

Effects of Phenol, Catechol, Chloro- and Metylphenol on Human Erythrocyte Membrane (*in vitro*)

B. Bukowska^{1*}, A. Marczak², J. Michałowicz¹, K. Wiśniewska¹

¹Department of Biophysics of Environmental Pollution,

²Department of Thermobiology

University of Łódź, Banacha 12/16, 90-237 Łódź, Poland

Received: 29 July 2008

Accepted: 24 March 2009

Abstract

This study continues our investigations concerning the interaction of phenol, catechol, 2,4-dichlorophenol (2,4-DCP), 2,4,5-trichlorophenol (2,4,5-TCP) and 2,4-dimethylphenol (2,4-DMP) with human erythrocytes. We focus on the effects of these compounds on erythrocyte membrane fluidity, as well as on their impact on membrane proteins. The fluorimetric method and fluorescent probes (ANS, DPH and TMA-DPH) were used to estimate the fluidity of erythrocyte membranes. SDS-gel electrophoresis was carried out to separate the proteins of the cell membrane. Additionally, an analysis of disturbances in size and shape of the erythrocytes by the application of the methods of flow cytometry and microscopic examination was performed.

It was observed that phenol derivatives like 2,4-DCP, 2,4,5-TCP, 2,4-DMP and catechol induced changes in membrane fluidity and perturbations in the content of a cell's membrane proteins. Changes in the level of spectrin, band 3 protein and low molecular weight proteins were also noted. Using three fluorescent probes we observed different changes in membrane fluidity on its different layers, depending on the structure and the concentration of the compound used. The application of flow cytometry and microscopic technique also demonstrated disturbances in the size and shape of erythrocytes. We concluded that chlorophenols induced more severe changes in erythrocyte membrane properties and phenol expressed the lowest toxicity.

Keywords: phenol, chlorophenol, methylphenol, erythrocytes, fluidity, morphology

Introduction

Phenol and its derivatives are widely used by the chemical industry as components or precursors of resins, plastics, disinfectants, dyes, antioxidants and perfumes [1]. Phenolic compounds are present in cigarette smoke and also exist in smoked food products [2]. Catechol is used as a topical

antiseptic, reagent, an antifungal preservative on potato seed, photographic developer and a developer in fur dyes, as well as an antioxidant in many branches of industry, including rubber, chemical, dye, photographic and pharmaceutical [3]. Moreover, catechol and hydroquinone are reactive metabolites of benzene [4]. Due to the common occurrence of these compounds in the environment and potential exposure of people and animals to their action, the mechanisms of their toxic activity towards cells are still being investigated [5-7].

*e-mail: bukow@biol.uni.lodz.pl

In this article, we continue the studies concerning the influence of phenol and its derivatives on human erythrocytes. The previous studies suggest that phenol derivatives have changed numerous biochemical parameters in erythrocytes, such as the activity of superoxide dismutase, glutathione peroxidase and catalase [8, 9], the activity of membrane acetylcholinesterase [10] and ATP-ase [11] and also the level of reduced glutathione [8, 9]. Other investigations showed that 2,4-dichlorophenol, 2,4-dimethylphenol and catechol revealed oxidative properties and caused ROS formation in the erythrocytes [6]. Moreover, it was observed that chlorophenols induced lipid peroxidation in red blood cells [12].

Erythrocytes represent a convenient model for investigation due to their susceptibility to lipid and protein peroxidation [13]. It was proved that oxidants produce alterations in erythrocyte membranes, decreasing cytoskeletal protein content, which can lead to abnormalities in erythrocyte shapes.

It has been suggested that some effects directly related to the toxicity of chemicals could be mainly connected with changes in membrane fluidity.

The concentrations of phenol and its derivatives which were used in our experiment are not commonly present in the human organism; however, they may be potentially administrated during its acute intoxication [14]. Moreover, it has been proved that congenial doses of phenols used in other experiments may provoke changes in human erythrocytes [6, 11, 12].

We wanted to look closer into the interactions of phenol and its derivatives with cell membrane proteins. Using fluorescent probes such as DPH, TMA-DPH and ANS, we were able to specify the membrane area, in which the investigated compounds are localized. As the proteins of the cytoskeleton and integral membrane proteins are responsible for the shape of red blood cells, the changes in protein fractions content in the erythrocytes exposed to phenol and its derivatives were also examined.

Material and Methods

Reagents

Catechol and phenol were obtained from Sigma (Poznań, Poland), but 2,4-dimethylphenol (purity 99%), 2,4-dichlorophenol (purity 98%), and 2,4,5-trichlorophenol (purity 98%), were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany). All other reagents were of analytical grade and were bought from ChemPur (Piekary Śląskie, Poland) and from Sigma-Aldrich (Poznań, Poland).

Preparation of Erythrocyte Suspensions and Cell Membrane

Human whole blood was obtained from the Blood Bank of Łódź. Blood was centrifuged (3,000 rpm × 10 min.), plasma and leukocyte buffy coat were aspired and the erythrocyte pellet was washed three times with phosphate-buffered saline (PBS), pH 7.4.

Erythrocytes were suspended to final hematocrit of 5% in PBS containing phenol and its derivatives at the concentrations from 10 ppm to 250 ppm. They were incubated at 37°C with continuous mixing for the appropriate time. Erythrocytes suspended in PBS were used as a control. For each experiment all samples (control and samples incubated with xenobiotics) were prepared from blood taken from a single individual.

