

# A physicochemical investigation on the effects of ozone on blood

Valter Travagli<sup>a</sup>, Iacopo Zanardi<sup>a</sup>, Antonella Silvietti<sup>b</sup>, Velio Bocci<sup>c,\*</sup>

<sup>a</sup> Department of Pharmaceutical Chemistry and Technology, University of Siena, Siena, Italy

<sup>b</sup> Central Laboratory of Clinical Analysis, Polyclinic, University of Siena, Siena, Italy

<sup>c</sup> Department of Physiology, University of Siena, Siena, Italy

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## Abstract

Ozonation of either human whole blood or saline-washed erythrocytes causes considerable damage to the latter and this result has opened a controversy. With the benefit of hindsight, it appears logical that once erythrocytes are deprived of the potent antioxidants of plasma, they become very sensitive to the oxidant effects of ozone. The aim of the present work was to perform a physical–chemical evaluation of some critical parameters able to clarify this issue. We have ascertained that when whole blood is exposed to the appropriate ozone doses used in human therapy, no damage ensues while saline-washed erythrocytes undergo conspicuous haemolysis. The dogma that ozone is always toxic is incorrect because its reactivity below the concentration of 80 µg/mL can be controlled by the plasmatic antioxidant system.

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## 1. Introduction

The first use of ozone in medicine begun during the first world war for treating Clostridial infections in German soldiers with gaseous gangrene. However, only in 1974, Wolff [1] published the classical method of exposing *ex vivo* human blood to oxygen–ozone for a few minutes in an ozone-resistant glass bottle and successive, prompt reinfusion in the donor patient. Since then, million of autotransfusions have been performed mostly in Europe [2–4], but there is not a general consensus regarding the biological effects and possible damage induced by ozone in blood. The oxygenation has never elicited problems because it does only lead to an increase of the pO<sub>2</sub> and complete saturation of haemoglobin to oxyhaemoglobin. Moreover the oxygenation effect is clinically irrelevant because blood is transfused slowly and mixes with an excess of venous blood. The main controversy is due to ozone that is an extremely reactive and unstable molecule: particularly in the past several authors [5–14] have exposed saline-washed erythrocytes to the gas mixture necessarily composed of oxygen–ozone. When erythrocytes are deprived of the plasma, washed three times with saline

and resuspended in a saline solution, they become extremely sensitive to the oxidizing activity of ozone with consequent modifications and breakdown of the cell membrane, intense formation of methaemoglobin and enzymatic inactivation. It is unfortunate that these data have been produced in artificial conditions because the plasma contains a wealth of antioxidants [15], which are able to tame the strong reactivity of therapeutic ozone doses. In unphysiological conditions, ozone, 10-fold more soluble than oxygen, readily dissolves in the water and attacks substrates such as the polyunsaturated fatty acids composing the phospholipids of the plasma membrane and easily oxidizes enzymes and intracellular reduced glutathione (GSH). Indeed, other studies, including our own [16–24] have clearly shown that when erythrocytes, protected by plasmatic antioxidants (uric acid, ascorbic acid and albumin), are exposed to experimentally defined therapeutic ozone doses (10–80 µg/mL of gas per mL of blood or 0.21–1.68 µmol/mL), neither peroxidation of membrane phospholipids nor generation of methaemoglobin are present. In practical terms, it appears that only one (likely an old erythrocyte) out of 200 erythrocytes undergoes breakdown, partly due to manipulation rather than peroxidation.

Unsurprisingly, Morgan et al. [25], by ozonating saline-washed erythrocytes showed a loss of deformability and decreased filterability. On the other hand, enthusiastic proponents of ozone therapy [26–28] have claimed that a slight

\* Corresponding author. Tel.: +39 0577 234226; fax: +39 0577 234219.  
E-mail address: bocci@unisi.it (V. Bocci).

peroxidation of the erythrocytic membrane induces favourable consequences on cell functions such as an increased fluidity of the membrane with enhanced cell deformability and filterability. Moreover ozonation will increase the negative charge of the erythrocytic membrane that, in conjunction with reduced plasma viscosity, due to concomitant reduced fibrinogen levels, would explain the improvement of rheologic parameters of ozonated blood in patients with peripheral occlusive arterial disease. These results, though favourable, are not consistent with reliable experimental data showing that ozonation of blood, particularly using low and medium ozone concentrations (10–40  $\mu\text{g/mL}$  of gas per mL of blood) do not involve any membrane peroxidation [16,19,21,23,24]. Moreover these data are in line with results showing that, erythrocytes indeed well protected by a multiform antioxidant defence system [29–33].

In order to clarify these controversial findings, we have performed a series of experiments using an ample range of ozone doses on whole human blood as well as saline-washed erythrocytes by evaluating critical plasma components, possible variations of plasma viscosity and haemolysis, electrophoretic mobilities of either ozonated or desialylated erythrocytes, spectroscopic determination and any possible variations of activity of crucial antioxidant enzymes in erythrocytes.

