^{1}H NMR spectra of liposomes formed with pure EYPC and EYPC with genistein at 1 mol%. $PrCl_3$ was added to the samples before measurement. The resonance line assignment and parameters used in spectral analysis are shown in the graph. The following parameters were determined: the full width at a half height (ν) and the I_{out}/I_{in} ratio.

Table 1
Parameters of NMR spectra of EYPC liposomes and EYPC liposomes with addition of 1 mol% genistein (EYPC + G).

Liposome composition	Parameter				
	ν CH_3 [ppm]	ν CH_2 [ppm]	ν $(N^+–CH_3)_{in}$ [ppm]	I_{in} [a.u.]	I_{out} [a.u.]
EYPC	0.108	0.221	0.080	3.140	0.650
EYPC + G	0.094	0.183	0.161	1.860	0.335

ν is the full width at a half height in different regions of membrane: hydrophobic (corresponding to CH_3 and CH_2) and polar head region (choline groups). Inner (I_{in}) and outer (I_{out}) liposome leaflets of the membrane from the choline head region are also included.

that shifted towards lower ppm values corresponds to the inner liposome surface. The ratio of the areas under the signal assigned to the outer layer to that assigned to the inner layer (I_{out}/I_{in}, outer to inner) is proportional to the number of choline heads in the outer and inner layers. It is obvious that the number of lipid molecules in the outer layer is greater than that in the inner layer, so for unilamellar liposomes the ratio I_{out}/I_{in} is greater than 1. The smaller the liposomes are the lower the number of the lipid molecules that can fit the inner layer of the liposome, so the higher the ratio I_{out}/I_{in} [27,28]. The I_{out}/I_{in} ratio smaller than 1 indicates formation of multilamellar liposomes.

Addition of genistein caused the change of the full width at a half height (ν) of the ^{1}H NMR. The decrease in the case of CH_3 group (13%) was observed, as well as for CH_2 groups (17%). On the other hand pronounced ordering effect on the motional freedom of lipids was observed in the head group region as it was manifested by an increase in the ν of the choline group in inner liposome surface. The presence of genistein caused the increase of ν by 101%. Moreover, the presence of genistein changed the I_{out}/I_{in} ratio from 0.21 in the case of pure EYPC to 0.18 in the case of liposomes with addition of isoflavonoid. Both ratios indicate the formation of multilamellar liposomes. The effect discussed about points out that genistein intercalates into polar head region.

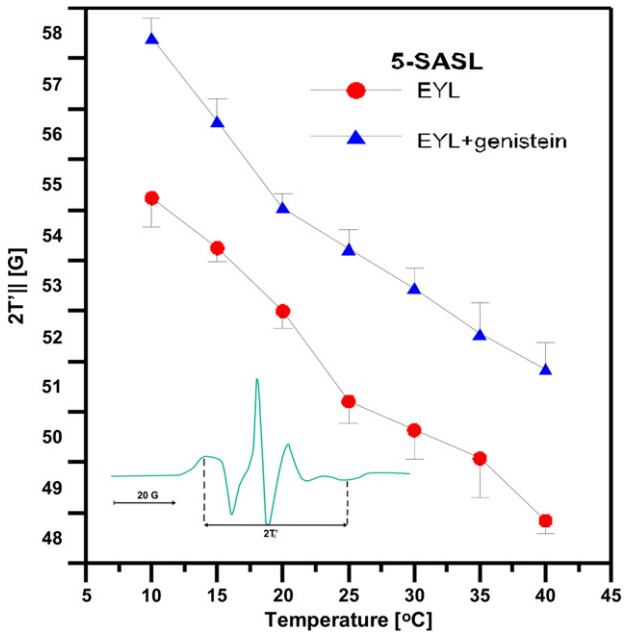

Fig. 2. The effect of genistein on a maximum splitting parameter ($2T'_{||}$) as a function of temperature of 5-doxylstearic acid (5-SASL) spin label doped into unilamellar egg yolk lecithin (EYPC) liposomes. (●) Liposomes of EYPC with addition of spin label at the concentration of 1 mol%. (▲) Liposomes with genistein at the concentration of 5 mol% and spin label at the concentration of 1 mol%. The concentration of lipid in 100 mM phosphate buffer was 10^{-5} M. The measurements were performed at various temperatures (10–40 °C). The spectrum was scanned at modulation amplitude 5 G.