Erythrocyte membrane ghosts were obtained by hypotonic lysis according to the procedure of Dodge et al. [15]. The erythrocytes were hemolyzed in 20 mM phosphate buffer, pH 7.4, and washed several times with 10 mM and then 5 mM phosphate buffer containing 0.1 mM EDTA and 0.1 mM PMSF (phenylmethylsulfonyl fluoride) till hemoglobin was released. All operations were carried out at about 4°C. Protein concentration was estimated using the method of Lowry and co-workers [16] with bovine serum albumin as a standard.

SDS-Gel Electrophoresis of Erythrocyte Membrane Proteins

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of erythrocyte membrane proteins was performed on a 5-20% running and 3% stacking gel using the method of Laemmli [17] with slight modifications. Forty milligrams of cell membrane proteins solubilized with β -mercaptoethanol were applied to polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue R-250 using the method of Fairbanks and co-workers [18] and analyzed with DESAGA CD-60 densitometer (Heidelberg, Germany).

Flow Cytometry

Control cells and erythrocytes incubated with phenol and their derivatives were analyzed with a flow cytometer (LSR II, Becton Dickinson). Cell size and shape were evaluated with simultaneous separate detection of low angle (FSC-A) and right angle (SSC-A) light scattering. The data obtained were displayed in the form of a diagram of cell number versus light scatter and were analyzed by the standard computer program WinMDI 2.8. The light scattered near the forward direction (low angle) is expected to be proportional to the size (volume) of the particle and is independent of cell refractive index and shape, whereas scattering at the right angle depends on the cell shape and internal properties of the scattered particles [19].

Fluorescence Measurements

Erythrocyte suspension aliquots were incubated for 10 min. at room temperature with the fluorescent probes DPH (1,6-diphenylhexatriene), TMA-DPH (4'-trimethyl-ammio-1,6-diphenyl-1,3,5-hexatriene) or ANS (8-anilino-1-naphthalene sulfonate). The final total concentrations of fluorescent probes were 2 μ M for DPH and TMA-DPH and also 1 μ M for ANS in 0.04% haematocrit, respectively.

Table 1. Per cent of anisotropy of ANS (part A), TMA-DPH (part B) and DPH (part C) in control erythrocytes and erythrocytes incubated with phenol, 2,4-DMP, 2,4-DCP, 2,4,5-TCP and catechol. Data are shown as means \pm SD for 8 - 12 independent experiments.

A	ANS anisotropy [%]	Phenol	2,4-DMP	2, 4-DCP	2, 4,5-TCP	Catechol
	Control	100	100	100	100	100
	10 ppm	93.4 \pm 5.5*	96.0 \pm 7.8	101.4 \pm 14.9	99.2 \pm 5.9	91.9 \pm 8.0
	50 ppm	92.1 \pm 5.1*	91.6 \pm 9.2	108.1 \pm 14.2	105.1 \pm 9.9	89.4 \pm 8.2*
	100 ppm	89.2 \pm 9.3*	89.1 \pm 7.9*	109.5 \pm 7.10*	109.5 \pm 7.1*	88.6 \pm 8.1*
	250 ppm	90.3 \pm 6.8*	90.5 \pm 5.4*	114.5 \pm 10.7*	110.8 \pm 6.8*	89.5 \pm 8.8*
	ANOVA I	F _{4; 44} = 4.71 P < 0.01	F _{4; 45} = 4.35 P < 0.001	F _{4; 60} = 3.52 P < 0.02	F _{4; 65} = 7.85 P < 0.001	F _{4; 49} = 4.72 P < 0.01
B	TMA-DPH anisotropy [%]	Phenol	2,4-DMP	2, 4-DCP	2, 4,5-TCP	Catechol
	Control	100	100	100	100	100
	10 ppm	100.7 \pm 9.6	99.5 \pm 6.6	97.4 \pm 7.5	100.0 \pm 10.4	101.1 \pm 12.9
	50 ppm	94.5 \pm 6.2	100.7 \pm 6.8	98.5 \pm 9.5	102.6 \pm 13.5	97.2 \pm 8.6
	100 ppm	99.7 \pm 5.6	97.6 \pm 10.8	98.1 \pm 5.8	103.1 \pm 10.3	100.5 \pm 13.5
	250 ppm	99.7 \pm 5.1	100.4 \pm 7.6	99.8 \pm 8.8	100.3 \pm 10.6	98.5 \pm 10.2
	ANOVA I	F _{4; 45} = 1.63 P > 0.05	F _{4; 55} = 0.35 P > 0.05	F _{4; 55} = 0.30 P > 0.05	F _{4; 55} = 0.28 P > 0.05	F _{4; 50} = 0.25 P > 0.05
C	DPH anisotropy [%]	Phenol	2,4-DMP	2, 4-DCP	2, 4,5-TCP	Catechol
	Control	100	100	100	100	100
	10 ppm	95.6 \pm 6.5	97.5 \pm 7.5	94.1 \pm 14.3	97.1 \pm 6.6	104.9 \pm 9.5
	50 ppm	93.6 \pm 10.2	95.6 \pm 8.5	87.1 \pm 8.22	93.9 \pm 8.9	102.0 \pm 9.3
	100 ppm	85.9 \pm 11.7*	91.2 \pm 8.1*	90.1 \pm 7.1*	87.8 \pm 7.7*	102.7 \pm 12.4
	250 ppm	85.2 \pm 10.4*	86.8 \pm 8.4*	86.4 \pm 6.4*	86.0 \pm 7.9*	97.9 \pm 6.4
	ANOVA I	F _{4; 45} = 3.99 P < 0.01	F _{4; 45} = 1.63 P > 0.05	F _{4; 55} = 5.24 P < 0.01	F _{4; 55} = 8.71 P < 0.001	F _{4; 51} = 0.92 P > 0.05