## 2. Materials and methods

### 2.1. Ozone generation and measurement

Ozone was generated from medical-grade oxygen ( $\text{O}_2$ ) using electrical corona arc discharge, by the  $\text{O}_3$  generator (Model Ozonosan PM 100K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and  $\text{O}_3$  concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International  $\text{O}_3$  Association. The photometer was periodically checked by using the iodometric titration according to Masschelein [34] in observance of the rules established by international ozone association (IOA). Tygon polymer tubing and single-use silicon treated polypropylene syringes (ozone-resistant) were used throughout the reaction procedure to ensure containment of  $\text{O}_3$  and consistency in concentrations.

### 2.2. Collection of human blood

#### 2.2.1. Experiment A (whole blood)

Blood samples of 60 mL were taken from three healthy, non-smoker, male blood donors in the morning, actually the authors (V.T., I.Z. and V.B.). Heparin (20 U/mL blood) or sodium citrate (1 mL/9 mL blood) were used as anticoagulants and blood samples were immediately subdivided and introduced (8 mL) in ozone-resistant syringes.

#### 2.2.2. Experiment B (saline-washed erythrocytes)

Blood samples of 40 mL were taken from the same donors as in (A). Heparin (20 U/mL blood) was used as an antico-

agulant and blood was immediately centrifuged at 2000 rpm for 5 min at room temperature to sediment erythrocytes, which were collected by discarding the supernatant. The erythrocytes were then washed with physiological saline solution (1:10) three times to remove trace of plasma. The erythrocytes were resuspended to 40 mL in phosphate buffered physiological saline (solution pH 7.4) samples were immediately subdivided and 5 mL were introduced in ozone-resistant syringes.

### 2.3. Gas delivery to biological samples

Gas delivery was carried out with a single dose of ozone (concentration per volume) as follows: a predetermined volume of a gas mixture composed of  $\text{O}_2$  (95–99%) and  $\text{O}_3$  (1–5%), at ozone concentrations 20, 40, 80 and 160  $\mu\text{g/mL}$  of blood (or 0.42–3.36  $\mu\text{mol/mL}$ ) was collected with a second syringe and immediately introduced into those containing the blood samples via a multidirectional stopcock. We always used a blood sample/gas volume at a 1:1 ratio. We have previously determined [19] that maintaining the syringes in a monodirectional oscillator (60 cycles/min) for 10 min allows a complete mixing of the liquid–gas phases with minimal foaming and that, within this period of time, ozone reacts completely with substrates, implying that all samples react with the ozone dose totally.

In order to obtain reproducible results, it needs to be emphasised that  $\text{O}_3$  is a very reactive gas so that extremely rapid and precise handling is required. The final gas pressure remained at normal atmospheric pressure.

Blood samples (whole and saline-washed) were treated with either oxygen ( $\text{O}_2$ ) and argon (Ar) as controls.

### 2.4. Biochemical determinations

- (a) Blood gases were determined with an IL-1620 blood gas analyser (Instrument Laboratory, Lexington, MA, USA) and an ABL 505 radiometer.
- (b) Haemocytometric determinations were made with a standard blood analyser.
- (c) Erythro sedimentation rate was evaluated for all whole blood samples treated with gas at 1 and 2 h.
- (d) Total antioxidant status was determined in plasma samples according to Rice-Evans and Miller [35] and expressed in mM of plasma.
- (e) Determination of thiobarbituric acid-reactive substances (TBARS): in order to evaluate the relevance of lipid peroxidation, TBARS were assessed in plasma as described by Buege and Aust's method [36].
- (f) Haemolysis concentrations were evaluated according to a standard curve using a lyophilised haemoglobin standard by the optical density at various wavelengths as reported in spectroscopic determination.
- (g) Erythrocytic enzymes glutathione peroxidase (GSH-Px), glutathione reductase (GSH-Rd) and superoxide dismutase (SOD) were measured on cell lysate with spectrophotometric Randox test kits (Randox Laboratories Limited, U.K.) and reported as U/ $10^6$  cells. Briefly, GSH-Px was mea-

sured according to Paglia and Valentine's method [37]; GSH-Rd according to Melissinos method [38]; SOD activity was determined by the inhibition of p-iodonitrotetrazolium (INT) reduction due to  $O_2^-$  generated by the combination of xantine and xantine oxidase [39]. Intracellular glucose-6-phosphate dehydrogenase (G6PDH) was detected on cell lysate, with a Sigma spectrophotometric kit (Sigma Diagnostic), according to a modification of the spectrophotometric method of Konberg and Horecker [40] and Lohr and Waller [41] and expressed as U/10<sup>12</sup> cells.

Enzymatic levels were determined only on blood samples treated with ozone at well-known therapeutic concentration.

- (h) Cholesterol, triglycerides, high density lipoprotein (HDL), low density lipoprotein (LDL) were determined by using a standard enzymatic colorimetric method (Roche).
- (i) Fibrinogen concentration was determined by the automatic Behring's method according to Rossi et al. [42].

