The Effect of Ozone on Plasmodium Falciparum-Infected Red Blood Cells

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Abstract

In the case of plasmodium-infected red blood cells we have been able to demonstrate for the first time that ozone has an effect on intracellular parasites without direct ozone/parasite contact, and without visible haemolysis. According to the initial parasitaemia, we found an inhibitive effect of ozone by factors between 3 and 7. The active mechanism involved can be explained via the reaction of ozone with the unsaturated fatty acids of RBC membrane and a consequent penetration of hydroxyhydroperoxides into the cells. The parasites are subjected to an increased oxidative stress, and their reproductive cycle is disrupted. Ozonation was carried out at a concentration of 80 µg/ml in a RBC suspension. Optimal growth inhibition was obtained by applying ozone twice, i.e. immediately before and after infection.

Introduction and Discussion of Problems

The highly efficient microbicide effect of ozone has been in worldwide use for over 100 years in waste water treatment and drinking water disinfection, and the literature on this subject is correspondingly extensive. The mechanisms of its bactericidal and virostatic or virus-inactivating effect \textit{in vitro} are well known, whereby the principal interest is directed at human pathogenic microorganisms (Masschelein 1996 and Brockmann and Botzenhardt 1999).

All studies named, however, relate to surface waters and infected aqueous solutions which have for the most part had ozone/oxygen gas mixtures passed through them for a specific time until obtaining the best result. In this way, it has been possible to determine relevant concentration-time concepts in the case of most microorganisms. In cases where these organisms are protected from ozone attack by external protein membranes or impurities, the combined application of ultrasound and ozone in addition to H$_2$O$_2$ with additional UV irradiation is effective.

We are faced with completely different problems in the case of intracellular microorganisms, which - protected by the outside cell membrane - are able to escape the direct attack of ozone, Plasmodium-infected red blood cells here being a typical example.

Where the aim is to affect the reproductive cycle of intracellular microorganisms by using ozone without destroying the cellular structure of the host cell, careful ozone dose-finding and a standardized cell model is necessary. As the highly reactive O$_3$ molecule is converted completely into peroxidic products in the red blood cell membrane, i.e. it is not able to penetrate into the intracellular space, direct contact between the ozone and the target microorganism is impossible.
The active mechanism required must therefore consist of how to influence the erythrocytic metabolism in such a way that the plasmodic metabolism is affected inside the host cell by a biochemical action also characteristic and known for ozone. A first report on the effect of ozone on Plasmodium falciparum in a standardized erythrocyte model, and thus on the ability to influence intracellular parasites through medical ozone was given (Lell, Viebahn, Kremsner 2001).

**The Reproductive Cycle of Plasmodium Falciparum**

As the cause of falciparum malaria (malaria tropica), Plasmodium falciparum is transmitted to humans via Anopheles mosquitoes. The pathogen involved - assigned to the Haemosporidia in the phylum Protozoa - is known to pass through 3 phases in its development, i.e.:

- A sexual phase forming sporozoites in the female Anopheles mosquito,
- Multiplication of the sporozoites in human liver cells, the preerythrocytic phase, and
- A so-called erythrocytic phase, whereby the merozoites originating from the liver cells develop into schizonts.

In the preerythrocytic and erythrocytic phases, we are primarily dealing with an asexual form of reproduction which, inside the red blood cells, can also pass into a sexual phase in the advanced stage of development; the half-moon shaped gametes are then formed.

Figure 1 shows the erythrocytic development phase in schematic form: In their 1st stage, the schizonts are recognizable from their circular “seal ring” shape, of which several may be present inside a single red blood cell. Mature schizonts with their pigimental accumulation at the center constitute the preliminary stage for a large number of merozoites occupying the entire red blood cell, which finally results in the lysis of the cell and subsequent reinfection. Simultaneously, sexual forms, i.e. the half-moon shaped gametes, are produced during this stage, filling the entire erythrocyte, which they finally destroy.