These values were selected for the fluorescence measurements in erythrocytes according to the membrane/water partition coefficient and fluorescence quantum yield of each probe. Measurements were made at room temperature using a Perkin Elmer LS-5B spectrofluorimeter. The excitation and the emission wavelengths were $\lambda_{exc/em} = 352 \text{ nm}/430 \text{ nm}$ for DPH, and $\lambda_{exc/em} = 358 \text{ nm}/428 \text{ nm}$ for TMA-DPH and $\lambda_{exc/em} = 337 \text{ nm}/480 \text{ nm}$ for ANS.

Membrane fluidity was assessed by fluorescence anisotropy measurements [20]. Fluorescence anisotropy was calculated according to equation:

$$r = (I_{vv} - I_{vh}G) / (I_{vv} + 2I_{vh}G)$$

...where I_{vv} and I_{vh} represent the components of light intensity emitted, respectively, parallel and perpendicular to the direction of the vertically polarized excitation light, and G is the correction factor ($G = I_{hv} / I_{hh}$).

Phase Contrast Microscopy

Erythrocytes incubated with phenol and their derivatives were fixed on a glass surface and the samples were evaluated using a phase contrast microscope (Olympus, Japan) at 600 x magnification.

Statistical Analysis

Statistical analysis was performed with STATISTICA data analysis software (2000 StatSoft, Inc., Tulsa, OK, USA). One-way analysis of variance (ANOVA) with post hoc multiple comparisons procedure (Tukey's test) was used to assess statistical differences in this study. The difference was considered to be significant for $P < 0.05$ and highly significant for $P < 0.001$. Mean values were calculated for 3 to 10 donors, whereas for each donor an experimental point was a mean value of three replications.

Results

Membrane Fluidity

Fluorescence anisotropy measurements are widely used as a simple means of estimating plasma membrane fluidity. The value of fluorescence anisotropy of the lipid probe points to its free rotation in the lipid bilayer and thus, it is an indicator of membrane fluidity. A decrease in anisotropy reflects a decrease in lipid order, which is associated with an increase in membrane fluidity.

To determine cell membrane fluidity, fluorescent probes ANS, TMA-DPH, and DPH were selected. Results are presented in Table 1.

Comparison of the anisotropy value for ANS probe showed that all tested compounds interacted with proteins and lipids near the surface of the cell membrane (Table 1A). 2,4-DCP and 2,4,5-TCP caused an increase in ANS anisotropy and thus a decrease in fluidity of this region of erythrocyte membranes. Stronger changes were noted for

2,4-DCP. The 2,4-DMP; phenol and catechol caused a decrease in this parameter and an increase in fluidity of this region of erythrocytes membrane.

TMA-DPH was used to monitor fluidity in the outer layer of the plasma membrane. The TMA-DPH molecules are believed to accumulate and remain almost exclusively in the outer leaflet of cell membranes, because their polar heads are anchored at the lipid-water interface while hydrocarbon moieties enter the lipid part of the membrane. The anisotropic orientation of TMA-DPH inside the membrane reflects the degree of the order of membrane lipid molecules. For this probe, no changes for any of the compounds examined were noted (Table 1B).

DPH intercalates between the lipid chains of the hydrophobic core of the membrane due to its hydrophobic character and this molecule is oriented perpendicularly to membrane plane. We observed a decrease in DPH anisotropy for all investigated compounds (excluding catechol) (Table 1C).

The Content of Cell Membrane Proteins

In this experiment we used only the highest concentration (250 ppm) of the individual compounds. This dose was selected as it caused changes in our earlier investigations [9-11].

After the incubation of human erythrocytes with phenol and its derivatives the content of the main protein fractions was obtained after SDS-PAGE and densitometric evaluation of the gels.

We identified the following protein fractions: spectrin 1 and 2, band 3 protein, band 4.1 protein, band 4.2 protein, actin (band 5), band 6 protein and low (small) molecular weight proteins (Fig. 1).

It was shown that 2,4-DMP, 2,4-DCP, 2,4,5-TCP and catechol caused changes in the level of the proteins. Fig. 2 shows that the strongest changes in the percentage of the protein contents were observed for spectrin, band 3 protein and low molecular weight proteins.