## 2.5. Spectroscopic determination

The absorbance was determined with a Perkin-Elmer Lambda 2B UV–vis spectrophotometer equipped with 10-mm quartz cells. The absorption spectra were obtained over the range 700–400 nm. A data interval of 1 nm was selected, with a signal averaging time of 0.5 s for each data point (resulting in a scan time of 150 s). All data were acquired with UV WINLAB software. Precisely, the adopted wavelengths for the determination of haemoglobin concentration were 414, 541 and 576 nm, while methaemoglobin was evaluated at 630 nm [24]. Before any determinations, samples were centrifuged at 3000 × *g* for 20 min in order to read the clear supernatants and, when necessary, the samples were appropriately diluted. All scans were performed by the same operator and carried out at 21 ± 0.5 °C.

As usual, all whole blood samples and saline-washed erythrocytes resuspended in PBS at the original haematocrit values after the gas treatment were evaluated [43].

## 2.6. Viscosity evaluation

Viscomate VM10AL (CBC Europe Ltd.) is an innovative torsional oscillating viscometer characterized by constant shear stress systems driven by a piezoelectric ceramic source. This instrument measure viscosity by sensing a change in oscillation amplitude of a liquid-immersed detector, based on constant input voltage. An original phase locked loop circuit maintains instrument resonant frequency of 1 kHz; the detector oscillation amplitude with no resistance is 1 µm. Angular acceleration of the detector is measured and reported as dynamic viscosity with the range 0.400–1000 mPa s. The probe dimension was 9 mm with respect to the diameter [44].

All the determinations were conducted into polystyrene Technicon® sample cups (Kartell, nominal capacity 2 mL). Temperature control was accurately monitored during the experiments (37.0 ± 0.1 °C). Viscosity values were recorded for six minutes (data collection every 5 s) by PC connection through a RS-232 port.

## 2.7. Capillary electrophoresis of erythrocytes

In order to estimate if the treatment with ozone influenced the superficial charge of erythrocytes we have carried out a capillary electrophoresis (CE) analysis [45,46]. Briefly, the samples firstly treated with gases (argon and oxygen as controls and various ozone concentrations) were centrifuged at 2000 rpm for 5 min at room temperature and then erythrocytes were washed with physiological saline solution four times. Next, the erythrocytes were resuspended (1:100) in electrode buffer as the sample for CE analysis. The electrode buffer selected was 50 mM phosphate buffer solution pH 7.4 with 6% sucrose. CE analysis was carried out by a P/ACE MDQ instrument (Beckman Coulter) with the anode at injection end and the cathode at the detector end. Separations (300 V/cm) were performed using 75 µm × 60.2 cm (50 cm to the detector) fused-silica capillaries (Beckman Coulter). The separations were monitored at 210 nm and 414 nm, in order to evaluate haemoglobin presence, with a photo diode array (PDA) detector and the data were analysed by 32 Karat™ Software (Beckman Coulter) with a PC. Throughout the experiments the capillary was maintained at 23 °C by a liquid cooling cartridge system, while the samples were conserved at 18 °C. Before each injection the capillary was washed for 2 min at 20 psi with a 0.1 M sodium hydroxide solution and with water and then rinsed with the electrode buffer for 5 min at 20 psi. As positive control, we have carried out a test with desialylated erythrocytes (from 500 µL of blood), previously washed and resuspended into 0.1 M phosphate buffer solution pH 7.4 with 6% glucose (w/v). 1.25 mL of neuraminidase (1 U/mL) was added to the sample incubated for 2 h at 37 °C. After this time, the erythrocytes were collected by centrifuge (2000 rpm for 5 min at room temperature) and therefore prepared for CE analysis. Moreover, another test, in order to estimate the effectiveness of the analysis, has been carried out making the CE of bovine and rat bloods.

## 2.8. Statistical analysis

Results were expressed as the mean ± S.D. of at least three independent measurements. ANOVA one-way performing the Bonferroni post-test (Instat software, version 3.0 GraphPAD Software Inc., San Diego, CA) were used for the statistical analysis of the results. Significance was defined as a *p* value less than 0.05 (\**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.001).

## 3. Results

The three human blood samples used in our experiments were characterized as follows: haemoglobin: 14.8, 15.1, 15.6 g/dL; erythrocytes: 4.90, 4.89, 5.02 × 10<sup>6</sup> mm<sup>3</sup>; haematocrit: 47.0, 47.8, 48.8; leukocytes: 5.8, 6.7, 6.4 × 10<sup>3</sup> mm<sup>3</sup>; platelets: 240, 228, 247 × 10<sup>3</sup> mm<sup>3</sup>; plasma proteins: 7.2, 6.9, 7.1 g/dL with albumin/globulin ratio of 1.7, 1.6, 1.7. Blood groups were: ORh+, ORh–, ARh–.