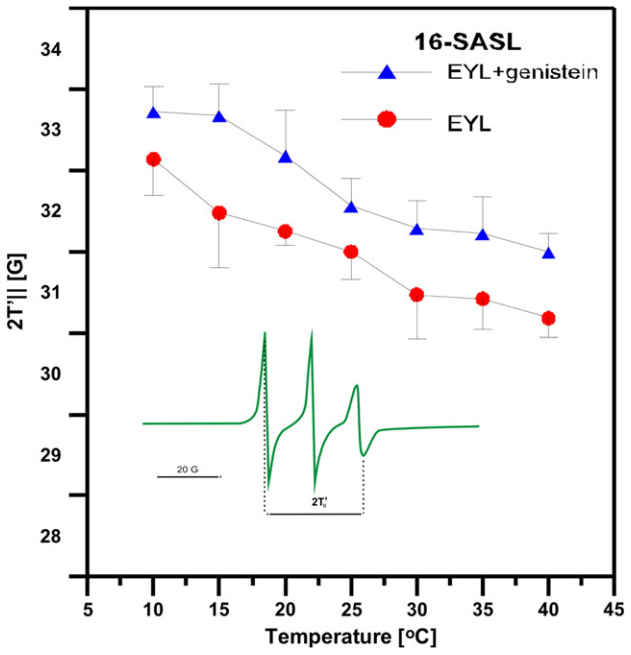

Fig. 3. The effect of genistein on a maximum splitting parameter ($2T'_{||}$) as a function of temperature of 16-doxylstearic acid (16-SASL) spin label doped into unilamellar egg yolk lecithin (EYPC) liposomes. (●) Liposomes of EYPC with addition of spin label at the concentration of 1 mol%. (▲) Liposomes with genistein at the concentration of 5 mol% and spin label at the concentration of 1 mol%. The concentration of lipid in 100 mM phosphate buffer was 10^{-5} M. The measurements were performed at various temperatures (10–40 °C). The spectrum was scanned at modulation amplitude 5 G.

3.2. Modification of structural and dynamic properties of EYPC liposomes by genistein

In our work we have also examined the effect of genistein on egg yolk lecithin liposomes representing unsaturated type of membranes. We applied spin labels doped into EYPC liposomes and examined by

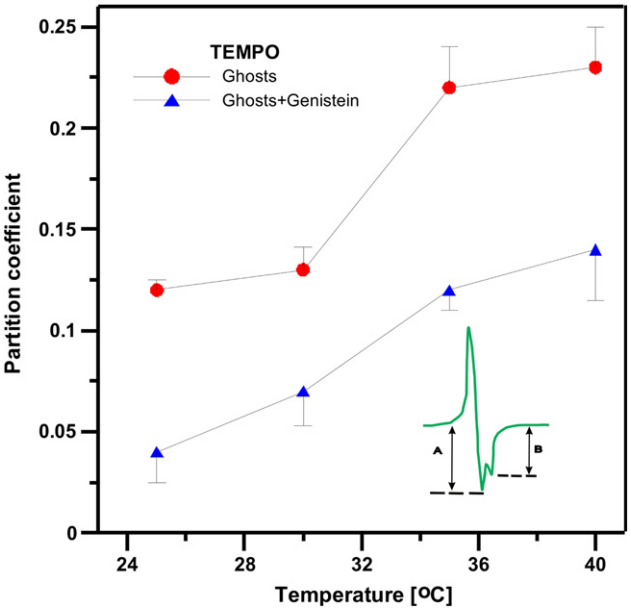

Fig. 4. Temperature dependence of partition coefficient B/A of Tempo spin label between lipid and water phase doped into ghost of human erythrocytes. (●) Control ghosts without genistein. (▲) Ghost with genistein addition. Tempo spin label was applied at a concentration of 2.5×10^{-4} mol per 1 mg of total protein in the membranes. Measurements were performed at various temperatures (25–40 °C).

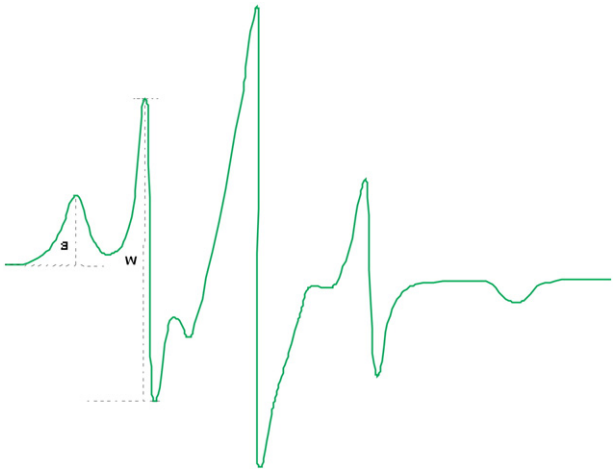

Fig. 5. A typical EPR spectrum of 4-maleimido-Tempo spin label in erythrocyte membrane. W represents peak of spin label weakly immobilized to the membrane and S shows strongly immobilized spin label.

EPR technique. We analyzed the maximum splitting parameter ($2\,T'_{||}$, the parameter closely related with the order parameter within membrane). The lower the fluidity of membrane in the vicinity of free radicals, the higher is the maximum splitting parameter. The results showed that modification of EYPC liposomes with genistein (5 mol%) had pronounced effect on the fluidity of hydrophobic core of the membrane at the depth monitored by 5-SASL. Genistein induced increase of the $2\,T'_{||}$ value at the depth penetrated by 5-SASL. The values of the maximum splitting $2\,T'_{||}$ for 5-SASL in liposomes with genistein were generally about 2.5–3 G higher (Fig. 2). The addition of genistein into the liposomes has changed the membrane structure into the more ordered one. Modulation of EYPC liposomes with flavonoid had very small effect on the fluidity at the hydrophobic core of the membrane at the depth monitored by 16-SASL (Fig. 3). The values of the maximum splitting parameter $2\,T'_{||}$ for 16-SASL in the liposomes with genistein were generally about 0.8 G higher.