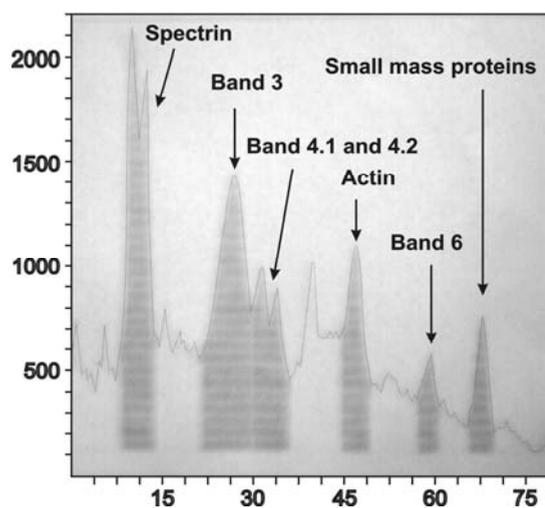


Fig. 1. Densitogram of control sample of protein fractions separated by the use of electrophoresis.

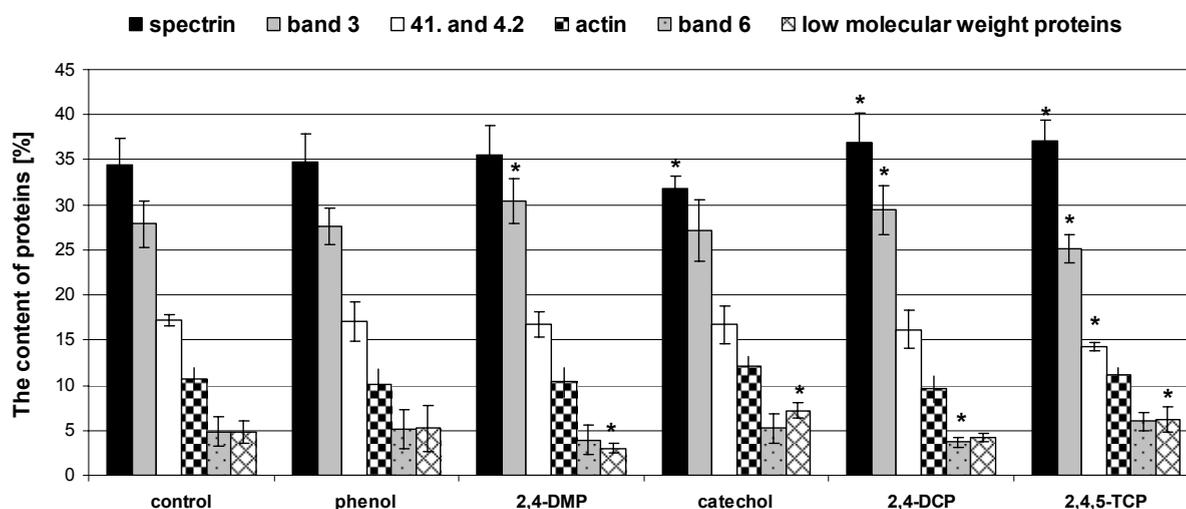


Fig. 2. The content of protein [%] in control erythrocytes and erythrocytes incubated with phenol and its derivatives in a concentration of 250 ppm. Data are shown as means \pm SD for 6 independent experiments.

2,4-DMP induced an increase in the percentage of content of band 3 and a decrease in low molecular weight proteins whereas catechol induced a decrease in spectrin and an increase in low molecular weight proteins. Chlorophenols induced the highest alteration in membrane proteins: spectrin, band 3, 4.1 and 4.2, band 6 and low molecular weight proteins. No statistically significant changes in the percentage of the contents of the erythrocyte membrane proteins were observed for phenol ($P > 0.05$).

Estimation of Morphological Changes in Erythrocytes

Flow cytometry technique was used to analyze erythrocyte size and shapes. Figs. 3 and 4 present quantitative changes in FSC-A and SSC-A parameter. After FSC-A histogram analysis, which gives information about cell size, it can be noticed that phenol and its derivatives caused changes in this parameter (Fig. 3). The strongest changes were observed for 2,4,5-TCP and catechol. Catechol ($F_{5;36} = 7.77$; $P < 0.001$), and 2,4-DMP ($F_{4;24} = 19.41$; $P < 0.001$), induced a statistically significant increase in FSC-A parameter after 1 h incubation. Phenol did not change this parameter for all tested concentrations. 2,4-DCP ($F_{4;30} = 8.23$; $P < 0.001$) and 2,4,5-TCP ($F_{4;26} = 25.97$; $P < 0.001$) induced a statistically significant increase in FSC-A values up to 20 ppm and the highest concentration (100, 250 ppm) decreased its value.

SSC-A histograms provide information about cell shape and the structure of the outside surface of cell membrane (Fig. 4). The analysis of histogram SSC-A shows that the strongest changes in the cell shape and the structure of the outside surface cell membrane were observed for 2,4,5-TCP ($F_{4;26} = 51.57$; $P < 0.001$). At the beginning an increase in SSC parameter value up to 50 ppm of 2,4,5-T was observed and then it was decreased from a dose of 100 ppm.

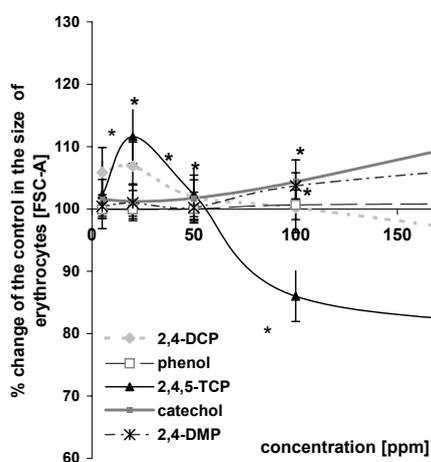


Fig. 3. Flow cytometry analysis of the percent changes of the control in the size of human erythrocytes incubated with phenol and its derivatives in doses of 10-250 ppm for 1 hour.