After oxygenation–ozonation of whole blood, the pO<sub>2</sub> increased from an average of 38 to an average of 420 mmHg with negligible modifications of the pCO<sub>2</sub> and pH. The TAS of

Table 1

TAS and TBARS values measured on whole blood samples immediately after exposure to argon, oxygen and progressively increasing ozone concentrations

	Control		Ozone ( $\mu\text{g/mL}$ )			
	Ar	O <sub>2</sub>	20	40	80	160
TAS value (mmol/L)	1.72 $\pm$ 0.061	1.70 $\pm$ 0.045	1.64 $\pm$ 0.056	1.49 $\pm$ 0.010	1.26 $\pm$ 0.041	1.01 $\pm$ 0.021
TBARS values ( $\mu\text{mol/L}$ )	0.28 $\pm$ 0.025	0.28 $\pm$ 0.015	1.00 $\pm$ 0.031	1.80 $\pm$ 0.047	2.93 $\pm$ 0.067	3.80 $\pm$ 0.100

the plasma was equivalent to 1.70 mmol/L, that corresponds to a normal value. Immediately after ozonation of whole blood, TAS values decrease to progressively lower values in relation to the ozone dose (Table 1). All values, except after ozonation with 20  $\mu\text{g/mL}$ , are statistically significant ( $p < 0.001$ ). The effect of ozonation of whole blood could be checked by determining the concentration of thiobarbituric reactive substances (TBARS) in the ozonated plasma: values increased from 0.28  $\mu\text{mol/L}$  in the controls up to 3.80  $\mu\text{mol/L}$  in the blood samples exposed to an ozone concentration of 160  $\mu\text{g/mL}$  per mL of blood. All values are statistically significant ( $p < 0.001$ ).

Fig. 1 shows that the levels of haemolysis assessed in human whole blood or saline-washed erythrocytes vary in relation to the ozone concentration from 20  $\mu\text{g/mL}$  per mL of blood or 0.41  $\mu\text{mol/mL}$  up to 160  $\mu\text{g/mL}$  or 3.36  $\mu\text{mol/mL}$ . Haemolysis was 0.19% in control values and increased up to 0.61% in whole blood samples exposed to 160  $\mu\text{g/mL}$  ozone. In comparison to heparin, the presence of citrate slightly reduced haemolysis, likely due to the physiological  $\text{Ca}^{2+}$  chelation. Haemolysis was far higher when erythrocytes had been washed with saline: from 5.61% in the controls, it increased from 6.56 to 15.88% showing to depend upon ozone concentration. Even values of haemolysis up to 30% have been determined in the past indicating the strong sensitivity of washed erythrocytes to ozone concentration of 240  $\mu\text{g/mL}$  (unpublished data). The significance of the data are indicated in Fig. 1 by asterisks. Similar results are evidenced also with respect to argon.

Evaluation of viscosity did not show significant variations between controls and ozone-treated samples (Fig. 2). It varied markedly in relation to the anticoagulant and expectedly decreased of about 40% in saline-washed erythrocytes resuspended in saline at their original haematocrit. No effect of ozonation became apparent.

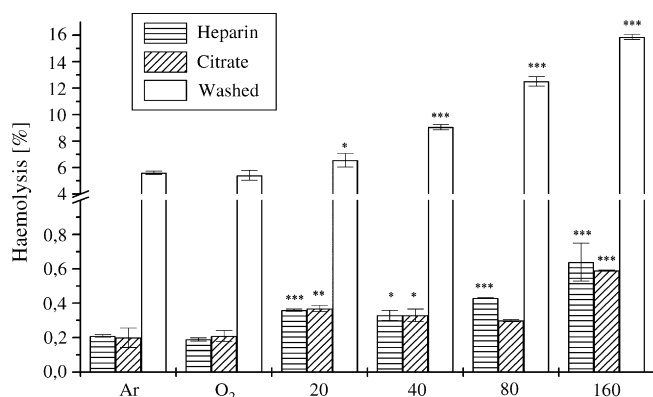


Fig. 1. Levels of haemolysis (%) in human blood samples after exposure to argon, oxygen and progressively increasing ozone concentrations.

The erythro sedimentation rate did not show any modification after 1 and 2 h in either controls or ozonated samples (data not shown).

Owing to previous data [27,28] claiming a decrease of fibrinogen and cholesterol after a mild blood ozonation, we determined the concentration of the fibrinogen, cholesterol, triglycerides, HDL and LDL in both controls and widely ozonated human blood samples either anticoagulated with heparin or citrate. In Table 2, no significant modifications among the various samples were detected, suggesting that ozone does not modify the concentration of these compounds even at the highest ozone concentration.

Similarly, in Table 3 no significant decrease of typical erythrocytic enzymes was detected after three levels of ozonation of heparin treated blood samples. Only the superoxide-dismutase (SOD) showed a 19% decrease that nonetheless was not significant probably because we tested twice the blood of the usual three donors.

