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Short communication

# A realistic evaluation of the action of ozone on whole human blood

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**Abstract**

We have clarified the role of the ozone concentration in relation to the resistance of human erythrocytes in whole human blood or in blood diluted either in saline or in distilled water.

Spectrophotometric data related to haemoglobin were evaluated by exposing samples of fresh human blood directly to ozone doses (ratio 1:1 volume), within the therapeutic range (0.21–1.68 mM) and to one toxic dose (3.36 mM). Furthermore, the same determinations have been carried out after previous dilution of the same blood with either pure water or physiological saline (1 ml blood + 29 ml diluent) followed by ozonation with the above reported ozone doses. Addition of either saline or water implies a dilution of plasma antioxidants and also total haemolysis after water dilution. Particularly the latter case represents a most unphysiological situation because the osmotic shock causes the solubilization of the erythrocytic content. While it is possible to demonstrate that after haemolysis there is an ozone-concentration dependent transformation of some oxyhaemoglobin to methaemoglobin, no such process occurs after ozonation of whole blood.

The results of this study fully confirm our previous data that judicious ozone doses neither damage erythrocytes, nor induce oxidation of intracellular haemoglobin. We hope that our conclusions will definitively clarify the absence of toxicity of ozonetherapy.


*Keywords:* Blood; Ozonation; Erythrocytes; Haemoglobin; Methaemoglobin; Antioxidants; Ozonetherapy

## 1. Introduction

The aim of this short communication is to clarify if the ozone concentrations used for therapeutic purposes damage erythrocytes in whole human blood and induce hemolysis. The parameters indispensable for performing a correct and atoxic ozonetherapy are as follows: (a) the precise concentration of ozone and the volume of gas containing an average of 96% oxygen and 4% ozone, hence to exactly determine the ozone dose, (b) the crucial need to expose to ozone only whole human blood because its dilution with distilled water (9.1 g of blood in 300 ml water), as performed by Cataldo and Gentilini [1–4] causes, not only total haemolysis, but, most importantly, lack of protection of blood constituents by physiological antioxidants and (c) the time of exposure and careful handling of blood to ozone.

In order to evidentiate the relevance of these parameters, we have purposefully exposed to several ozone doses fresh human blood either as such or at first diluted with distilled water or with physiological saline, which is able to prevent haemolysis and consequently protect the intracellular haemoglobin. The measurements of electronic absorption spectra for detecting the Soret band, oxyhaemoglobin and methaemoglobin show how ozone behaves in physiological conditions.

## 2. Materials and methods

### 2.1. Ozone generation and measurement

Ozone was generated from medical-grade oxygen ($O_2$) using electrical corona arc discharge, by the $O_3$ generator (Model Ozonosan PM 100 K, Hansler GmbH, Iffezheim, Germany), which allows the gas flow rate and $O_3$ concentration to be controlled in real time by photometric determination, as recommended by the Standardisation Committee of the International $O_3$ Association. The ozone flow-rate was kept constant at 3 l/min in all experiments. Tygon polymer tubing and single-use silicon treated polypropylene syringes (ozone-resistant) were used

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throughout the reaction procedure to ensure containment of $O_3$ and consistency in concentrations.

### 2.2. Collection of human blood and gas delivery to biological samples

A blood sample of 30 ml was taken from a healthy, non-smoker, male blood donors in the morning. The donor was fully informed of the purpose of the study. Heparin (20 U/ml blood) was used as an anticoagulant and blood samples were immediately subdivided and introduced (2.5 ml) in ozone-resistant syringes: equal aliquots were exposed directly to either air, or oxygen only or oxygen–ozone with different ozone concentrations (20, 40, 60, 80 and 160 μg/ml of gas per ml of blood). Gas delivery was carried out with a single dose (concentration per volume) of ozone as follows: a predetermined volume of a gas mixture composed of $O_2$ (∼96%) and $O_3$ (∼4%), at various concentrations, 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 [5] that maintaining the syringes in a monodirectional oscillator (60 cycles/min) for 20 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. The concentrations of ozone ranged between 20 and 160 μg/ml per ml of the sample corresponding to 0.42 and 3.36 mM, respectively.

Other blood samples were divided into two equal aliquots of 1 ml each to be diluted with 29 ml of either physiological saline or bidistilled water. In order to obtain experimental data comparable with Cataldo and Gentilini's technique [1,2] samples of whole blood (1 ml) were gently diluted before ozonation with either bidistilled water or saline (29 ml), respectively. The diluted blood samples were then treated with either air or oxygen or ozone as just specified.