Fig. 2 shows infected erythrocytes in a blood smear, Fig. 2a showing as an example the asexual development of Plasmodium falciparum, and Fig. 2b showing its female gametes (according to Lieske et al., 1991).

**The Influence of Plasmodia on RBC Metabolism**

Infected red blood cells are first and foremost considerably restricted in their property as an oxygen transport system. In animal experiments, Schmidt and coworkers (1994) were able to determine, in Plasmodium berghei-infected red blood cells, that the oxygen transport capacity was reduced by 30 % versus non-infected cells. The increase in haemoglobin /oxygen affinity here probably underlies this effect as the sequel of a reduced 2,3-DPG (2,3-diphosphoglycerate) content. At the same time, the oxygen bonding curve of the infected erythrocytes is shifted to the left of the equation, as shown in Fig. 3 (Schmidt et al. 1994).
Fig 1: The erythrocytic development phase of Plasmodium falciparum shown schematically (Lieske et al., 1991)

Fig. 2a: Ring-shaped plasmodia and mature schizonts

Fig. 2b: Female Plasmodium falciparum gametes

Fig. 2: Infected erythrocytes in a blood smear (according to Lieske et al., 1991).
2,3-DPG, an intermediate product of RBC glycolysis, exerts an influence on haemoglobin-oxygen affinity by bonding itself as an allosteric molecule with the haemoglobin molecule, whose conformation is changed, thus releasing oxygen:

\[
\text{Hb(O}_2\text{)}_4 + 2,3\text{-DPG} \quad \leftrightarrow \quad 2,3\text{-DPG} \cdot \text{Hb} + 4 \text{O}_2
\]

By contrast, a reduction in 2,3-DPG implies that this equation experiences a shift to the left, i.e. at the expense of the free oxygen.

In addition to this, the MetHb content of 19 % in the infected erythrocytes (compared with 0.7 % in healthy cells) contributes substantially to a restriction of the oxygen transferal function of the affected cells.

In order to pass through their development cycle in the red blood cell with as little hindrance as possible, the plasmodia, which are sensitive to oxidation, are obliged to depend on an intact glutathion system, as it protects them from the reactive oxygen species which would otherwise act on them as oxidants (approx. 5 % of the oxygen absorbed via respiration on a day-to-day basis is metabolized to reactive oxygen species such as superoxide anions, hydrogen peroxide, hydroxyl radicals, or organic peroxides).

In the course of RBC metabolism, the glutathion redox cycle is built up via the pentose/phosphate path and maintained by the availability of reduction-equivalent NADPH (see Figs. 4 and 5). A defective or absent antioxidant system prevents the plasmodia from attaining maturity due to increased oxidative stress. This means that a lack of glucose-6-phosphate dehydrogenase, the enzyme at the beginning of the pentose/phosphate path, or its presence in a defective form, can result in resistance to malaria in areas where the disease is endemic.

On this basis, Krauth-Siegel and coworkers discuss the synthesis of a molecule tailored in accordance with modern drug design which is capable of blocking glutathion reductase, and thus making the reductive effect of glutathion ineffective: the plasmodia would then be exposed to the reactive oxygen species, thus interrupting their reproductive cycle (Krauth-Siegel et al. 1989).

The Ozone Concept

In fact, the “effective ozone” model is based on a similar idea. This is because the action of ozone on the erythrocytic metabolism could be demonstrated via a considerable number of parameters, and confirmed both in vivo and in vitro (Buckley et al. 1975, Washüttl et al. 1986, Viebahn 1992 and 1995, Bocci 1993, Hoffmann et al. 2000), see also Fig. 3 and Fig. 4.

