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The influence of ozone on the domain structure of human erythrocyte membranes: an EPR study

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ABSTRACT

Introduction. Autotransfusions of ozonised blood or infusions of gaseous ozone into blood vessels and body cavities are believed to exert therapeutic effects in some pathological states. Investigations on the reaction of ozone with biological molecules and membrane structures are a subject of crucial importance. The present study aimed to yield more precise data about the alterations, which occur in different erythrocyte membrane regions subjected to medical ozone. This could provide some additional information about the structural changes in the membrane at a molecular level.

Material and methods. Blood was obtained from 22 healthy volunteers (aged 21 to 63) by vein puncture and mixed with 1/10 volume of 0.13 M trisodium citrate. Erythrocytes were isolated from fresh blood by centrifugation at 4°C, at 1,500 × g and purified by three cycles of resuspension and washing with PBS. Ozone was generated by passing pure gaseous oxygen at 30 l/h through an apparatus producing silent electric discharges. EPR spectra were obtained at X-band (9.4 GHz), at modulation frequency of 100 kHz. The scan time was 4 min, and the time constant 0.3 s. Since biological membranes are heterogeneous systems composed of several coexisting domains, EPR spectra are superimpositions of several spectra with different fluidity parameters.

Results. The effects of ozone at two concentrations (10 and 45 g/m^3) on fluidity and phospholipid domain structure of erythrocyte membranes were investigated by electron paramagnetic resonance (EPR). At increased ozone concentration (45 g/m^3), the portion of the least ordered domains (WLO) increased, with a corresponding decrease of ordered (WMO, WO), more rigid regions. The order of lipid acyl chains of two ordered (MO and O) as well as the least ordered (LO) domains diminished, as expressed by smaller order parameters. For ozone concentration of 10 g/m^3 values of order parameter were slightly increased, which indicates a tendency to rigidisation of lipid bilayer at this ozone concentration. This change was, however, statistically insignificant. There were no statistically significant differences in the thermotropic behaviour of weight factor of ordered domains (WMO, WO) between ozonised and control red cells.

Conclusion. The obtained data shows that ozonation of erythrocytes results in cell membrane fluidisation and an increase in red cell deformability. On the other hand, these results could suggest that ozonation of erythrocytes leads to structural changes in the membranes, especially in cytoskeletal proteins, but this effect is probably dose-dependent.

Key words: ozonized blood, plasma membrane, therapeutic ozon action, thermotropic behaviour

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Introduction

Autotransfusions of ozonised blood or infusions of gaseous ozone into blood vessels and body cavities are believed to exert therapeutic effects in some pathological states. Ozonotherapy has been criticised by numerous investigators, on the grounds that the mechanism of therapeutic ozone action is still unknown [1, 2]. Therefore, investigations on the reaction of ozone with biological molecules and membrane structures are a subject of crucial importance. It is well known that ozone is one of the most powerful oxidants. During ozonotherapy, plasma membranes may be the major sites of damage [1, 3].

The reaction of ozone with biological membranes has been the subject of enduring controversy. It has been reported that lipid peroxidation caused by ozone decreases as well as increases the molecular ordering of biological membranes [4–6].

In previous studies of the influence of medical ozone on erythrocytes, the heterogeneous structure of plasma membrane, composed of several coexisting domains of different fluidity, was not taken into account [7].

Therefore, with respect to the heterogeneity of the membrane structure, the present study aimed to yield more precise data about the alterations, which occur in different erythrocyte membrane regions subjected to medical ozone. This could provide some additional information about the structural changes in the membrane at a molecular level.

Membrane fluidity was studied using the electron paramagnetic resonance (EPR) spin-labelling technique. The line shape of experimental spectra was compared to calculated spectra by the model based on the lateral heterogeneity of cell membrane with several coexisting domains of different fluidity and dynamics [8].

Materials and methods

Fatty acid spin label 5 doxylstearic acid (5-DSA) was purchased from Sigma (St. Louis, MO, USA). Blood was obtained from 22 healthy volunteers (aged 21 to 63) by vein puncture and mixed with 1/10 volume of 0.13 M trisodium citrate. Erythrocytes were isolated from fresh blood by centrifugation at 4°C, at 1,500 × g and purified by three cycles of resuspension and washing with PBS (phosphate buffered NaCl solution, 310 mOsm, pH 7.4), after careful removal of the buffy coat.

Ozone was generated by passing pure gaseous oxygen at 30 l/h through an apparatus (ATO3, Krio Metrum, Poland) producing silent electric discharges. The ozone/oxygen mixture at two concentrations: 10 and 45 g/m^3 of O_2/O_3 mixture was passed for 5 min over 5 mL of stirred blood sample.

EPR spectra were obtained at X-band (9.4 GHz), at modulation frequency of 100 kHz. The scan time was 4 min, and the time constant 0.3 s.