In order to obtain reproducible results, it needs to be emphasised that $O_3$ is a very reactive gas so that extremely rapid and precise handling is required. The final gas pressure remained at normal atmospheric pressure.

### 2.3. 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) Total antioxidant status (TAS) was determined in plasma samples according to Rice-Evans and Miller [6] and expressed in mM of plasma.
(d) 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 [7].
(e) Haemoglobin concentrations were evaluated according to a standard curve using a lyophilized haemoglobin standard by the optical density at various wavelengths, as below reported.

### 2.4. Electronic absorption spectra

The absorbance was determined with a Perkin-Elmer Lambda 2B UV–vis spectrophotometer equipped with 1-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 achieved with UV WINLAB software. Precisely, the adopted wavelengths for the determination of free haemoglobin concentration were 414, 541 and 576 nm, while methaemoglobin was evaluated at 630 nm. 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.

## 3. Results and discussion

### 3.1. Blood characterization

The human blood sample used in our experiments was characterized as follows: haemoglobin: 15.6 g/dl; erythrocytes: $4.94 \times 10^6$ mm$^3$; haematocrit: 48.2; leukocytes: $6.7 \times 10^3$ mm$^3$; platelets: $240 \times 10^3$ mm$^3$; plasma proteins: 7.2 g/dl with an albumin/globulin ratio of 1.7.

After oxygenation–ozonation of whole blood, the $pO_2$ increased from 39 to an average of 420 mmHg with negligible modifications of the $pCO_2$ and pH. The TAS of the plasma was equivalent to 1.67 mmol/l that corresponds to a normal value. Immediately after ozonation of whole blood, TAS values decreased to progressively lower values depending upon the ozone dose (Table 1). In previous experiments we have observed that TAS values return to normal within 20 min upon blood incubation at 37 °C owing to a rapid reduction of oxidized compounds [5,8]. TAS values could not be determined in the blood samples diluted with pure water or saline before ozonation.

The effect of ozonation of whole blood was checked by determining the concentration of TBARS in the ozonated plasma: values increased from 0.28 μmol in the control up to 3.6 μmol in the blood sample exposed to an ozone concentration of 160 μg/ml per ml of blood (Table 1).

Table 1
TAS and TBARS values measured on whole blood samples immediately after exposure to air, oxygen and progressively increasing ozone concentrations

| | Control | | Ozone [μg/ml] | | | | |
|---|---|---|---|---|---|---|---|
| | Air | $O_2$ | 20 | 40 | 60 | 80 | 160 |
| TAS values [mmol] | 1.67 | 1.65 | 1.58 | 1.48 | 1.39 | 1.21 | 1.03 |
| TBARS values [μmol] | 0.28 | 0.31 | 0.97 | 1.78 | 2.35 | 2.88 | 3.64 |

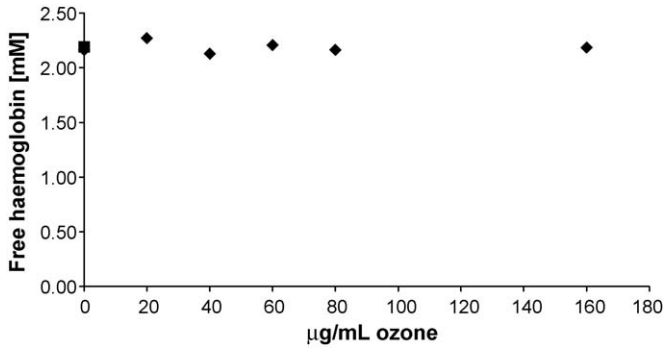


Fig. 1. Ozonation of whole blood. Haemoglobin concentration as evaluated after dilution of the samples with water. Solid square represents the control after exposition to air.