Catechol ($F_{5;36} = 13.23$; $P < 0.001$), 2,4-DMP ($F_{4;27} = 8.76$; $P < 0.001$) and 2,4-DCP ($F_{4;27} = 29.69$; $P < 0.001$) induced a statistically significant decrease in SSC-A after 1 h incubation. No statistically significant changes in SSC-A were observed for all tested concentrations of phenol ($P > 0.05$).

Phase Contrast Microscopy

The observed changes using the method of flow cytometry concerning the size (volume) and shape of erythrocytes were confirmed by visual observations (microscopic photographs) (Fig. 5).

Phenol and 2,4-DMP did not cause statistically significant changes in erythrocyte morphology. We did not observe echinocyte and stomatocyte formations in cases of the activity these compounds.

Chlorophenols provoked a formation of echinocytes with the highest effect for 2,4,5-TCP [9]. They also induced follicle formation (tearing away of membrane fragments), which suggested that oxidative processes appeared in red blood cells. Catechol changed erythrocyte morphology with the formation of a few echinocytes.

Discussion

In this article we tried to compare the effect of phenol and its derivatives on erythrocyte membrane. Phenol derivatives changed the fluidity of the erythrocyte membrane and interacted with its proteins. Additionally, we estimated the changes in the size and shape of red blood cells after treatment with the tested compounds.

Fluid properties of biological membranes are essential for numerous cell functions, including cell growth, solute transport, signal transduction and membrane-associated enzymatic activities. Even slight changes in membrane fluidity may cause an aberrant function and pathological processes [21].

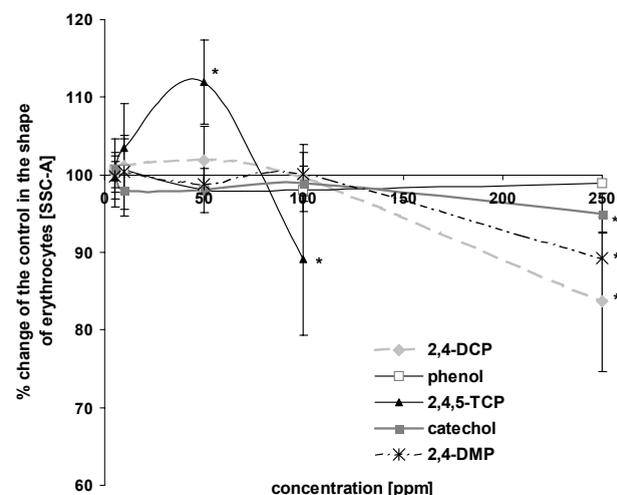


Fig. 4. Flow cytometry analysis of the changes [%] in the shape of control erythrocytes and erythrocytes incubated with phenol and its derivatives in doses of 10-250 ppm for 1 hour.

We used three fluorescent probes to study cell membrane fluidity at different depths of the lipid bilayer.

ANS was a very important probe that we used. This molecule is not able to immerse itself deeply in lipid bilayer because its negatively charged sulphonic residue has to remain on the surface. ANS may be bound to proteins, thus it provides information about surface fluidity (of this part) of the membrane. In our studies we observed two kinds of

effects. The 2,4-DCP and 2,4,5-TCP caused an increase in the anisotropy parameter for higher concentrations of these compounds. For the other tested compounds we noted a decrease in the anisotropy parameter (Table 1A).

The results obtained with the TMA-DPH probe showed that phenol and its derivatives did not cause changes in the fluidity in hydrophilic regions of the lipid bilayer (Table 1B).