The order parameter S was calculated from the formula of Gaffney [9]:

$$S = \frac{A_{II} - (A_{\perp} + C)}{A_{II} + 2(A_{\perp} + C)} \times 1.723$$

$$C = 1.4–0.53\ (A_{II} - A_{\perp})$$

where and are parallel and perpendicular hyperfine splitting parameters of the spectrum, respectively. Changes of the order parameter values correspond to changes of membrane local viscosity — i.e. its increase means a decrease of viscosity.

Since biological membranes are heterogeneous systems composed of several coexisting domains, EPR spectra are superimpositions of several spectra with different fluidity parameters. Therefore, the line-shape of the EPR spectra $I(B_r)$ can be expressed as $I(B_r) = \Sigma W_i I_i(B_{ri})$, where $I_i(B_{ri})$ is the EPR spectrum line-shape of domain i and W_i is the EPR spectrum intensity weight factor which is proportional to the number of spin probes located in a particular domain in the membrane. The line-shape simulations and calculations of the number of domains, the relative portion (W) and the fluidity parameter (S) of each domain were performed by a program for line shape simulation and optimisation (EPRSIM v. 4.0) [8].

Results and discussion

The best fit to the experimental spectrum was obtained when the heterogeneous membrane structure was taken into account with the superimposition of three types of domains with different order parameters (Fig.1): two regions with a high degree of order of the

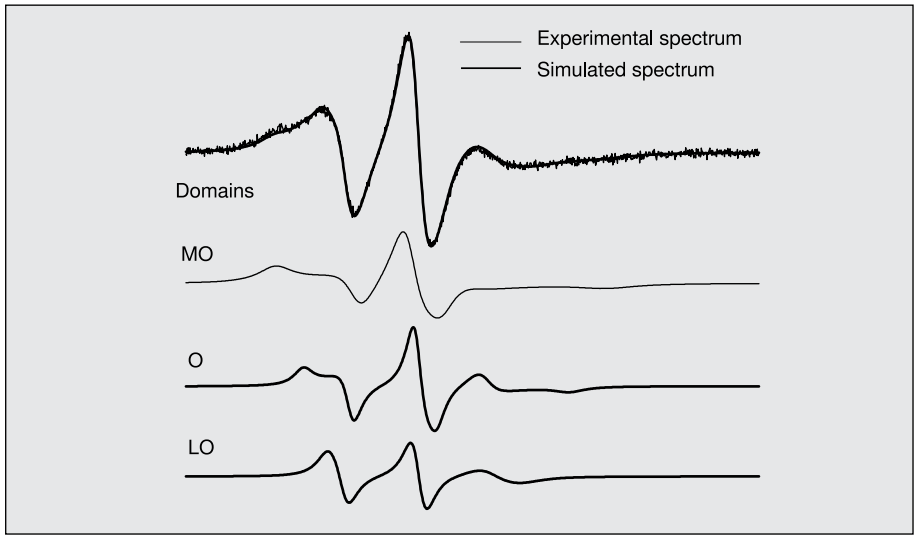

Figure 1. Experimental and simulated EPR spectrum of the spin-labelled erythrocyte membrane. The simulated spectrum is decomposed into three spectral components which correspond to three membrane domains of different fluidity characteristics (MO — the most ordered domain, O — ordered domain, and LO — the least ordered domain)

lipid acyl chains (one of them most ordered — MO and the second one ordered — O) and one region with poor alignments of hydrocarbon chains (least ordered — LO, with a small order parameter). From the best fits of simulated to experimental spectra calculations of number and portions of the coexisting membrane domains (W) and their fluidity characteristics were made by a program for line shape simulation and optimisation (EPRSIM v. 4.0) [8].

The treatment of erythrocyte membrane with ozone revealed that increase in ozone concentration affected the fluidity characteristics and the portion (W) of membrane domains (Tab. 1). At high ozone concentration, the portion of the least ordered domains (W_{LO}) was increased, with a corresponding decrease of ordered (W_{MO}, W_O), more rigid regions. The order of lipid acyl chains of two ordered (MO and O) as well as the least ordered (LO) domains diminished, as expressed by smaller order parameters S (Tab. 1). For ozone concentration of 10 g/m^3 values of the order parameter (S) were slightly increased, which indicates a tendency to rigidisation of lipid bilayer at this O_3 concentration. This change was, however, statistically insignificant.

To obtain a better insight into the dynamics of the processes occurring in cell membrane domains of ozonised red cells, the thermotropic behaviour of fluidity characteristics have also been analysed. There were no statistically significant differences in thermotropic behaviour of weight factor of ordered domains (W_{MO}, W_O) between ozonised and control red cells (data not shown). The temperature dependence of the relative weight factor representing the least ordered domains (W_{LO}) in erythrocyte membrane differed for non-treated (control) and ozonised red cells, especially at an O_3 concentration of 45 g/m^3 (Fig. 2). At 37°C, a marked increase in the portion of the least ordered domains (W_{LO}) was observed.