In Fig. 3, we would like to show the electrophoretic pattern of human erythrocytes exposed to only control gases or to four ozone concentrations. The experiments reported in Panel A were repeated three times for the blood of the three donors and in all cases both controls and the differently ozonated erythrocytes came out with the same electrophoretic mobility. A sample of normal erythrocytes, after undergoing a desialylation of about 85% with neuraminidase showed a marked decrease of mobility. Similarly, bovine and rat erythrocytes showed a different pattern (Panel B) because their content of sialic acid is different from the human ones. Furthermore, Tables 4 and 5 summarize the retention time of all the samples tested including those relative to saline-washed erythrocytes after undergoing ozonation. We were somewhat surprised to observe no modification of the

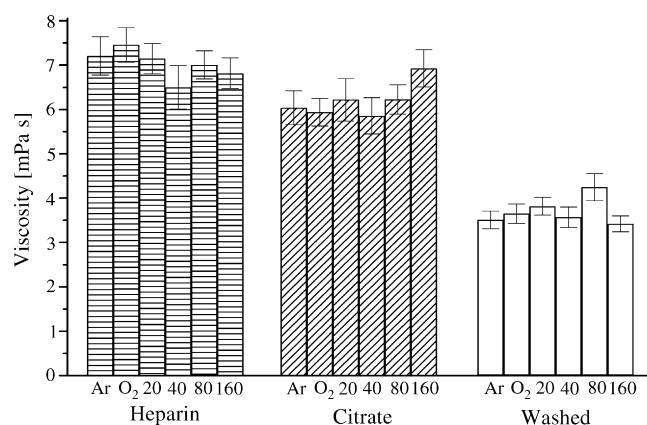
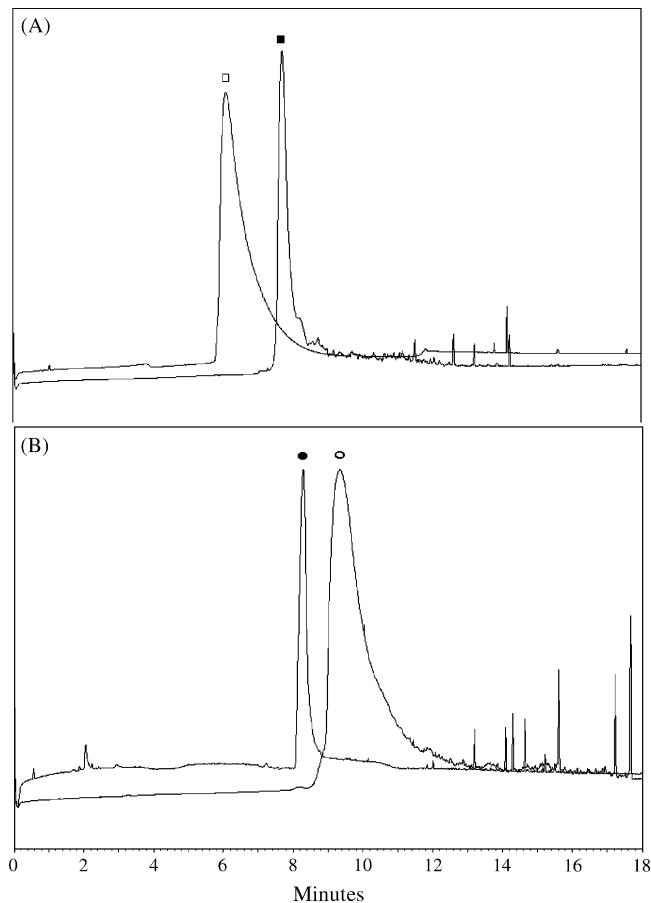


Fig. 2. Blood viscosity variation in human blood samples after exposure to argon, oxygen and progressively increasing ozone concentrations.

**Table 2**  
Determination of plasma levels (mean  $\pm$  S.D.) of the following parameters on whole blood samples immediately after exposure to argon, oxygen and progressively increasing ozone concentrations