Glutathion GSH

Due to the high reactivity of ozone, a direct exposure of the microorganism to it is out of the question:

Via the specific reaction of ozone with the double compounds in unsaturated fatty acids, the phospholipids in the cell membrane are broken down and are able to penetrate the cell in the form of short-chain hydroxylhydroperoxides. To meet this oxidative stress process, the cell responds with enzymatic peroxide breakdown via the glutathion system, an effect which can be followed in a measurable way via the consistent decrease in GSH concentration immediately after administering medical ozone (Bocci 1993).
A disturbance of the GSH oxidative protection system implies an increase of oxidative stress on the parasite, with a consequent restriction or disruption of its development cycle.

2,3-Diphosphoglycerate
At the same time we find an increase in 2,3-DPG, a fact which could also be demonstrated in vivo and, more recently, in red blood cell concentrates (Washüttl et al. 1986, Viebahn 1992 and 1995, Hoffmann et al. 2000).
An increase in 2,3-DPG reduces haemoglobin/oxygen affinity, i.e. it shifts the oxygen bonding curve to the right in the direction of released oxygen.
Ozone or the “ozone peroxides” can be understood as genuine antagonists to the effect plasmodia otherwise exerts inside the red blood cell: the ozone produces an increase in 2,3-DPG without producing methaemoglobin and without haemolysis; as the GSH is the first point of attack, this means that the antioxidant cycle - of vital importance to the plasmodia - is interrupted.
Intensive work is being conducted on concepts as to the synthesis and biological efficacy of stable ozonides, peroxides and epoxides with their antimalarial activity, particularly in the context of the increasing modes of resistance of malaria pathogens to pharmaceuticals used either preventively or therapeutically in areas where malaria is endemic (de Almeida Barbosa et al. 1996).

![Fig. 3: The effect of ozone on the erythrocytic metabolism](image-url)
**Fig. 4: The Pentosephosphat Path**

**Influence on the Immune System**

The immunomodulatory effect of ozone also presents a possible model for inhibiting plasmodial growth: by applying the relevant concentrations of ozone, it is possible to stimulate immune-competent cells to produce their specific cytokins (Haddad et al. 1996, Bocci et al. 1990-1998).

In animal experiments, interleukin-12, interleukin-6, IL-10, TNF and IFN-γ have been found effective in the field of malaria prevention, whereby a mechanism via reactive oxygen species is under discussion (Kremsner, Nüssler et al. 1992; Hoffmann, Crutcher et al. 1997; Kössodo, Monso et al. 1997).

The direct influence of reactive oxygen species such as superoxide radicals, hydrogen peroxide and OH radicals has been described in malaria infected children as regards the treatment, immune resistance and pathology of malaria by Kremsner and co-workers (Kremsner et al. 2000).

**Material and Methods**

**Plasmodium Falciparum**

As described by Binh et al. (1997), the laboratory-adapted *Plasmodium falciparum* strain BINH was cultivated in 50 ml cell culture flasks. As culture medium, RPMI 1640 with additional glutamine (Seromed, Berlin) and 1.5 % (wt./vol.) AlbuMax (Gibco BRL, Paisley, Scotland), and a low-IgG bovine serum extract as substitute for human serum were used. The medium was adjusted to a pH value of 7.4, and filtered (0.22 µm filter; Millipore, Bedford MA).

Donor red blood cells of group 0 were added, and the culture adjusted to a haematocrit of
2 %, with a total volume of 10 ml. Cultivation took place at 37°C in an atmosphere of 5 % O₂, 5 % CO₂, and 90 % nitrogen. The culture medium was changed every second day.

**Ozone Source**

For treatment of the infected erythrocytes, an ozone/oxygen mixture at a concentration of 80 μg/ml was used. This had proven itself to be an effective concentration in preceding series of tests, without resulting in detectable haemolysis. Medical ozone was provided by an ozone generator [Ozonosan PM 100, Dr. J. Hänsler GmbH, Iffezheim, Germany] producing pure ozone via silent electric discharge under continuous photometric measurement of the O₃ concentration.

