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*Scientific paper*

# Effect of Ozone on Oxygen Transport and Pro-Oxidant-Antioxidant Balance of Red Blood Cell Suspension

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## Abstract

**Introduction:** Ozone affects blood oxygen transport and the pro-oxidant-antioxidant balance. However, the role of blood formed elements and gas transmitters in these processes still remains unclear. The aim of the present study was to investigate the effect of ozone on oxygen transport and the pro-oxidant-antioxidant balance in a red cell suspension.
**Methods:** The red cell suspension was incubated with ozone at a concentration of 6 mg/l and substances affecting the synthesis of gas transmitters (nitroglycerin, sodium hydrosulfide). Parameters of blood oxygen transport and pro-oxidant-antioxidant balance were determined.
**Results:** The effect of ozone on blood oxygen transport was found which was manifested in an increase in oxygen partial pressure and the degree of oxygenation. The index of hemoglobin –oxygen affinity $p50_{actual}$ was raised and a shift of the oxyhemoglobin dissociation curve rightwards was noticed. The addition of the gas transmitter donor, nitric oxide, enhanced the effect of this gas on the parameters of oxygen transport in the erythrocyte suspension
**Conclusion:** We found that ozone induced a change in oxygen-binding properties of the erythrocyte suspension which is a mechanism of action of the above gas on the adaptive processes in the body realized directly at the level of erythrocytes.

**Keywords:** Ozone, red blood cells, gas transmitter, hemoglobin oxygen affinity, nitric oxide, hydrogen sulfide

## 1. Introduction

Ozone ($O_3$) exerts various physiologic effects on the organism: increases the rate of erythrocyte glycolysis, improving oxygen delivery to tissues, and activates the enzymatic link of the antioxidant system (glutathione, peroxidase, catalase and superoxide dismutase).[1] Our earlier studies demonstrated the effect of $O_3$ on blood oxygen transport, which was manifested by a distinct shift of the oxyhemoglobin dissociation curve (ODC) rightwards and elevated concentrations of the gas transmitters hydrogen sulfide ($H_2S$) and nitric oxide (NO).[2] An effect of ozone on hemoglobin – oxygen affinity (HOA) due to activation of the gas transmitter system is suggested.[3] Red blood cells possess their own mechanisms of NO synthesis and can serve as an essential source of NO under hypoxia.[4] Moreover, erythrocytes were shown to contain 3-mercaptopyruvate sulfotransferase, contributing to $H_2S$ production.[5]

However, NO-synthase activity is not only inherent to red blood cells; it is also a characteristic of leukocytes and thrombocytes. Thrombocytes were demonstrated to contain two isoforms of NO-synthase (inducible and endothelial), and the application of flow cytometry allowed Mahaj et al. to detect inducible NO-synthase in leukocytes.[6,7] Therefore, it was necessary to investigate the ability of red blood cells to respond to the action of ozone.

The purpose of this work was to study the effect of ozone on oxygen transport in a red blood cell suspension.

## 2. Materials and Methods

### 2. 1. Materials and Study Design

Venous blood was drawn with a heparin-pretreated syringe (50 U/ml).

This study was conducted, using a suspension of red cells from the blood of albino Sprague Dawley male rats fed a standard laboratory diet. The experimental protocol was approved by the Ethical Committee of Grodno State University (approval No1 of January 14, 2019).

To separate blood plasma and erythrocytes, blood samples were centrifuged at 3000 r.p.m. over 10 min and washed twice with a cold isotonic solution. Thereafter the

obtained red blood cell suspension was divided into 4 groups of samples of ten 1.2 ml samples in each group (the hematocrit level was 40%) (Figure 1). An isotonic solution of sodium chloride was enriched with an ozone-oxygen mixture for 4–5 minutes using an ozone generator UOTA-60-01 (Medozon, Russia), which makes it possible to measure ozone concentrations by an optical method in the ultraviolet range, which is provided by the technical capabilities of the device.