3.3. The effect of genistein on structural and dynamic properties of erythrocyte membranes — an EPR spin label study

To examine the influence of genistein on the fluidity of erythrocyte membranes the spin label technique was applied. The shape of EPR spectra is strongly dependent on the motional freedom of the free radical segment of spin label molecule within the membrane [11,13,14,21]. The effect of genistein was observed in the region of polar head group penetrated by polar spin label Tempo (Fig. 4). High field EPR spectra of Tempo spin label show two peaks: the one correspondent to a

Table 2
The effect of genistein on 4-maleimido-Tempo spin label binding to ghost of human erythrocytes.

Genistein concentration (μg/ml)	W/S ratio ± SD
Control	5.01 ± 0.35
10	4.31 ± 0.05*
25	4.10 ± 0.29**
50	3.31 ± 0.40***

Control are membranes incubated in the absence of genistein. Red blood cell ghosts were incubated for 1 h at 37 °C with various concentrations of genistein (10, 25, 50 μg/ml). Labeling with 4-maleimido-Tempo spin label was at a ratio 1 mg of label per 25 mg of membrane proteins. EPR spectra were recorded at room temperature. Five separate experiments were performed.
SD — standard deviation.
* $p \le 0.02$.
** $p \le 0.01$.
*** $p \le 0.005$.

Table 3
The influence of genistein on the size of red blood cells.

Concentration of genistein (μg/ml)	Diameter of cell (μm ± SD)
Control	8.21 ± 0.62
10	6.82 ± 0.81*
25	6.72 ± 1. 05*
50	5.9 ± 0.81*

Control cells were incubated in isotonic saline solution for 1 h at 20 °C in the dark. All the remaining cells were incubated with different genistein concentrations in the same experimental conditions. After fixation with glutaraldehyde the diameters of 200 cells were measured. Three independent experiments were performed.
* $p \leq 0.001$; $n = 3$.

relatively mobile fraction of spin label in the water phase (A) and the second fraction of spin label immobilized within membrane (B) [12]. This parameter is connected with the membrane fluidity [22]. As it can be seen in Fig. 4, genistein caused a decreased penetration of spin label into membranes by lowering partition coefficient. The smallest incorporation of Tempo spin label was observed at 35 °C. The membranes were also labeled with maleimido Tempo spin label and analyzed by EPR in order to check the effect of genistein on proteins of the membranes. It is known that maleimido Tempo spin label binds covalently to membrane proteins giving rise to EPR absorption which represents, respectively, weakly (W) and strongly (S) immobilized state of the labeled sites in the membrane proteins (Fig. 5). The W/S ratio has been used to monitor the changes in protein conformation and the environment within the membrane [11,29]. An increase in the W/S ratio indicates conformational changes hence a decrease in protein–protein interactions. Exposure of ghosts to genistein caused a decrease in W/S ratio with the increasing concentration of isoflavonoid (Table 2). The values altered from 5.01 in control to 3.31 for 50 μg/ml genistein. Thus genistein produced lowering of the segmental motion of spin-labeled binding sites in all the concentrations.