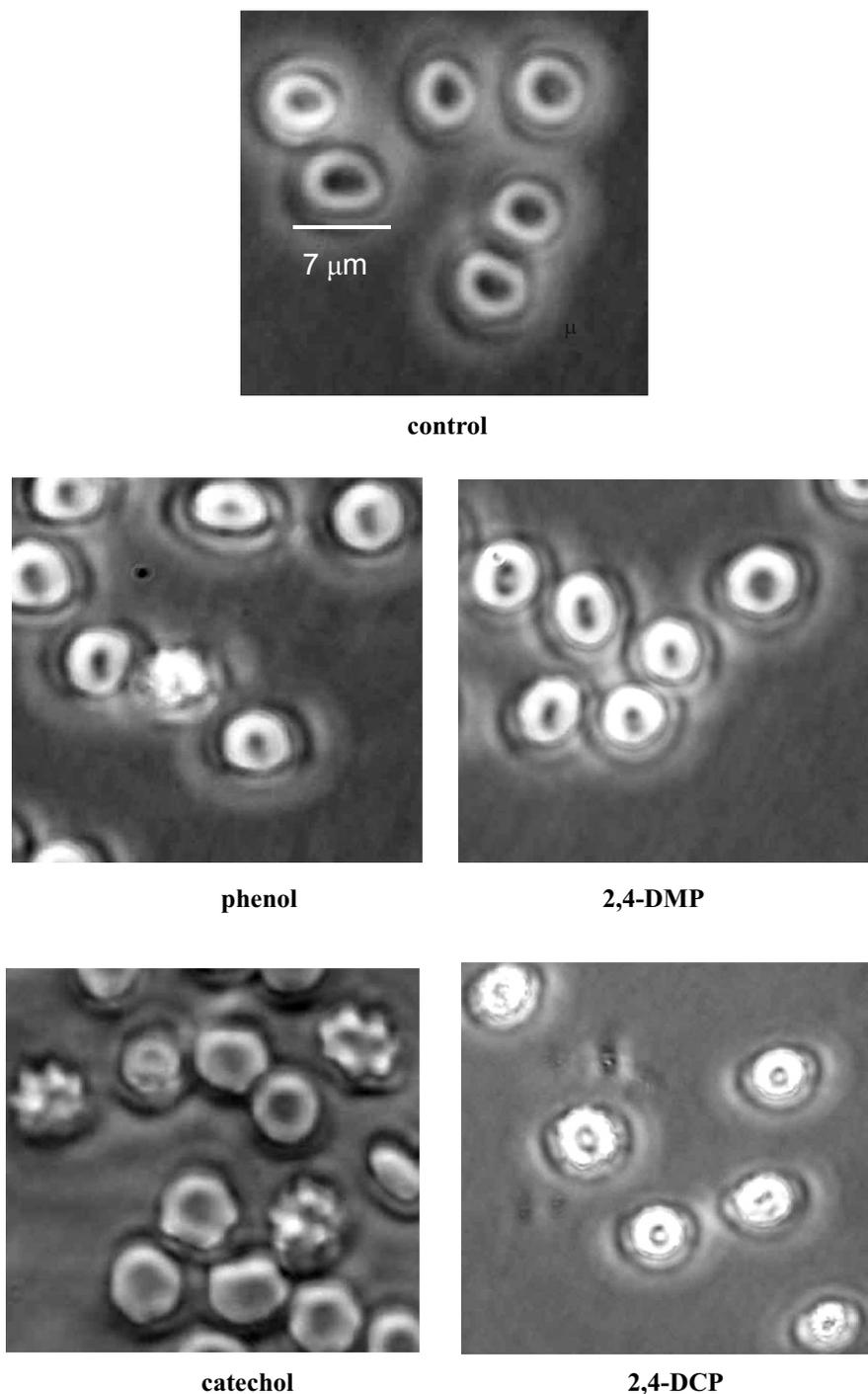


Fig. 5. Micrographs of human control erythrocytes and erythrocytes incubated with 250 ppm of phenol, 2,4-DMP, catechol and 2,4-DCP. Bukowska in Cell Biology International 2004 published photo with 2,4,5-TCP. 2,4,5-TCP induced drastic shape changes, i.e. to cell shrinkage [9].

Our study showed the effect of phenol and its derivatives on membrane fluidity, mainly in the deeper hydrophobic part of the lipid bilayer (Table 1C). This region was tested with a DPH probe. Excluding catechol, all the tested compounds caused a decrease in the anisotropy parameter. Duchnowicz and Koter also investigated the fluidity of erythrocyte membrane using 16-doxylstearic acids using ESR [12]. This probe study estimated the changes in lipid fluidity at the level of the 16th carbon atom in the fatty acid moiety that may also be measured by the use of DPH in the fluorometric method. They observed a decrease in the τ_B and τ_C correlation times in erythrocytes incubated with 2,4-DCP, 2,4,5-TCP and 2,4-DMP, which suggested an increase in membrane fluidity. Our measurements confirmed these findings.

Because changes in the anisotropy fluorescence of ANS inform us about perturbation in both lipids and proteins present in cell membranes, we tried to compare the changes in membrane proteins using the electrophoresis technique. Protein-protein and protein-lipid interactions are thought to play a vital role in the maintenance of the shape and deformation of red blood cells [22].

Changes in the content of proteins in erythrocyte membranes were observed in electrophoretic gels (Fig. 2). Catechol and 2,4,5-TCP increased the content of low molecular weight proteins as compared to control, suggesting fragmentation of main membrane proteins. The highest changes were observed for 2,4,5-TCP, which caused statistically significant changes in the relative amounts of some fractions: spectrin (α and β), band 3, band 4.1 and 4.2 and low molecular weight proteins. 2,4-DCP caused changes in the relative amounts of spectrin and band 3 and band 6 proteins, and catechol changed the relative amounts of spectrin and lower molecular weight proteins.

Every change in the content of spectrin will provoke a transformation in the cell shape, leading to a decreased cellular deformability and survival [23-25]. Therefore, the observed changes in spectrin and band 3 protein prompted us to examine erythrocyte shape and size.

We may conclude that catechol, phenol, and 2,4-DMP increased FSC-A parameter, which was accompanied by a decrease in SSC-A and, reversely, a decrease in FSC-A (size/volume) accompanied by an increase in SSC-A (shape). The exceptions are chlorophenols that at lower doses up to 50 ppm, increased both SSC-A and FSC-A parameters, but decreased them at higher doses. The highest changes both for SSC-A and FSC-A parameters were observed for 2,4,5-TCP. We suppose that the effect on the shape of erythrocytes was determined by the presence of three chlorine atoms in this phenol (Figs. 3 and 4).