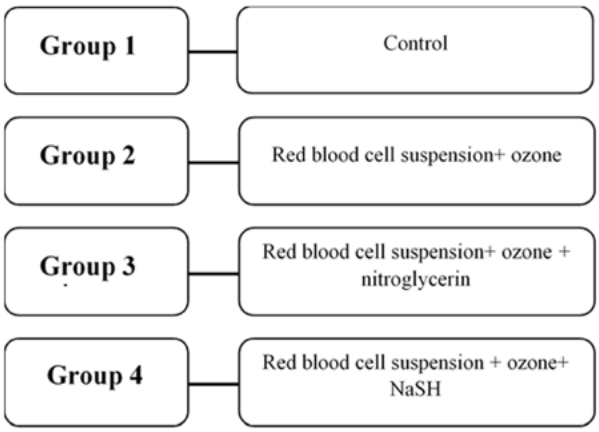


**Figure 1:** Study design

Group 1 samples (control) contained the erythrocyte suspension (1.2 ml) + isotonic solution of sodium chloride (1.1 ml). The contents of Group 2 samples were as follows: the red blood cell suspension (1.2 ml) + 1 ml of the ozonized isotonic solution of sodium chloride ($O_3$ concentration was 6 mg/l) + 0.1 ml of the isotonic solution of sodium chloride. The samples of Group 3 were composed of the red blood cell suspension (1.2 ml) + 1 ml of the ozonized isotonic solution of sodium chloride ($O_3$ concentration was 6 mg/l) + 1 ml of the solution containing the gas transmitter nitroglycerin (SchwarzPharma AG) at the final concentration of 0.05 mmol/l. Group 4 samples contained the red blood cell suspension (1.2 ml) + 1 ml of the ozonized isotonic solution of sodium chloride ($O_3$ concentration was 6 mg/l) + 0.1 ml of the solution containing the gas transmitter sodium hydrosulfide (Sigma-Aldrich) at the final concentration of 0.38 mmol/l. The contents of each sample were mixed. The incubation time was 60 min. Thus, we had control without ozonation (Group 1), the ozonized red blood cell suspension (Group 2) and the red blood cell- and gas-transmitter-containing suspensions (Groups 3 and 4).

## 2. 2. Methods

### 2. 2. 1. Blood Oxygen Transport (Hemoglobin-Oxygen Affinity)

Parameters of blood oxygen transport and acid-base status were measured using a Stat-Profile pHOx plus L gas analyzer at 37°C. Partial pressures of oxygen ($pO_2$) and carbon dioxide ($pCO_2$), the degree of oxygenation ($SO_2$), standard bicarbonate (SBC), actual base excess/standard base excess (ABE/SBE), hydrogen carbonate ($HCO_3^-$), pH and total plasma carbonic acid ($TCO_2$) were determined. Hemoglobin-oxygen affinity was assessed spectrophotometrically by $p50_{actual}$ ($pO_2$ corresponding to 50% Hb saturation with oxygen). The Severinghaus formulas were used to calculate $p50_{standard}$ and ODC position.[8]

### 2. 2. 2 Pro-Oxidant/Antioxidant System

The activity of free radical processes was evaluated by the contents of primary (diene conjugates, DC) and intermediate (malondialdehyde, MDA) products of lipid peroxidation (LPO) in the red blood cell suspension. The level of DC was measured spectrofluorimetrically (an SM 2203 spectrofluorimeter, SOLAR) by a method based on the intensity of absorption of diene structures of lipid hydroperoxides at 233 nm, in comparison with the blank samples in which the biological material was substituted by distilled water.[9] The DC content was expressed as U/ml. The concentration of MDA (TBARS) was assessed by the interaction with 2'-thiobarbituric acid (TBA) which, when heated in acidic medium, causes the formation of a pink trimethine complex.[10] The intensity of color was determined spectrophotometrically at a wavelength of 55 nm with a PV12 51 SOLAR spectrophotometer and compared to control. The MDA concentration was expressed as μmol/l.

To determine catalase activity in hemolysates, we used the method of Koroliuk based on spectrophotometrical recording of the amount of the colored product of the reaction of $H_2O_2$ with ammonium molybdate having a maximum absorption at a wavelength of 410 nm.[11] The activity of catalase was expressed as mmol $H_2O_2$/min/g Hb. The amount of the enzyme catalyzing the formation of 1 mmol of the product per 1 min under the experimental conditions was taken as the unit of activity.