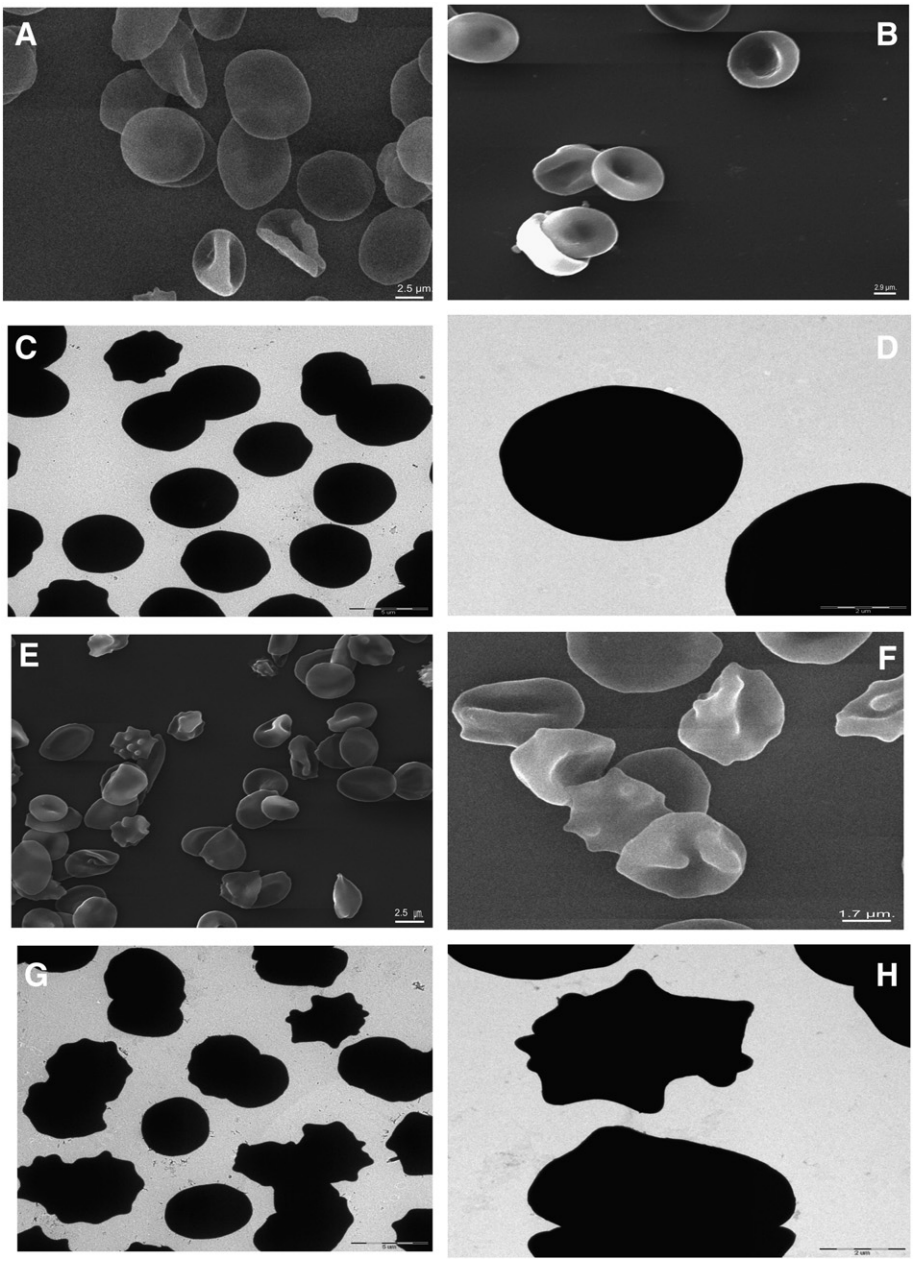

Fig. 6. Scanning and transmission electron micrographs of erythrocytes treated with genistein. A–D represent control cells. E–H are the cells incubated with genistein in the concentration of 50 μg/ml.

3.4. Erythrocyte size and shapes after genistein treatment

For analysis of the erythrocyte shapes and measurements of erythrocyte diameter light, transmission and scanning electron microscopy techniques were applied. Genistein caused changes in size and shape of cells. Measurements showed a reduced diameter of red blood cells (Table 3). The diameter of erythrocytes incubated with genistein at the concentration of 10, 25 and 50 μg/ml was smaller by 17, 18 and 28% in regard to control cells. Scanning electron microscopy studies revealed the changes in the shape of examined cells. Among erythrocytes treated with genistein, at all the concentration studied, irregular cells with numerous extrusions on their surface or cells with ruffled edges were found (Fig. 6). The number of deformed erythrocytes was 15, 25 and 35% for 10, 25 and 50 μg/ml genistein, respectively (Table 4).

3.5. The effect of genistein on membrane arrangement in human cervix carcinoma cells

The cells treated with genistein were examined under an electron and fluorescence microscope to get deeper insight into the changes caused by genistein on membranous structures within cells. No cytotoxicity towards the examined cells was observed after 4 h of incubation with genistein in the concentration of 15 μg/ml (cell viability equaled 106.27 ± 7.01) whereas cell viability after 24 h of incubation with genistein in the same dose, was decreased by 25% (Table 5). The control cells showed typical intracellular organization of membranous structures (Fig. 7 A–C). Prominent cisternae of endoplasmic reticulum were visible with ribosomes attached to the surfaces (Fig. 7 B–C). Mitochondria were uniformly shaped with intact cristae. In control cells well discernible nuclear envelope was noticed with inner and outer membranes easily recognizable (Fig. 7 A). In cells treated with genistein with granular cytoplasm local widening of nuclear envelope was observed (Fig. 7 D–F). Ribosomes were dispersed from RER and the cisternae of the endoplasmic reticulum were dilated. The mitochondria revealed membrane swelling. Fluorescence microscopy investigation has supported the influence of genistein on membranous architecture of the examined cells. DIL-cell labeling solution was added directly to culture media to selectively label all intracellular membranes among them endoplasmic reticulum [30–32]. The fluorescence of this pigment was assessed by a fluorescent microscope. After incorporation into membranes, control cells exhibited high fluorescence level in the whole cell with the highest intensity in the adjacent region of the nuclei (Fig. 7 G). The cells treated with genistein in the concentration of 15 μg/ml revealed much lower fluorescence intensity, especially in the vicinity of nucleus (Fig. 7 H–J) when compared to control cells. In cells treated with genistein with granular cytoplasm local widening of nuclear envelope was also observed (Fig. 7 D, F).

Table 4
The effect of genistein on the shape of red blood cells.

Concentration of genistein (μg/ml)	Deformed cells (% ± SD)
Control	0.9 ± 0.15
10	15 ± 1.15*
25	25 ± 1. 52**
50	35 ± 2. 51**

Control cells were incubated in isotonic saline solution for 1 h at 37 °C in the dark. All the remaining cells were incubated with different genistein concentrations in the same experimental conditions. All the cells were fixed with 4% glutaraldehyde and then observed under the light microscope. Three independent experiments were performed with 1000 cells in each sample.

* $p \leq 0.002$; $n = 3$.

** $p \leq 0.001$; $n = 3$.

Table 5
HeLa cell viability after incubation with genistein.

Cell culture	Time of incubation	Genistein concentration (μg/ml)	% of viability
HeLa	4 h	Control	100
		5	101.85 ± 5.90
		10	104.06 ± 6.64
		15	106.27 ± 7.01
	24 h	Control	100
		5	79.53 ± 5.66
		10	76.04 ± 4.33
		15	74.71 ± 4.99

Neutral red uptake assay. The results are presented as a percentage of control, arbitrarily set to 100%.