### 3.2. Spectrophotometric results

Our results show that the potent oxidative action of ozone can be fully tamed by the blood antioxidant system provided that human blood is directly exposed to safe ozone doses, which have been experimentally verified to be within 10–80 μg/ml (0.21–1.68 mM) of gas per ml of blood. It is noteworthy that even a concentration beyond the therapeutic window (160 μg/ml or 3.36 mM) has neither yielded a significant haemolysis, nor presence of methaemoglobin at 630 nm (Fig. 1). Dilution of previously ozonated blood in pure water (1 ml ozonated blood in 29 ml water) has not shown the presence of methaemoglobin suggesting that ozonation of whole blood in our conditions does not allow the penetration of ozone into the erythrocytes, hence the oxidation of oxyhaemoglobin to methaemoglobin. In the Cataldo and Gentilini's studies, the initial dilution of bovine blood in pure water caused a massive haemolysis so that ozone, at concentration in most cases beyond the therapeutic window, could easily oxidize the solubilized and unprotected oxyhaemoglobin. Our methodological approach to dilute the ozonated blood also in saline (Fig. 2) has proved to be very demonstrative. In fact, only the blood sample exposed to a potentially toxic ozone concentration (3.36 mM) has shown a modest increase of haemoglobin at 414 nm (Soret band). For all the other determination neither haemolysis, nor presence of methaemoglobin has become appreciable.

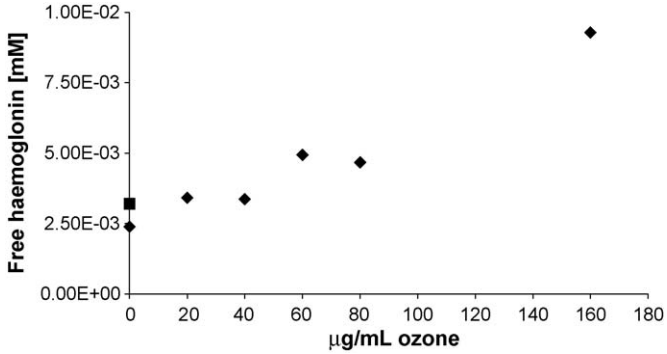


Fig. 2. Ozonation of whole blood. Haemoglobin concentration as evaluated after dilution of the samples with saline. Solid square represents the control after exposition to air.

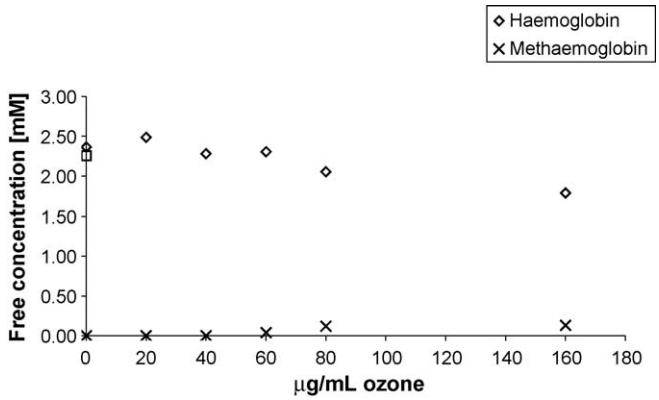


Fig. 3. Ozonation of whole blood after dilution with water. The empty square represents the haemoglobin concentration control after exposition to air. Methaemoglobin formation (×) as evaluable at 630 nm.

The experiments shown in Figs. 3 and 4 were devised for reproducing Cataldo and Gentilini's approach, that is whole blood was diluted first (1 plus 29, v/v) in pure water and also in saline in our study. It is interesting to observe that dilution of blood in pure water has allowed the release of oxyhaemoglobinin solution so that we can observe a small but definite ozone-concentration dependent increase of methaemoglobin that is accompanied by a minimal decrease of haemoglobin as evaluated at 575 nm (Fig. 3). However, when this extreme dilution was performed in saline, the erythrocyte structure was preserved up to the ozonation dose of about 40 μg/ml where only compounds solubilized in the diluted plasma underwent a modest oxidation. Above this concentration, ozone causes the breakdown of a few erythrocytes with the consequent release of a small amount of haemoglobin and the contemporary methaemoglobin transformation. The validity of our interpretation is supported by Fig. 4, where the ordinate scale is 10-fold less than in Fig. 3.