### 2. 2. 3. Statistical Analysis

The correspondence of the study data to the normal distribution law was tested by the Shapiro-Wilk test. With consideration for this criterion, non-parametric statistics with application of Statistica 10.0 software (StatSoft Inc., Tulsa, Oklahoma, USA) was used. Three and more independent groups were compared by the Kruskal-Wallis one-way analysis-of-variance-by ranks test. Allowing for the small sample and multiple comparisons, the significance of the data obtained was evaluated using the Mann-Whitney U-test. The Wilcoxon signed ranks test was applied for paired in-group comparisons of the indices levels using repeated measures ANOVA. The results were presented as a median (Me), the interquartile range between the 25$^{th}$ and the 75$^{th}$ percentile. The data were considered significant at the level of $P < 0.05$.

## 3. Results and Discussion

The $O_3$ treatment of the red blood cell suspension resulted in an increase in the fundamental indices of blood oxygen transport: $SO_2$ by 121.8 % ($P < 0.05$), $pO_2$ by 74.1% ($P < 0.05$) (Table 1). Under these conditions, the value of HOA parameter $p50_{actual}$ was increased by 21.4% ($P < 0.05$) and the ODC was shifted rightwards (Figure 2) compared to the control group. The value of $p\ 50_{standard}$ was also observed to rise. No significant changes were found when analyzing the acid-base balance parameters. Nitroglycerin enhanced the effect of ozone on the oxygen transport in the red blood cell suspension. The values of $SO_2$ and $pO_2$ increased by 12.5% and 21.0 ($P < 0.05$), respectively, in comparison with the samples pretreated with the ozonized isotonic solution of sodium chloride. Under these conditions, the HOA parameter $p50_{actual}$ increased by 7.5% ($P < 0.05$) and the ODC was shifted rightwards more distinctly (Figure 2). The $H_2S$ donor (sodium hydrosulfide) did not exert a similar effect.

The treatment of the red blood cell suspension with the ozonized saline solution caused an 85.3% elevation of the MDA content in the red blood cell suspension and the concentration of DC was increased by 77.4% ($P < 0.05$) in comparison with the control group (Table 2), whereas catalase activity was decreased by 44.5% ($P < 0.05$). The addition of the gas transmitter donors, nitroglycerine and sodium hydrosulfide, did not cause any changes in the LPO indices. However, it raised catalase activity by 46.1% ($P < 0.05$) and 43.8% ($P < 0.05$), respectively compared to the group of samples containing the ozone –treated red blood cell suspension.

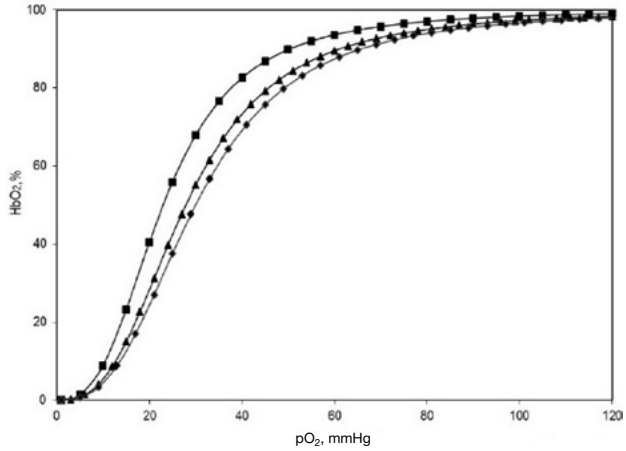


**Figure 2**: Effect of ozone on the position of the oxyhemoglobin dissociation curve at real pH and $pCO_2$ values. ▪ – control; ▲ – red blood cell suspension + ozone; ♦ – red blood cell suspension +ozone +NO.

In comparison with other blood formed elements, red blood cells are an important target for the action of ozone. Due to the hexose monophosphate shunt, ozone promotes activation of 2,3-diphosphoglycerate mutase (DPGM), which finally results in conversion of erythrocyte1,3-DPGM to 2,3-DPGM that, binding to the hemoglobin β-chain, may bring about an ODC shift right-