3.6. Antioxidant enzymes and radical level in HeLa cells

Activities of selected antioxidant enzymes in HeLa cells are shown in Table 6. In the cells treated with hydrogen peroxide alone an increase in superoxide dismutase (SOD) and catalase (CAT) activity as well as rise in SAR level was observed. In the cells exposed to genistein and oxidative shock simultaneously, superoxide dismutase (SOD) activity was decreased in all the concentrations studied. The biggest decrease (by 13%) was observed for the cells incubated with genistein at the concentration of 15 μg/ml. Similarly catalase (CAT) activity was (not statistically lowered) in cells exposed to genistein and oxidative shock simultaneously in all the concentrations studied. The enzyme activity was lowered by about 29, 35 and by 24% at the concentrations of 5, 10 and 15 μg/ml, respectively. Cells treated with H_2O_2 demonstrated a big increase in superoxide anion radical (SAR) level. More than 3-fold higher level of SAR was observed in H_2O_2 treated HeLa cells. When cells where preincubated with genistein statistically significant, dependent on the genistein concentration decrease in SAR level was noted. The biggest decline in the level of superoxide anion radicals (by 49%) was noted in the cells treated with flavonol at the concentration of 15 μg/ml in relation to control (Table 6).

The antioxidant activity of genistein was additionally determined spectrophotometrically by the 1,1-diphenyl-2-picrylhydrazyl (DPPH$^{\bullet}$) radical scavenging assay. The radical scavenging activity of genistein at the doses of 5, 10 and 15 μg/ml was expressed as percentage of reduction of DPPH. As shown in Table 7, scavenging activity against DPPH$^{\bullet}$ radical was 1.65-fold higher than the values obtained for Trolox, a synthetic vitamin E, as a reference compound. The highest scavenging activity was noted for isoflavonoid at the dose of 15 μg/ml.

4. Discussion

One of the targets for natural anti-cancer drugs such as flavonoids are cell membranes as well membranous organelles [33]. In the current investigation we studied the effects of genistein on liposomal membranes made of EYPC and erythrocyte membranes to evaluate its interaction with membrane lipids and proteins. Additionally we tried to gain further insight into flavonoid effects on human cervix carcinoma and red blood cells. The range of concentration used in the present study is similar as compared with studies of other authors [1,11,33–36]. Physiological concentrations of genistein are lower. As it was found by some authors plasma concentration of genistein in humans was in the range of 1 to 10 μmol/l [37] with average 1.8 ± 0.3 μmol/l. However, after supplementation of purified isoflavones, blood concentration may increase up to 28 μmol [38]. On the basis of the principles of pharmacology and the elimination half-life of genistein it can be predicted that steady state plasma concentration would be more readily maintained by repeated ingestions of isoflavones throughout the day [39]. In vitro experiments do apply higher concentration of examined isoflavone, similar to our doses, in investigations concerning cells. But it is worth noting that