Gas treatment	Fibrinogen (mg/dL)		Cholesterol (mg/dL)		Triglycerides (mg/dL)		HDL (mg/dL)		LDL (mg/dL)	
	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate	Heparin	Citrate
Ar	272 $\pm$ 29.5	260 $\pm$ 11.2	156 $\pm$ 8.5	150 $\pm$ 10.7	61 $\pm$ 19.3	44 $\pm$ 6.8	63 $\pm$ 5.7	61 $\pm$ 8.5	75 $\pm$ 10.1	72 $\pm$ 3.1
O <sub>2</sub>	260 $\pm$ 28.1	263 $\pm$ 3.5	156 $\pm$ 9.4	150 $\pm$ 11.4	62 $\pm$ 18.8	43 $\pm$ 4.4	64 $\pm$ 5.7	62 $\pm$ 7.7	76 $\pm$ 9.4	70 $\pm$ 6.3
20	255 $\pm$ 31.8	254 $\pm$ 7.0	156 $\pm$ 7.1	156 $\pm$ 7.5	62 $\pm$ 19.1	45 $\pm$ 4.4	64 $\pm$ 4.9	63 $\pm$ 8.2	76 $\pm$ 7.4	73 $\pm$ 11.2
40	250 $\pm$ 24.3	264 $\pm$ 10.4	157 $\pm$ 8.8	158 $\pm$ 9.2	64 $\pm$ 19.3	48 $\pm$ 5.1	62 $\pm$ 6.5	63 $\pm$ 9.3	76 $\pm$ 6.6	76 $\pm$ 6.6
80	248 $\pm$ 27.5	268 $\pm$ 5.7	158 $\pm$ 11.0	157 $\pm$ 2.5	65 $\pm$ 19.0	48 $\pm$ 6.4	61 $\pm$ 5.0	65 $\pm$ 7.9	77 $\pm$ 8.8	78 $\pm$ 6.8
160	262 $\pm$ 7.0	262 $\pm$ 10.1	156 $\pm$ 8.1	159 $\pm$ 5.7	61 $\pm$ 19.6	49 $\pm$ 6.6	63 $\pm$ 4.5	64 $\pm$ 7.6	75 $\pm$ 8.0	79 $\pm$ 6.4



**Fig. 3.** Capillary electrophoresis. Panel A: (■) human blood and (□) human blood treated with neuraminidase. Panel B: (●) bovine blood and (○) rat blood.

**Table 3**  
Enzymatic levels (U/g Hb) in human blood after exposing blood samples to either O<sub>2</sub> or O<sub>3</sub>

Treatment	Enzymatic levels (U/g Hb)			
	SOD	GSH-Px	GSH-Rd	G6PDH
Ar	830.2 $\pm$ 249.6	24.4 $\pm$ 5.8	2.1 $\pm$ 0.5	5.2 $\pm$ 2.4
O <sub>2</sub>	836.9 $\pm$ 293.6	19.1 $\pm$ 5.8	2.0 $\pm$ 0.5	5.3 $\pm$ 2.4
20	726.8 $\pm$ 197.5	25.2 $\pm$ 6.4	2.0 $\pm$ 0.6	5.7 $\pm$ 2.6
40	694.7 $\pm$ 140.9	25.8 $\pm$ 6.1	2.1 $\pm$ 0.5	5.2 $\pm$ 2.7
80	670.8 $\pm$ 185.5	27.2 $\pm$ 6.1	2.1 $\pm$ 0.4	5.6 $\pm$ 3.0

Values represent mean  $\pm$  S.D. ( $n=6$ ).

**Table 4**  
Retention time (min) of samples after exposure to argon, oxygen and progressively increasing ozone concentrations (mean  $\pm$  S.D.)

Sample	Heparin	Citrate	Washed
Ar	7.65 $\pm$ 0.01	7.70 $\pm$ 0.02	7.72 $\pm$ 0.02
O <sub>2</sub>	7.67 $\pm$ 0.05	7.71 $\pm$ 0.02	7.75 $\pm$ 0.03
20	7.65 $\pm$ 0.05	7.70 $\pm$ 0.05	7.73 $\pm$ 0.01
40	7.60 $\pm$ 0.04	7.75 $\pm$ 0.03	7.75 $\pm$ 0.03
80	7.63 $\pm$ 0.03	7.72 $\pm$ 0.01	7.76 $\pm$ 0.04
160	7.67 $\pm$ 0.05	7.69 $\pm$ 0.04	7.73 $\pm$ 0.06

**Table 5**  
Retention time of different blood samples (mean  $\pm$  S.D.)

Sample	Retention time (min)
Human blood	7.67 $\pm$ 0.05
Human blood treated with neuraminidase	6.11 $\pm$ 0.03
Bovine blood	8.30 $\pm$ 0.02
Rat blood	9.34 $\pm$ 0.03

retention time even after the highest ozone concentration. Thus it appears that ozonation of human blood samples has minimally modified the electric charge of the surviving erythrocytes indicating that the ghosts are lost during the preparation of the samples.

#### 4. Discussion

The present work was undertaken for definitively clarifying whether a brief exposure of human blood to a gas mixture composed of oxygen (95–99%) and ozone (1–5%) using concentration of ozone within the therapeutic range of 20–80  $\mu$ g/mL of gas per mL of blood (0.42–1.68  $\mu$ mol/mL) as previously determined by several authors [1,3,19,21,26,47] causes the peroxidation of the erythrocyte membrane or/and induces modification of their negative charge. This aspect has been controversial because two different approaches have been used: the first has examined the effect of oxygen–ozone on repeatedly saline-washed human erythrocytes, while the second has been performed in human whole blood. The unsolved question is whether ozone is, in any case, able to attack the erythrocyte membrane and cause peroxidation of the PUFA composing the phospholipid bilayer. If the latter occurs, some of the unsaturated fatty acids are broken down with possible fragmentation, exit of haemoglobin and possible modification



of the negative charge of the membrane. Several years ago, it was determined that the erythrocyte membrane during physiological ageing, undergo remodelling, a reduced surface to volume ratio but no modification of the electrophoretic charge [48].