**Table 1:** Effect of ozone on oxygen transport in red blood cell suspension (median [25th; 75th percentile])

| Parameter | Control (n = 10) | Red blood cell suspension + ozone (n = 10) | Red blood cell suspension + ozone + NO (n = 10) | Red blood cell suspension + ozone + $H_2S$ (n = 10) |
|---|---|---|---|---|
| $SO_2$, % | 26.96 [26.00; 32.10] | 59.80 [56.30; 62.20]* | 67.30 [62.70; 67.70]*# | 58.10 [57.90; 58.70]*Ψ |
| $pO_2$, mmHg. | 18.15 [17.40; 19,60] | 31.60 [30.10; 34.50]* | 38.25 [37.40; 39.20]*# | 31.05 [28.70; 31.30]*Ψ |
| pH, units | 7,305 [7.287; 7.356] | 7.331 [7.321; 7.352] | 7.313 [7.293; 7.315]# | 7.318 [7.312;7.353] |
| $pCO_2$, mmHg. | 5.25 [4.70; 5.90] | 4.70 [4.50; 5.10] | 6.45 [4.30; 8.70] | 6.45 [5.20; 8.20]# |
| $HCO_3^-$, mmol/L | 2.70 [2.30; 3.30] | 2.45 [2.30; 2.70] | 3.25 [2.30; 4.20] | 3.30 [2.80;4.18]# |
| $TCO_2$, mmol/L | 2.85 [2.40; 5.90] | 2.60 [2.40; 2.90] | 3.5 [2.40; 4.50] | 3.45 [2.83;4.50]# |
| ABE, mmol/L | –23.60 [–24.60; –20,40] | –23.65 [–23.80;–23.60] | –23.30 [–24.10; –22.40] | –23.20 [–23.48;–22.30]# |
| SBE, mmol/L | –19.90 [–21.00; –17.10] | –20.60 [–20.70; –20.30] | –20.05 [–20.30; –19.50]# | –20.80 [–21.15; –20.53]Ψ |
| SBC, mmol/L | 9.00 [8.50; 10.80] | 8.60 [8.60; 8.90] | 8.85 [8.40; 9.40] | 8.75 [8.40;9.38] |
| $p50_{actual}$, mmHg | 22.96 [22.40; 23.97] | 27.88 [27.49; 27.92]* | 29.99 [29.79; 31.13]*# | 27.55 [27.49; 27.85]*Ψ |
| $p50_{standard}$, mmHg | 20.63 [20.20; 21.60] | 25.40 [23.00; 26.90]* | 28.35 [27.40; 29.00]*# | 23.40 [23.40; 23.60]*Ψ |

Note: changes compared to control (*), red blood cell suspension + ozone (#), Red blood cell suspension + ozone + NO (Ψ).

**Table 2.** Effect of ozone on indices of pro-oxidant-antioxidant balance of red blood cell suspension (median [25th; 75th percentile])

| Parameter | Control (n = 10) | Red blood cell suspension + ozone (n = 10) | Red blood cell suspension + ozone + NO (n = 10) | Red blood cell suspension + ozone + $H_2S$ (n = 10) |
|---|---|---|---|---|
| MDA, μmol /L | 8.73 [7.63; 9.21] | 16.18 [11.84; 17.57]* | 14.60 [11.57; 17.84]* | 15.55 [14.73; 16.31]* |
| DK, U/mL | 16.62 [15.50; 18.23] | 29.48 [17.54; 34.87]* | 28.48 [25.29; 29.26]* | 28.50 [27.12; 29.89]* |
| Catalase, mmol $H_2O_2$/min/g Hb | 13.53 [12.19; 14.02] | 7.51 [6.18; 9.88]* | 10.97 [10.23; 11.47]*# | 10.80 [10.04; 11.60]*# |

Note: changes compared to control (*), red blood cell suspension + ozone (#).

wards.[12] We believe that in addition to the above mechanism, other mechanisms can be involved in this process, in particular those mediated through gas transmitters. Red blood cells contain the constitutive isoform of NO synthase, which produces NO.[13] Blood plasma shows nitrites/nitrates, but during inhibition of erythrocyte NO synthase, their concentration considerably decreases, which proves that red blood cell NO is exported via anion exchanger 1 in the form of secondary nitrogen species to which hemoglobin is unlikely to bind, thus providing for a free NO pool.[14] Our findings show that the addition of the exogenous donor nitric oxide (nitroglycerin) increases the effect of $O_3$ on blood oxygen transport in the red blood cell suspension. However, the hydrogen sulfide donor (sodium hydrosulfide) does not have this effect. It is known that NO is capable of changing HOA.[15] Its release from red blood cells is controlled by the blood $pO_2$ level, whereas the $O_3$ treatment promotes an increase in this parameter.[16] According to our findings, red blood cells directly respond to the effect of ozone, and this response is manifested with involvement of the gas transmitters, independently of leukocytes and thrombocytes.