Via a sterile bacterial filter (pore width: 0.22 μm), the ozone/oxygen mixture was drawn up into a silicon-coated disposable syringe, at a total volume corresponding to that of the cell suspension. To obtain a homogeneous distribution of the ozone within the culture medium, the flask containing it was gently swirled for 10 minutes. Controls were treated in the same manner, though with air in place of the ozone/oxygen mixture.

**Parasitic Growth**

Growth of the parasites was determined via counting a total of at least 1,000 red blood cells in a smear colored with Giemsa, parasitaemia then being expressed in percent.

**Mononuclear Leukocytes**

Mononuclear leukocytes (MNCs) were obtained via density gradient centrifuging from whole blood (Ficoll-Hypaque, Pharmacia Fine Chemicals, Uppsala, Sweden). Ozonization of the leukocytes took place either in whole blood at a concentration of 80 μg/ml and subsequent isolation of the mononuclear cells. In a second preparation, the mononuclear cells were isolated first, and ozonization was performed in a suspension of 2 million cells per ml and a concentration of 30 μg ozone per ml cell suspension.

In 96-hole cell culture plates, 200,000 mononuclear cells were cultivated per hole, with a parasite : MNC ratio of 1 : 1, and a haematocrit of 2 %. In a negative control, the leukocytes were exposed to sterile air and, in a positive control, either to lipopolysaccharides of E. coli (LPS; Sigma, St. Louis, USA), or phytohemagglutinin (PHA, Sigma).

Measurement values were obtained as mean values from 3 measurements in each case. For statistical evaluation, the Student t test was applied at a significance level of 0.05.

**Measurement Results**

**The Effect of Ozone on Parasitic Growth**

BINH Erythrocytes

1. The growth of Plasmodium falciparum in standardised RBC cultures compared with the control group (air) can be seen in Fig. 5. The initial parasitaemia level was 0.5 % (infected erythrocytes). In this series, the RBC suspension was ozonized after addition of the parasites (P + O).
Already after the 2nd day, ozonization produced a significant inhibition of growth, reaching its maximum point on the 4th day with a factor of 6.7 (1.3 % parasitaemia after ozonization versus 8.7 % without ozonization).

2. A second trial preparation with a higher parasitaemia level of 5 % also produced a significant growth inhibition: a factor of 2.6 was reached on the 1st day, i.e. with a parasitaemia level of 5.6 % after ozonization versus 14.6 % in the control group.

3. In a further test series, we were able to clarify the question as to whether ozonization of the red blood cells produced a similar growth inhibition before being infected with plasmodia, i.e. whether the time factor has an influence in the context of ozone effect. This involved an ozonization of the erythrocytes before adding the parasites and, in a second sample, after adding them, thus enabling a pre-/post-ozonization comparison to be made. The results have been summarized in Fig. 6: Both test models produced a growth inhibition of the parasites, though the inhibitive effect was somewhat less before adding the parasites than in the case of ozonization after actual infection.

4. The best result, i.e. yet another increase in growth inhibition, is obtained by pre- and postozonization of the infected erythrocyte suspension. This effect is particularly noticable on days 3, 4 and 5 (see Fig. 6).

5. Initially, the level of maturity of the plasmodia at each specific ozone application immediately before or after infection was disregarded; the following test series was performed to clarify a possible influence: Determination of parasitaemia via the ozonization of erythrocyte suspensions infected with plasmodia in the stages of ring-shaped and mature trophozoites (48-hour life cycle). At first sight, Figs 7 and 8 do not indicate different active mechanisms, in both cases we find approximately the same level of growth inhibition compared with the controls, which is particularly noticable on days 4 and 5.
Fig. 5: The effect of ozone on the intraerythrocytic growth of Plasmodium falciparum. Ozone applied after infection. Parasitaemia in %.