The dynamic of the events occurring during ozonation of whole human blood have been recently clarified [2,3]: the first step is the physical solubilization of the two gases in the water and there is no doubt that they dissolve in relation to their solubility coefficient, relative partial pressure and temperature in the water of either the saline solution or the plasma. At a normal gas pressure of 760 mmHg, only 2.0 mL of oxygen dissolve in 100 mL of water and allows complete oxygenation of haemoglobin. Ozone, one of the strongest oxidants, is 10-fold more soluble than oxygen and, even more important, it reacts immediately with hydrophilic antioxidants present in plasma such as uric acid (4–7 mg/dL), ascorbic acid (15–20 µg/mL), the abundant sulphhydryl groups of albumin (~45 mg/mL) and a variety of PUFA (~5 mg/mL) bound to albumin. It is well known that the plasma contains a wealth of antioxidants [15,31], the content of which can be measured [35] and expressed as the total antioxidant status. When erythrocytes are suspended in saline and their membrane has been totally deprived of the albumin protection, the ozone can only react with NaCl forming some NaOCl, but mostly acts on the double bonds of PUFA, possibly oxidizes cholesterol and the external glycoproteins and, depending on the ozone dose, it may even enter the cell. The sequence of reaction was well determined by Ueno et al. [20], who detected DMPO-spin adduct of hydroxyl radical together with the spin adduct of carbon-centered radical. In the extreme experiment by Cataldo and Gentilini [14], where bovine erythrocytes have been lysed in distilled water, also haemoglobin was heavily oxidized. On the contrary, if plasma is present, provided that the total antioxidant status is within the normal range of 1.28–1.83 mmol/L [35], and the concentration of ozone tested is below 80 µg/mL of gas per mL of blood, all the ozone reactivity is quenched by the plasma antioxidants and the small formation of spin adduct of carbon-centered radical is due to the peroxidation of PUFA albumin-bound. All the ozone was consumed within 30 s in Ueno et al. experiments [20] while we have allowed the reaction to be completed in 10 min because we prefer to use a gentle mixing of the blood–gas phases. As a consequence, the membrane of erythrocytes (as well as other blood cells) that is shielded by albumin molecules remains intact when we use the therapeutic concentrations of ozone. The reaction can be summarized as follows:

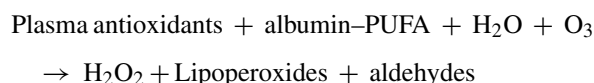


Fig. 4 shows a scheme exemplifying the sequence of events occurring when the ozone continues to dissolve in the water of plasma until it is exhausted, while hydrophilic antioxidants undergo oxidation and the content of liperoxides increases in relation to the ozone dose. However, in saline-washed erythrocytes resuspended in saline, with relatively high levels of ozonation the phospholipid Criegee ozonide

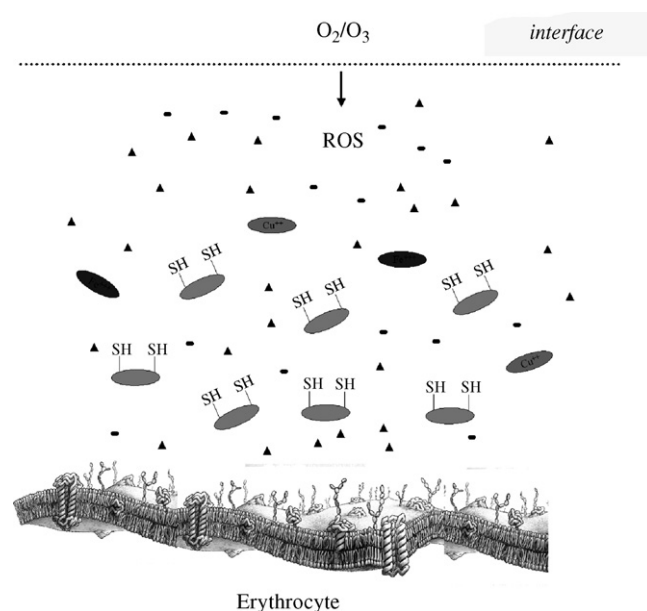


Fig. 4. Scheme exemplifying the sequence of events occurring when the ozone continues to dissolve in the water of plasma.