Exposure of blood to $O_3$ leads to production of reactive oxygen species, inducing activation of lipid peroxidation in cell membranes and the development of oxidative stress.[17] Continuous exposure of red blood cells to the multitude of different oxidants contributes to the formation of their potent intracellular antioxidant defense system, with gas transmitter mechanisms occupying a special place in the hierarchy of these processes.[18] Due to its oxidative activity, ozone stimulates the antioxidant system of erythrocyte defense and improves cell deformability.[19] Reactive oxygen species are neutralized to give hydrogen peroxide, which finally results in an increase in catalase activity.[20] However, in our studies, the activity of the enzyme decreased, thus providing evidence for an imbalance between antioxidants and free radicals. In the membrane fraction of red blood cells, ozone, as a source of oxygen, reacts with NO to form the powerful oxidant peroxinitrite.[21] Subsequent oxidation of methemoglobin by peroxinitrite may induce synthesis of globin radicals, which enhance pro-oxidant activity in red blood cells.[22] In turn, NO and hydrogen sulfide are also capable of affecting the metabolism of antioxidants due to the gas transmitter enzymes and their reaction products and this feature was noticed in the groups of samples with nitroglycerin and hydrogen sulfide which showed elevated catalase activity compared to the group containing the ozone-treated red blood cell suspension.[23]

Thus, we found ozone-induced changes in parameters of blood oxygen transport of the erythrocyte suspension, which is one of the mechanisms of the action of ozone on adaptive processes in the body which are directly realized by red blood cells.

## 4. Conclusion

1. The exposure of the red blood cell suspension to ozone improved its oxygen transport indices: the increase in $pO_2$ and $SO_2$ as well as the ODC shift rightwards were found.
2. Although the addition of nitroglycerin enhanced the effect of ozone on the oxygen transport in the red blood cell suspension under the above experimental conditions (the distinct ODC shift rightwards), sodium hydrosulfide did not exert a similar effect.
3. Ozone induced an elevation of the DC and MDA concentrations as well as a decrease of catalase activity. The gas transmitter donors did not enhance oxidative stress, but activated catalase.

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### Author contributions

VZ designed, organized, and wrote the article; designed the outline; solved queries related to scientific publications from the journals. EB performed Pubmed searches, aided in writing, and critiqued the literature. All authors have read and approved the manuscript provided.

**Institutional Review Board Statement**

The study was conducted according to the guidelines of the Declaration of Helsinki, and Ethical Committee of the Grodno State Medical University (approval No. 1) approved the study on January 14, 2019.

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## Povzetek


**Uvod**: Ozon vpliva na prenos kisika v krvi in na prooksidativno-antioksidativno ravnovesje, vendar vloga sestavin kri in plinskih prenašalcev pri teh procesih še vedno ostaja nejasna. Namen te raziskave je bil proučiti vpliv ozona na prenos kisika in prooksidativno-antioksidativno ravnovesje v suspenziji rdečih krvničk.

**Metode**: Suspenzijo eritrocitov smo inkubirali z ozonom v koncentraciji 6 mg/l in snovmi, ki vplivajo na sintezo plinskih prenašalcev (nitroglicerin, natrijev hidrosulfid). Določeni so bili parametri prenosa kisika v krvi in prooksidativno-antioksidativno ravnovesje.

**Rezultati**: Ugotovili smo vpliv ozona na prenos kisika v krvi, ki se je kazal v povečanju delnega tlaka kisika in stopnje oksigenacije. Indeks dejanske afinitete hemoglobina do kisika p50 se je povečal, opazen pa je bil tudi premik disociacijske krivulje oksihemoglobina v desno. Dodatek donorskega plinskega prenašalca, dušikovega oksida, je povečal učinek tega plina na parametre prenosa kisika v suspenziji eritrocitov.

**Zaključek**: Ugotovili smo, da je ozon povzročil spremembo lastnosti vezave kisika v suspenziji eritrocitov, kar predstavlja mehanizem delovanja omenjenega plina na prilagoditvene procese v telesu, ki se dogajajo neposredno na ravni eritrocitov.