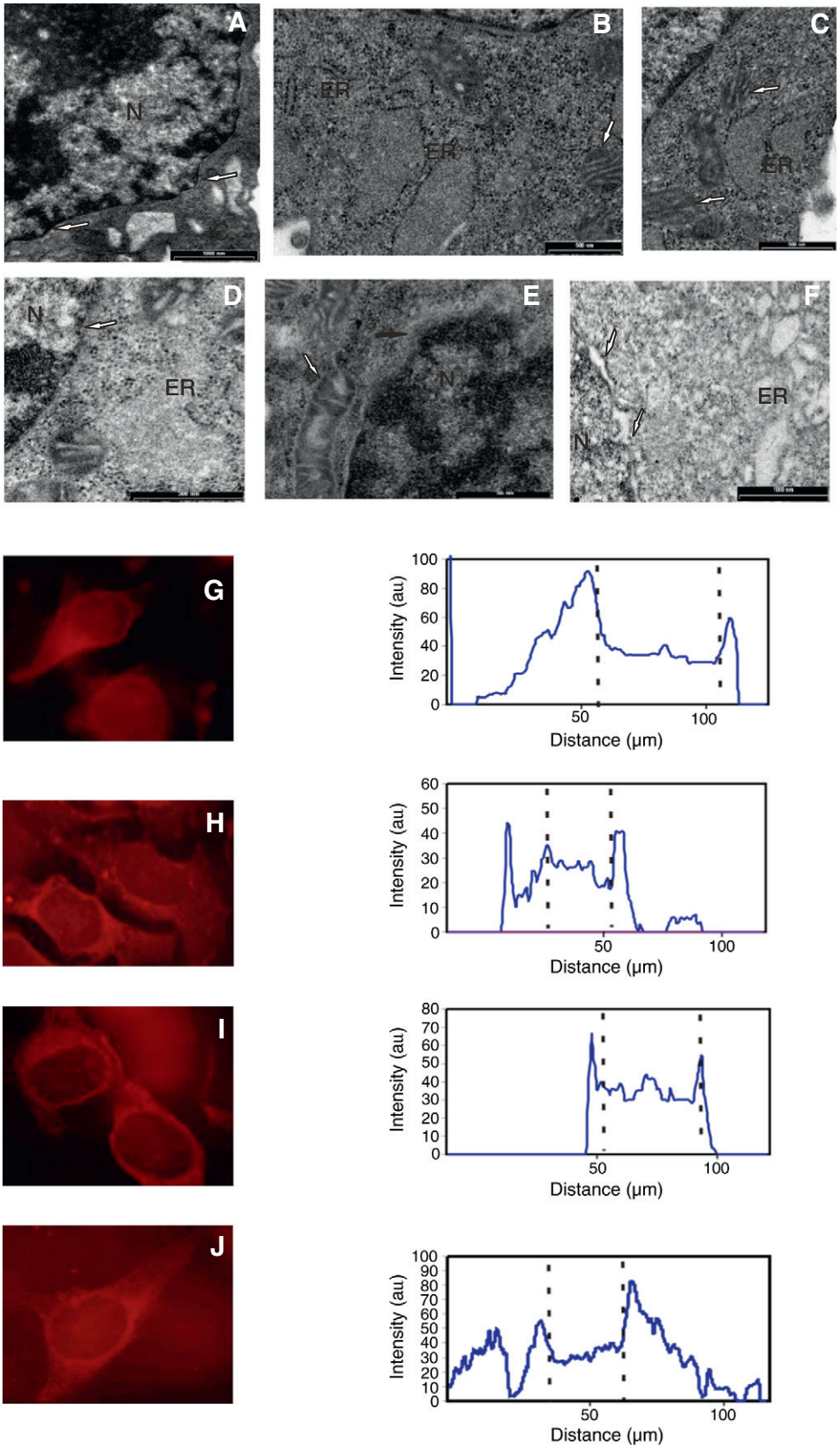

Fig. 7. The influence of genistein on membranous structures of HeLa cells (A). The changes in ultrastructure of HeLa cells. Control cells are represented in A–C. D–F represent cells treated with genistein. (B) DIL staining of human negroid carcinoma cells. The graphs on the right hand side present fluorescence intensity measured along the long diameter of the respective cells. G represents control cells and H–J represent cells treated with genistein. Between dashed lines there is nuclear region of the cells. The profile of DIL fluorescence was performed under a confocal scanning microscope (LSM 5 PASCAL) with an Ar laser at an excitation wavelength of 549 nm.

Table 6
CAT, SOD and superoxide anion radical (SAR) in HeLa cells.

Antioxidant marker (unit)	Control	Os	G5/Os	G10/Os	G15/OS
CAT (U/mg)	36.50 ± 0.19	79.75 ± 5.65**	56.75 ± 1.53	52.02 ± 0.97	60.64 ± 0.69
SOD (U/mg)	400.00 ± 69.81	531.61 ± 16.53**	500.08 ± 15.95	484.48 ± 38.76	461.05 ± 33.26*
SAR	0.0019 ± 0.0006	0.0063 ± 0.0003*	0.0038 ± 0.004*	0.0049 ± 0.0004**	0.0031 ± 0.0003**

Values are means ± S.D. of two independent experiments. The relative level of SAR was measured spectrophotometrically at 560 nm. Control cells; Os — hydrogen peroxide treated cells; G/Os — cells preincubated with genistein at different concentrations and then shocked.
* $p < 0.01$; $n = 2$.
** $p < 0.03$; $n = 2$.

the method used to investigate biochemical processes has certain thresholds of analytical sensitivity. Therefore, to demonstrate certain genistein activity to a measurable extent, the compound has to be applied at a concentration high enough. At the same time in vivo experiments do support pharmacological and physiological effects of genistein after it had been orally taken [1,40].

4.1. Genistein interacts with lipids and proteins of erythrocyte membranes and alters size and shape of red blood cells

The range of concentrations used in the present experiments concerning red blood cells (10, 25, 50 μg/ml) was similar as compared with the studies of other authors who had also examined red blood cells [11,34–36]. In our experiment we have observed the reduced diameter of red blood cells together with some changes in their surface appearance. We have observed echinocytic (spiculated) forms after incubation with genistein. According to the classical bilayer couple theory echinocytes are formed when a compound incorporates into and expands the outer leaflet of the membrane lipid bilayer [41,42]. Our EPR data on ghosts supported the concept on incorporation of genistein into head group zone of lipids. Similarly the EPR data obtained by Ajdzanowicz and coworkers gave evidence on genistein fluidity decreasing activity in the region near the hydrophilic surface [43]. Such shape-altered human erythrocytes exhibit changed membrane permeability [44]. This may suggest an increased surface area to volume ratio of the erythrocytes with respect to its volume. In consequence this can explain inhibition of hemolysis by genistein as it was observed in the work of Simao et al. [45]. Genistein hindered hemolysis possibly by interacting within the membrane as those authors postulated. Red blood cell membrane is composed of lipid bilayer and an underlying protein network. The proteins of the cytoskeleton and integral membrane proteins contribute to the maintenance of shape, size and elasticity of the cell [46,47]. Therefore, the interaction of genistein with membrane proteins may lead to changes of the shape and size of erythrocytes. In our experiments with ghost of humans we observed the changes in W/S ratio that supports our interpretation on the interaction of this compound at the level of cytoskeletal proteins. Our studies indicate that genistein binding to proteins is responsible for increased protein–protein interaction. The preferential binding of maleimido spin label to SH groups of cytoskeletal proteins: spectrin, actin, bands 2.1, 4.1 and to cytoplasmic region of band 3 proteins has been reported by some authors [29,48].