1-palmitoyl-2-[8-(5-octyl-1,2,4-trioxolan-3-yl)-8-octanoyl]-sn-glycero-3-phosphocholine (PC-Criegee ozonide) could be formed. This compound could relay the toxic effects of ozone into the cellular membrane [49] eventually leading to many other degradation compounds [50] (see Fig. 5). Table 1 shows that peroxidation values (TBARS) increases about 10-fold at the highest therapeutic ozone concentration while the TAS value decreased of about 30% in comparison to the original value. This means that about 70% of the total antioxidant capacity remains available in spite of the acute oxidative stress induced by ozone. It is important also to note that, thanks to the very rapid recycling of dehydroascorbate to ascorbate and GSSG (oxidized glutathione) to GSH in the erythrocyte, the antioxidant status return to normal within 20 min [32,33]. The minimal rise of free haemoglobin (0.19% and 0.64% in heparinized and ozonated blood, respectively), the absence of significant modifications of the plasma levels of fibrinogen, cholesterol, triglycerides, HDL and LDL (Table 2), as well as the normal levels of crucial antioxidant enzymes in the erythrocytes support our view. Similar data by Zimran et al. [22] and Biedunkiewicz et al. [51] are in line with our statement.

These data are consistent with the absence of any significant modification of the blood viscosity, as already seen in Fig. 2. It seems reasonable to postulate that a variation of the fibrinogen levels and of the erythrocytic membrane would modify the viscosity pattern only in a slight manner.

Finally it is important to note that the electrophoretic mobility of erythrocytes of the whole series of samples of whole blood did not minimally change after ozonation suggesting that the carboxyl group of the *N*-acetyl-neuraminic acid exposed in the membrane remained intact during ozonation. Eylar and Edwin [52] beautifully established that in human erythrocytes the sialic acid is responsible for the negative surface charge rather than the lipid phosphate of phosphatidylserine. Only after exten-

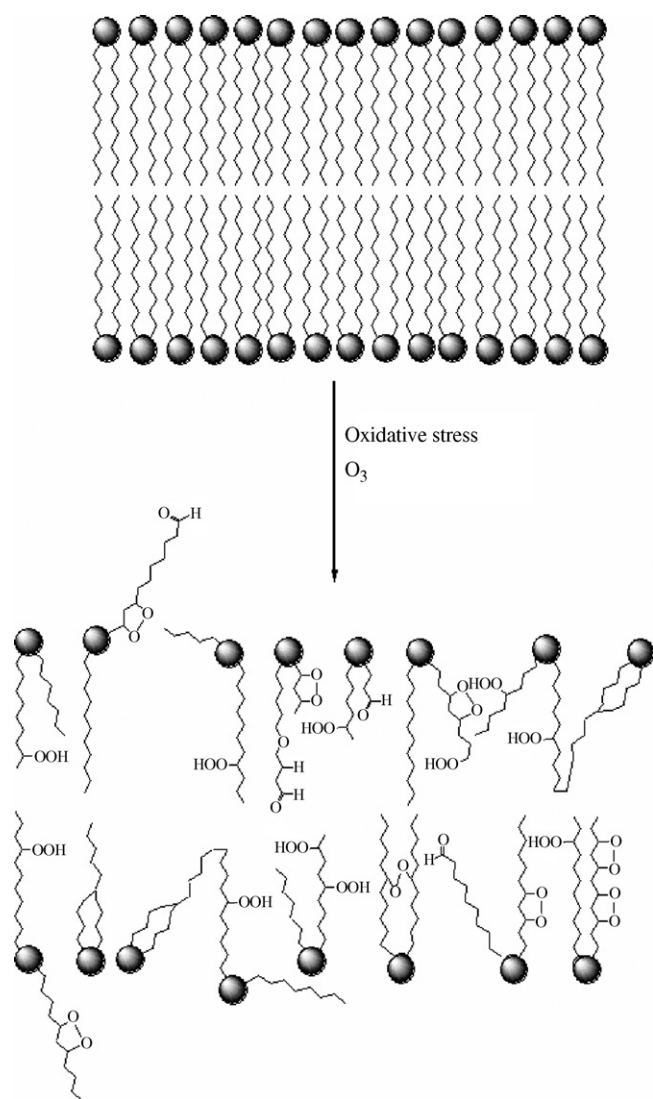


Fig. 5. The scheme shows the damage of erythrocytic membrane when saline-washed red blood cells are resuspended in saline and exposed to oxygen–ozone gas mixture.

sive desialylation, the electrophoretic mobility of our normal erythrocytes was markedly modified.

On the other hand, saline-washed erythrocytes resuspended in saline, even if treated with control gases, showed a significant increase of free haemoglobin (up to 5.61%). Moreover a progressive increase from 6.56 up to 15.88% was measured and determined to be ozone-dose-dependent clearly showing a damage of the erythrocytes. We expected that washed and ozonated erythrocytes will display a very broad range of mobilities because we envisaged a more or less partial oxidation of the terminal sialic acid. This does not appear to happen and most likely the heavily oxidized erythrocytes break down and are excluded during the electrophoretic run. Nonetheless, it is clear that ozonation of saline-washed erythrocytes is absolutely unphysiological and leads to artful results. Therefore, in human therapy, ozonation of blood must be performed only using whole blood and the appropriate ozone concentrations in order to exclude any cell damage or toxicity [4].

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