Erythrocyte membranes have been reported, using the EPR spin labelling method, to undergo three thermotropic phase transitions at 8, 20 and 40°C. Transitions at 8 and 40°C depend on the interactions of cytoskeletal proteins with membrane; protein 4.1 is involved in the 8°C transition of red blood cells, whereas spectrin seems to be involved in a structural membrane transition at 40°C. The 20°C transition is related to the components of the lipid phase of the membrane [10].

Our previous results showed that for the ozone concentration of 45 g/m^3 the disappearance of the low temperature break and the appearance of two breaks at 37°C and 16°C were observed, which could indicate that O_3 at this concentration selectively affects membrane protein band 4.1 [7].

Table 1. Order parameter (S) and the portion of a specific domain (W) used in the calculation of the EPR spectra of spin probe 5-DSA in control and ozonised erythrocyte membrane, which gave the best fit to the experimental spectra

Control erythrocytes				**Ozonised erythrocytes**					
				Concentration 10 g/m^3			**Concentration 45 g/m^3**		
Domain	MO	O	LO	MO	O	LO	MO	O	LO
S	0.630 ± 0.005	0.421 ± 0.002	0.185 ± 0.003	0.635 ± 0.005	0.425 ± 0.005	0.187 ± 0.003	0.610 ± 0.003[b]	0.410 ± 0.003[c]	0.169 ± 0.005[a]
W	0.46 ± 0.04	0.33 ± 0.03	0.21 ± 0.02	0.47 ± 0.03	0.33 ± 0.04	0.20 ± 0.03	0.42 ± 0.04[b]	0.31 ± 0.01[c]	0.27 ± 0.03[a]

The best fit to the experimental spectrum was obtained when the heterogeneous membrane structure was taken into account with the superimposition of three types of domains with different order parameters (S). The relative portion of the distinct domain is given by the EPR spectra intensity weight factor (W).
[a]Significantly different from values obtained for domain LO in control erythrocytes, $p < 0.005$
[b]Significantly different from values obtained for domain MO in control erythrocytes, $p < 0.05$
[c]Significantly different from values obtained for domain O in control erythrocytes, $p < 0.05$

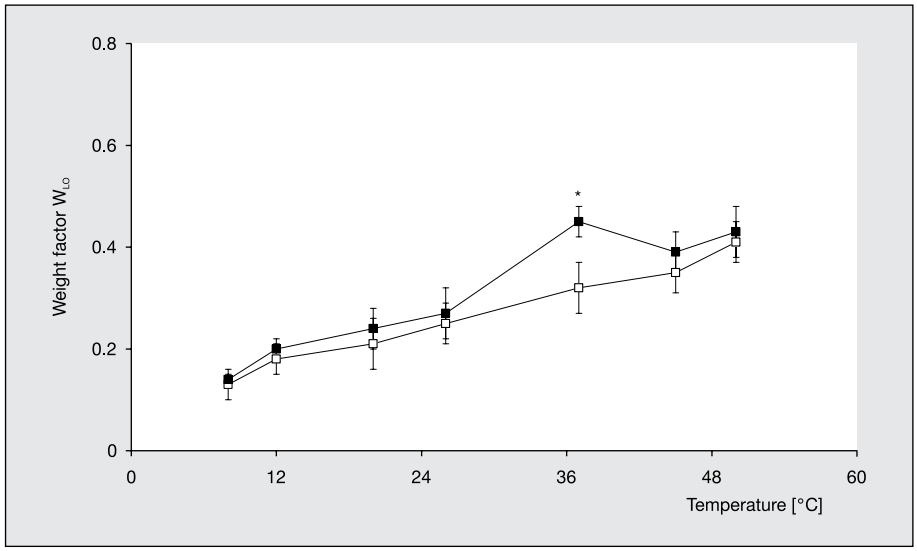

Figure 2. Temperature dependence of the relative weight factor representing the less ordered membrane domains (W_{LO}) in erythrocyte membranes labelled with 5-DSA spin probe. (□) — untreated cells; (■) — erythrocyte membranes with ozone at concentration of 45 g/m^3. Each point represents the mean of five repeats; typical results ± S.D. are indicated

Observed in this experiment was a marked increase in the portion of the least ordered domains (W_{LO}) at 37°C. This may indicate structural changes in spectrin moiety, because this protein molecule is involved in a high temperature transition [11]. Some observations have shown that the strong oxidation of erythrocyte membrane at an O_3 concentration higher than 10 g/m^3 leads to the destruction of protein molecules [12]. On the other hand, degradation of membrane proteins seems to be conducive to lower ordering of lipids and leads to an increase of its motional freedom. Therefore, the phenomenon we observed in our experiments, i.e. an increase in the portion of more fluid (LO) membrane region, may result from the destruction of membrane proteins, especially at an O_3 concentration of 45 g/m^3. We also observed in this experiment the influence of ozone on the organisation of membrane constituents; this agrees with previously described results [6, 7, 12, 13].

The obtained data shows that ozonation of erythrocytes results in cell membrane fluidisation and an increase in red cell deformability. On the other hand, these results could suggest that ozonation of erythrocytes leads to structural changes in the membranes, especially in cytoskeletal proteins, but this effect is probably dose-dependent.

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