Furthermore, the light microscope technique was used to analyze erythrocyte shapes. The comparison of a series of erythrocyte photos revealed that, in contrast to biconcave disc shape of normal red cells, erythrocytes incubated with chlorophenols and catechol displayed remarkable changes in morphology. Among normal erythrocytes, irregular cells were found. According to Sheetz and Singer's [26] hypothesis, the shape changes induced in erythrocytes arise from a

differential expansion of two monolayers in the membrane lipid bilayer. Thus, echinocytes are produced by the insertion of the added compound in the outer monolayer, whereas stomatocytes are obtained by its location in the inner monolayer. In our experiments, echinocytes were formed after a treatment with catechol and chlorophenols. After incubation with erythrocytes with 2,4-DMP and phenol we did not observe the formation of stomatocytes and echinocytes (Fig. 5).

Comparing the actions of the investigated compounds on erythrocyte membrane, we proved that chlorophenols induced the strongest changes in erythrocyte membrane properties and phenol induced the relatively lowest changes. Phenol did not change the level of carbonyl groups in human erythrocytes [6] and the percentage of membrane protein fractions (Fig. 2), the value of the size of human erythrocytes (Fig. 3) and the activity of acetylcholinesterase membranes in erythrocytes [10]. This compound did not induce lipid peroxidation [27] and did not increase the amount of free radicals in erythrocytes after 3 hours (only after 24 hours) [6].

The 2,4-dimethylphenol increased the level of carbonyl groups [6], induced haemoglobin oxidation [28] and changed the value of SSC-A and FSC-A (Figs. 3 and 4) but did not induce lipid peroxidation [28], and did not change erythrocyte morphology (Fig. 5). The observed changes in erythrocyte morphology incubated with 2,4-DMP differ from those observed under the influence of chlorine-containing compounds (Fig. 5). The presence of the methyl group within the phenol molecule did not cause severe oxidative changes in erythrocyte membrane or changes in composition of the lipid-protein bilayer structure.

Chlorophenols oxidize lipids [28] and proteins [6], cause ROS formation [29, 30], change protein conformation [12] and induce the highest changes in the percentage of membrane protein fractions (Fig. 2). They also disturb membrane fluidity (Table 1) and, finally, change the size and shape of red blood cells (Figs. 3 and 4) and in a consequence the morphology of erythrocytes (Fig. 5). The additional chlorine atom in 2,4,5-TCP is most probably responsible for significant changes in erythrocyte size and shape (Figs. 3 and 4), which may lead to cell shrinkage and hemoglobin leakage [9].

One major functional consequence of such damage is the breakdown of the membrane barrier and thus hemolysis of erythrocytes. Cruz Silva et al. [31] suggested that hemolysis may be the result of the modifications of erythrocyte membrane proteins and changes of the framework of cytoskeleton and/or plasma membrane proteins. Disturbance of the structure of the erythrocyte membrane caused by the intercalation of phenol derivatives into membrane may lead directly to the loss of membrane integrity and facilitate the penetration of other toxic factors into the bilayer of other agents. The earlier investigations performed by Duchnowicz et al., [28] and also Bukowska and Kowalska [27] confirmed that phenol and its derivatives induced hemolysis. The stronger lysis was observed for catechol, lower for chlorophenols and methylphenol and the lowest for phenol.

In conclusion, phenol derivatives induced disturbances in the structure of erythrocyte membranes, leading to changes in normal erythrocyte size and shape. They also decreased mechanical stability of red blood cells.

Abbreviations:

2,4-DCP – 2,4-dichlorophenol;
 2,4,5-TCP – 2,4,5-trichlorophenol;
 2,4-DMP – 2,4-dimethylphenol;
 DPH – 1,6-diphenylhexatriene;
 TMA-DPH – 4'-trimethyl-ammonio-1,6-diphenyl-1,3,5-hexatriene;
 ANS – 8-anilino-1-naphthalene sulfonate;
 ROS – Reactive Oxygen Species;
 FSC-A – size, volume of erythrocytes (low angle light scattering);
 SSC-A – shape of erythrocytes (right angle light scattering).

Acknowledgments

This work was partly funded from costs of a statutory investigation admitted for the Department of Biophysics of Environmental Pollution in 2007.