Fig. 6: The effect of ozone on the intraerythrocytic growth of Plasmodium falciparum: ozone applied after infection (Par+Ozon), ozone applied before infection (Ozon+Par), ozone applied before and after infection (Oz+Par+Oz). Parasitaemia in %.
Fig. 7: The effect of ozone on the intraerythrocytic growth of Plasmodium falciparum: P. falciparum + ozone, juvenile ring-shaped forms. Parasitaemia in %.

Fig. 8: The effect of ozone on the intraerythrocytic growth of Plasmodium falciparum: P. falciparum + ozone, adult trophozoites. Parasitaemia in %.
The Effect of Ozonized Leukocytes on Parasitic Growth

It was not possible to determine completely as to how far the immunological aspects of ozone play a part in the test design just described. Thus, in order to determine a possible effect on a culture of malaria parasites containing activated mononuclear white blood cells, the leukocytes of two different donors were first ozonized and then added to the culture. No effect of ozone could be recognized. Nevertheless, the known effect, although not yet explained up to now, of growth stimulation of the plasmodia by non-stimulated MNCs (Binh et al. 1997) could also be observed here, whereas the PHA- and LPS-stimulated mononuclear cells produced, as expected, a significant growth inhibition.

Discussion

In our investigations, we have been able to demonstrate for the first time that ozone has an effect on intracellular parasites - without direct ozone/parasite contact, and without visible haemolysis.

The sensitivity of Plasmodium falciparum to reactive oxygen species is known and has been well investigated as regards the antiparasitic effect of two classes of substance, i.e. artemisinin derivates and trioxanes, with endoperoxides functioning as mediators (Meshnick 1994). The immune resistance here involved is, to a certain extent, based on the effect of reactive oxygen compounds, such as those produced by granulocytes in the form of different oxygen radicals in order to attack intracellular microorganisms.

Naturally, not only the parasite itself but also the host cell is sensitive to reactive oxygen compounds. For example, it has recently been possible to demonstrate that an increasing formation of oxygen radicals produces the severest forms of malaria-induced anaemia in children (Kremsner et al. 2000).

In the investigations described here, a marked effect of medical ozone on the growth of Plasmodium falciparum can be demonstrated in vitro. According to the initial parasitaemia, we find an inhibitive effect of ozone by factors between 3 and 7.

The active mechanism involved can quite feasibly be explained via the reaction of ozone with the unsaturated fatty acids of the erythrocyte membrane and a consequent penetration of hydroxyhydroperoxides into the cells. In the further course of intracellular processes, various mechanisms are to be discussed. A direct reaction of the short-chain peroxides with the parasites; in the case of Plasmodium falciparum, two membranous systems come into consideration: the actual plasmodium membrane itself, or the membrane of the parasitophorous vacuole. Over and beyond this, by-products formed from the peroxides could also function as reactive agents.

The second reaction path probably takes place via the glutathion system which, as an effective antioxidant system, reduces the peroxides formed from ozone and is itself converted into an oxidized form (GSSG). Consequently, the parasites involved are subjected to an increased oxidative stress, and their reproductive cycle is disrupted.

In addition to this, an ozone-induced increase in 2,3-DPG counteracts the influence of the plasmodia on the erythrocytic metabolism, so that a further active principle of medical ozone here also plays its part.

The measurement results obtained in this study agree with the active mechanisms just discussed:
A significant inhibition of parasite growth produced by ozone is found when ozonization is carried out at a concentration of 80 μg/ml in a red blood cell suspension, independently of whether ozonization takes place before or after infection with plasmodia. In fact, the level of maturity of the plasmodia also has no influence, at least within the 48-hour cycle. Under the conditions given, an optimal growth inhibition is obtained by applying ozone twice, i.e. immediately before and immediately after infection.

The authors are certain that the results obtained up to now can be improved, both by repeated ozonization of the red blood cell suspension before and after introducing parasites, and by optimizing the concentration of medical ozone per ml suspension.

The same applies for a possible immunomodulatory effect which, however, in the investigation here described using activated mononuclear cells, showed no results.

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