Genistein, as it was shown in the current study, really affects lipid membrane. One should however remember that the extent to which the examined isoflavonoid acts depends on the type of membrane. Erythrocyte membranes contain also proteins. Hence mutual interaction between proteins and lipids will play important role in such complex system in terms of fluidity. One should also keep in mind that lipid composition of erythrocyte membrane varies a lot from pure lipid system represented by liposomal membranes.

In this study we found that genistein acted mainly at the region of polar head groups and below it. It made the state of membrane more compact. The results obtained in the current study correspond well with our previous results concerning DPPC membranes [13]. As it was shown in the latter manuscript genistein really affected physical properties of membranes formed with dipalmitoylphosphatidylcholine (DPPC). It showed rigidifying effect especially with respect to polar head groups. FTIR analyses of the liposomes gave evidence that genistein incorporates into DPPC membranes via hydrogen bonding between the lipid polar head groups in the C–O–P–O–C segment and its hydroxyl groups.

4.2. Genistein ordering effect on liposomal membranes made of EYPC

The effect of genistein on the erythrocyte membranes can be better elucidated by analysis of the effects of this isoflavonoid in phospholipid membranes made of EYPC, representing unsaturated type of membranes, as was studied by ^{1}H NMR and EPR techniques in the current study. The effect of genistein on the polar head group region was analyzed in Fig. 1 where proton resonance in choline group of pure membranes and membranes with addition of examined isoflavone was compared. The inclusion of genistein to the membrane pronouncedly decreased the motional freedom of polar head group region. Simultaneously, genistein induced slight motional freedom of alkyl chains that was manifested by narrowing of spectral peaks of CH_2 and of terminal CH_3 groups of lipid alkyl chains in genistein-containing samples.

EPR technique has supported the finding obtained by NMR measurements. We applied two types of spin labels that provided information about the membrane. 5-SASL tested the order of alkyl chains in the hydrophobic region adjacent to the polar heads whereas 16-SASL enabled the examination of the membrane in the hydrophobic core of the lipid membrane. We found no (very slight) changes in deep hydrophobic region of membranes. Simultaneously we found big changes in the fluidity of hydrophobic region close to the polar heads of the membrane at the depth penetrated by 5-SASL. The modification of liposomes with genistein has resulted in ordering the membrane structure as compared with pure liposomes that is in accordance with the conclusion drawn from NMR measurements. Our findings are well correlated with the

Table 7
Free radical scavenging activity in DPPH test.

Genistein	5 μg/ml	10 μg/ml	15 μg/ml
% of reduction as compared to control (0% of reduction)	0.94 ± 0.63	2.27 ± 0.58*	6.03 ± 1.03*
Reduction value, which corresponds to the following Trolox concentration (μg/ml)	1.007 ± 0.330	1.007 ± 0.330	3.648 ± 0.543

Values are means ± S.D. of three independent experiments. The absorbance of the solutions was measured spectrophotometrically at 515 nm. Trolox was used as a reference for free radical scavenging activity. Results are means ± SD. Data were analyzed using ANOVA one-way analysis of variance with Dunnettt *post hoc* test.
* $p < 0.01$; $n = 3$.

observations of other authors [16,18]. They used different techniques (fluorescent probes, calorimetry) and gave evidence for genistein intercalation into membranes. Once incorporated genistein caused a decrease in lipid fluidity in all the regions of membrane-interior as well as in exterior portion.

NMR results of the current study provided evidence of genistein as isoflavone that restricts motility mainly in the polar head group region. Our EPR data pointed also genistein incorporation at the level penetrated by 5-SASL (so below head group) and at the polar head zone (ghost of the erythrocytes). One should remember about sensitivity of the methods applied. In comparison to EPR technique NMR is much more sensitive to local changes caused by addition of flavonoid.

Such behavior of genistein is consistent with the results obtained by Furusawa and co-workers in another study [49]. As it was shown in this paper, genistein rigidified the tumor cell model membranes. From the previous study on DPPC membranes we know that genistein incorporates into membranes via hydrogen bondings [13] and shows broad distribution in membranes with the highest propensity to polar head group zone and below the head group. Observations of other authors [7,50] who suggested interaction of polyphenols at the surface of bilayers by hydrogen bonding agree with our result. Scheidt and coworkers have also supported evidence for maximal distribution of genistein in the lipid–water interface in monounsaturated POPC model membranes [51].

4.3. The changes in morphology and viability of HeLa cells under the influence of genistein

The results obtained with the use of fluorescent and electron microscope have supported the presence and activity of genistein in cell membrane and internal membranes. Genistein intercalates to these membranous structures of the cells and changes their arrangement and in consequence modifies their activities. It was found that genistein changes proliferation of the cells [52,53], affects membrane transport, inhibits cell cycle, induces apoptosis or prevents lipid peroxidation and free radical formation [5,54–56].

Signaling pathways, one of the genistein antitumour mechanism, can be changed by a compound that alters membrane fluidity and in aftermath causes growth inhibition of tumor cells. Our data from NR assay supported the influence of genistein on cell viability of human cervix cancer cells in vitro. We observed growth inhibition by 25% in the examined cells. Similarly, decrease in cell viability was observed for genistein by Gacche and coworkers in HeLa-B75 cell line [57].