References

- MICHAŁOWICZ J., The occurrence of chlorophenols, chlorocatechols and chlorinated methoxyphenols in drinking water of the largest cities in Poland. *Pol. J. Environ. Stud.* **14**, 327, **2005**.
- KJÄLLSTRAND J., PETERSSON G., Phenolic antioxidants in alder smoke during industrial meat curing. *Food Chem.* **74**, 85, **2001**.
- SCHWEIGERT N., ALEXANDER J., ZEHNDER J., Chemical properties of catechols and their molecular modes of toxic action in cells from microorganisms to mammals. *Environ. Microbiol.* **3**, 81, **2001**.
- WAN J., WINN L.M., The effects of benzene and the metabolites phenol and catechol on c-Myb and Pim-1 signaling in HD3 cells. *Toxicol. Appl. Pharmacol.* **201**, 194, **2004**.
- HORI T.S.F., AVILEZ I.M., IWAMA G.K., JOHNSON S.C., MORAES G., ALFONSO L.O.B., Impairment of the stress response in *Brycon amazonicus* juveniles exposed to low concentrations of phenol. *Comp. Biochem. Physiol. Part C* **147**, 416, **2008**.
- BUKOWSKA B., MICHAŁOWICZ J., KROKOSZ A., SICIŃSKA P., Comparison of the effect of phenol and its derivatives on protein and free radical formation in human erythrocytes (in vitro). *Blood Cell Mol. Dis.* **39**, 238, **2007**.
- MICHAŁOWICZ J., BUKOWSKA B., DUDA W., The differences in phenolic content in rivers exposed and non-exposed to anthropogenic contamination. *Chemosphere* **71**, 735, **2008**.
- BUKOWSKA B., Effects of 2,4-D and its metabolite 2,4-dichlorophenol on antioxidant enzymes and level of glutathione in human erythrocytes. *Comp. Biochem. Physiol. Part C* **135/4**, 435, **2003**.
- BUKOWSKA B., 2,4,5-T and 2,4,5-TCP induce oxidative damage in human erythrocytes: role of glutathione. *Cell Biol. Int.* **28**, 557, **2004**.
- BUKOWSKA B., HUTNIK K., Phenoxyherbicides and their derivatives: Effect on the activity of erythrocytes acetylcholinesterase (in vitro). *Pest. Biochem. Physiol.* **85**, 174, **2006**.
- DUCHNOWICZ P., SZCZEPANIAK P., KOTER M., Erythrocyte membrane protein damage by phenoxyacetic herbicides. *Pest. Biochem. Physiol.*, **82**, 59, **2005**.
- DUCHNOWICZ P., KOTER M., Damage to the erythrocyte membrane caused by chlorophenoxyacetic herbicides. *Cell Mol. Biol. Lett.* **8**, 25, **2003**.
- CARINI M., ALDINI G., BOMBARDELLI E., MORAZZONI P., FACINO R.M., UVB-induced hemolysis of rat erythrocytes: protective effect of procyanidins from grape seeds. *Life Sciences* **67**, 1799, **2000**.
- MICHAŁOWICZ J., DUDA W., Phenols – the sources and toxicity. *Pol. J. Environ. Stud.* **16**, 78, **2007**.
- DODGE J.T., MITCHELL C., HANAHAN D.J., The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch. Biochem. Biophys.* **100**, 119, **1963**.
- LOWRY O.H., ROSENBROUGH N.J., LEWIS FARR A., ROSE J.R., Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265, **1951**.
- LAEMMLI U.K., Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680, **1970**.
- FAIRBANKS G., STECK T.L., WALLACH D.F.H. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. *Biochemistry* **10**, 2606, **1971**.
- De GROOTH B.G., TERSTAPPEN L.W., PUPPLES G.J., GREVE J., Light-scattering polarization measurements as a new parameter in flow cytometry. *Cytometry* **8**, 539, **1987**.
- LAKOWICZ J.R., Principles of Fluorescence Spectroscopy. Kluwer Academic Publishers, New York, **1999**.
- SINGER S.J., NICOLSON G.L., The fluid mosaic model of the structure of cell membranes. *Science* **175**, 720, **1972**.
- MARCZAK A., JÓŹWIĄK Z., The interaction of DNR and glutaraldehyde with cell membrane proteins leads to morphological changes in erythrocytes. *Cancer Lett.* **26**, 118, **2008**.
- SOSZYŃSKI M., SCHUESSLER H., Effect of X-irradiation on erythrocyte membrane proteins. Primary radicals. *Int. J. Radiat. Biol.* **60**, 859, **1991**.
- MARCZAK A., WALCZAK M., JÓŹWIĄK Z. The combined effect of IDA and glutaraldehyde on the erythrocyte membrane proteins. *Int. J. Pharm.* **335**, 154, **2007**.
- SUWALSKY M., RAMOS P., VILLENA F., AQUILAR F., SOTOMAYOR C.P., The Organophosphorus Insecticide Parathion Changes Properties of natural and Model membranes. *Pest. Biochem. Physiol.* **70**, 74, **2001**.
- SHEETZ M.P., SINGER S.J., Biological membranes as bilayer couples. A mechanism of drug-erythrocyte interactions. *Proc. Natl. Acad. Sci. USA*, **71**, 4457, **1974**.
- BUKOWSKA B., KOWALSKA S., Phenol and catechol induce prehemolytic and hemolytic changes in human erythrocytes. *Toxicol. Lett.* **52**, 73, **2004**.

28. DUCHNOWICZ P., KOTER M., DUDA W., Damage of erythrocyte by phenoxyacetic herbicides and their metabolites. *Pest. Biochem. Physiol.* **74**, 1, **2002**.
29. LI F., JI L., OH K., Hydroxyl radical generation and oxidative stress in *Carassius auratus* liver as affected by 2,4,6-trichlorophenol. *Chemosphere* **67**, 13, **2007**.
30. BUKOWSKA B., RYCHLIK B., KROKOSZ A., MICHAŁOWICZ J., Phenoxyherbicides induce production of free radicals in human erythrocytes: Oxidation of dichlorodihydrofluorescein and dihydrorhodamine 123 by 2,4-D-Na and MCPA-Na. *Food Chem. Toxicol.* **46**, 359, **2008**.
31. CRUZ SILVA M.M., MADEIRA V.M., ALMEIDA L.M., CUSTODIO J.B., Hemolysis of human erythrocytes induced by tamoxifen is related to disruption of membrane structure. *Biochim. Biophys. Acta* **1464**, 49, **2000**.