The whole of our data indicate that Cataldo and Gentilini's dramatic conclusion that ozone can be classified as a blood poison is unacceptable because it is based on a unphysiological and harsh methodological approach, which prevents the blood antioxidant system from protecting its components. After reading their papers [1,2], we realized that their experimental methods, albeit correct for showing the strong oxidative action

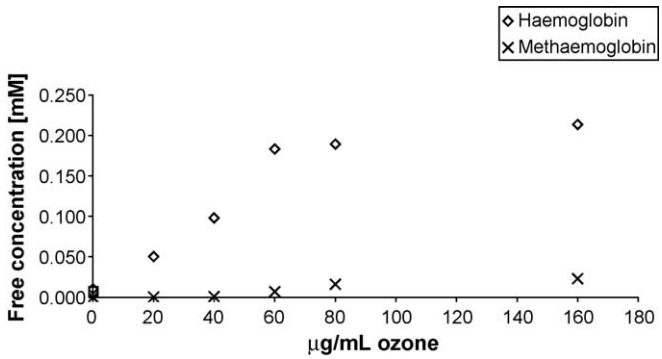


Fig. 4. Ozonation of whole blood after dilution with saline. The empty square represents the haemoglobin concentration control after exposition to air. Methaemoglobin formation (×) as evaluable at 630 nm.

of ozone on extremely water-diluted bovine blood, were so harsh and different from those used in medical ozonetherapy, that could not allow any reasonable conclusion on ozone toxicity. Our letter to the Editor [9] was meant to clarify the issue but Cataldo answered [10] that their "study was not to demonstrate that blood ozone therapy is dangerous" but wanted to show "that ozone reacts selectively with the prosthetic groups of haemoglobin exactly as it happens with CO and HCN". In his counterpoint [10], he finally insinuated that ozonetherapy could be assimilated to a sort of homeopathic treatment, like the one based on the administration of trace of arsenic. His suggestion is however untenable because the mechanisms of action of ozone as a therapeutic agent are based on well-defined biochemical reactions [11,12] and it is regrettable that relevant chemical reactions could be used for improperly judging ozonetherapy without knowing its methodology carefully crafted on the principle "*primum non nocere*". The last 2.5 billion years of earth's life in a oxygen atmosphere have permitted the evolution of a potent, almost redundant antioxidant system represented by hydrophilic and lipophilic compounds, by a number of cooperative antioxidant enzymes and proteins able to chelate transition metals. Besides annihilating the antioxidant system with water dilution, Cataldo and Gentilini [1,2] have also used ozone doses that are four-fold higher than ours because, if ozone concentrations are similar, the added gas volume to the diluted blood was in a different ratio (4:1 and not 1:1 as in our experiments), thus markedly increasing the cumulative dose.

Ozone is not only 10-fold more soluble in water than oxygen but, once dissolved in the undiluted plasma, it reacts instantaneously with biomolecules (uric and ascorbic acids, albumin-thiol groups, polyunsaturated fatty acids, etc). As a consequence, in contrast with oxygen, ozone does not follow Henry's law and the mixing of the gas with the liquid phase is almost immediately followed by the consumption of ozone with the simultaneous generation of oxidized antioxidants, hydrogen peroxide, as a typical reactive oxygen species and a heterogenous mixture of low molecular weight lipid oxidation products. All of these compounds have biological activities and triggers crucial biochemical reactions in erythrocytes, leukocytes, platelets and endothelial cells. The reaction can be precisely controlled by knowing the total antioxidant capacity [6] of the plasma (ranging between 1.28 and 1.83 mM) and the ozone dose (gas volume × ozone concentration as μg/ml). Therefore, in order to avoid any adverse effects, it is imperative to correctly calibrate the ozone dose to the blood's antioxidant capacity: experimental data performed in the last decade [5,11,12] have indicated that the safe range of ozone concentration is within 10–80 μg/ml of gas per ml of blood. An ozone concentration below 10 μg/ml is hardly effective because the ozone dose is instantly quenched by hydrosoluble antioxidants, whereas a concentration above 80 μg/ml (depending upon the individual TAS value) may partly overwhelm the antioxidant capacity, causes some erythrocytic damage and generates methaemoglobin as shown by Cataldo's [4] and our data. Our results have been fully confirmed by Shinriki et al. data [13], demonstrating that, within the therapeutic range, there is neither oxidation of the erythrocytic membrane, nor formation of methaemoglobin.

Finally, we could not agree more with Timbrell, who, in his recent book [14] "The poison paradox" has clearly pointed out that chemicals can behave as friends or foes depending upon the dose and the biological system. This concept fits well with current biochemical data, which have shown that other gases such as NO and CO in physiological amount behave as crucial messengers and maintain a normal vascular tone, whereas they become noxious during inflammation. The same behaviour has been attributed to ozone by Wentworth et al. [15] and Bocci [16], who have compared ozone to the Roman God Janus.

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