4.4. Antioxidant action of genistein in HeLa cells treated with hydrogen peroxide

Biological activity of flavonoids is partly connected with their antioxidative action. Oxidative stress leads to cancer, aging, inflammation and other serious diseases. It is also a common apoptotic mediator [58,59]. In the present paper we examined genistein effect on SOD, CAT activity as well as on radicals' level in cultured human negroid cells.

We found that treatment of HeLa cells with 2 mM H_2O_2, a precursor of other reactive oxygen species, caused statistical increase in activities of SOD, CAT and the level of anion radicals. Results obtained by Mansour and coworkers are in accordance with our data. They found an increase in catalase activity in HeLa cells exposed to hydrogen peroxide [60]. A slight increase in CuZnSOD activity, the enzyme that is cytosolic form and is regulated by oxidative stress, was also noticed in HeLa cells after 24 h of incubation with hydrogen peroxide [61]. When cells were pretreated with genistein we observed a decrease in ROS level in comparison to hydrogen peroxide-challenged cells. Therefore genistein alleviated oxidative shock by improving redox balance as indicated by the decrease in superoxide anion radicals. In accordance with our study is the other one concerning kinetics of free radical scavenging activity. Genistein was shown to have promising scavenging activity against superoxide radical [57]. In another work, genistein downregulated oxidative stress level by decreasing the level of ROS in genioglossus muscle [62]. Flavonoids are able to protect cells against oxidative stress by different mechanisms. They can scavenge free radicals. Flavonoids prevent the loss of antioxidant by ROS or can regulate endogenous antioxidant defenses. Antioxidant activities of genistein have been shown in many chemical oxidation systems. It was found to protect neurons against oxidative stress [63]. Genistein reduced significantly the oxidative stress in diabetic persons induced by glucose self-oxidation radical generation [64]. Genistein inhibited membrane protein oxidation in human erythrocytes and hindered membrane lipid peroxide formation. Genistein has C2–C3 double bond in conjugation with 4-oxo in the C-ring, 3- and 5-hydroxy groups and the 4-oxo atom in the A and C rings. These traits are very crucial in antioxidant activity of genistein [65]. Our data obtained in DPPH assay showed some scavenging activity of genistein in comparison to Trolox. Similarly to our data the results of Gacche and coworkers have shown that genistein was effective in DPPH reducing agent (but to lesser extent) than other flavonoids [57]. In the work of Kruk and coauthors it has been demonstrated that genistein possesses antioxidant activity showing two fold greater activity than the standard Trolox. At the same time this isoflavonoid affected absorption of DPPH radical but was effective to some extent in the direct reaction with this radical [65].

We observed that pre-incubation of the cells with genistein has no significant effect on CAT activity. Similarly Suzuki and coworkers have observed no significant changes in catalase (and superoxide dismutase) in prostate cancer cells [66]. The same effect of genistein was found by other authors examining skeletal muscle cells subjected to oxidative stress [67]. Simultaneously these authors found protective effect of genistein on the examined cells against oxidative damage that was exhibited by lowering radicals' level.

Similarly in our study we have shown that preincubation with genistein diminished significantly SAR level in hydrogen-induced HeLa cells with the smallest effect observed for the concentration of 10 μg/ml. Such genistein treatment did not affect viability of cells (after 4 h of incubation) as was determined by NR assay that demonstrates the absence of cytotoxic effects. At the same time SOD activity that dismutase superoxide anion radicals, was lowered so we can suppose that elevated level of SAR in this experimental variant was not associated with SOD activity. At the same time catalase activity was the lowest at this dose indicating probably that genistein did not detoxify hydrogen peroxide efficiently and the superoxide anion radical could be bigger at such conditions. Some other explanation cannot be excluded. Genistein might act by altering the regulation of the activity of other enzyme. We did not check the activity of glutathione peroxidase (GPx), another very crucial enzyme that converts hydrogen peroxide into water and thereby stops creation of radicals. GPx acts as antioxidants in detoxification system and deals with ROS together with other antioxidant enzymes including SOD and CAT [66]. Suzuki and coauthors have found that genistein showed no statistically significant changes in activity of superoxide dismutase and catalase (similarly to our results) but strongly affected glutathione peroxidase in human prostate cancer cell lines LNCAP and PC3. Protective properties of genistein can also be connected with other mechanisms not discussed above. Our data from the current study give evidence for genistein incorporation into membranes and in consequence changing their properties. Therefore incorporation followed by stabilization of plasma membrane can be crucial for physiological benefits of genistein. The lowest DM (dipole moment) was calculated for isoflavone genistein (1.639 D) which indicates its hydrophobic nature [57]. Such substance can include into and later cross biological membrane and thereby reach their targets within cells.

4.5. Conclusions

In the present work we pointed the localization of genistein within EYPC and human erythrocyte membranes with higher propensity

towards upper region of membrane e.g. lipid water interface. We correlated membrane effects of genistein with its in vitro effects on the cells, among them with its in vitro inhibition of cell proliferation, the changes in internal membranes arrangement, the changes in the shape and morphology of the cells and antioxidant activity of examined flavonoid. Such correlations are of interest in terms of genistein mechanism as membrane modulating compound and in aftermath heavily contributing to the changes inside cells that are important for pharmacological activity of genistein in many illnesses.

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