The Influence of Ozone on 2,3-Diphosphoglycerate Synthesis in Red Blood Cell Concentrates

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Abstract

The longer blood preserves are stored, the more haemoglobin loses, after transfusion, its ability to transfer oxygen from the lungs to the tissue. This is due to an increase in a physiologically abnormal oxygen affinity of haemoglobin from a rapid loss during storage of 2,3-diphosphoglycerate (DPG), causing a reduction in blood preserve quality. A rise in the oxygen affinity of Hb in patients with functionally restricted compensatory mechanisms is of pathophysiological importance. Developing methods for restoring the function of stored RBCs is important for tissue oxygen supply. This investigation aimed at shifting the oxygen affinity of the RBCs back within a therapeutically favorable range by suitable treatment. Refunctionalisation of RBCs in preserves is described in a patent by Viebahn (1992) describing a treatment of stored preserves with an O_3/O_2 mixture producing an increase in erythrocytic DPG content. To examine this effect, cooled concentrates stored between 15 and 35 days received defined and controllable treatment, samples being transferred to a medium simulating post transfusion conditions. To assess the function and metabolic status of the RBCs, parameters were recorded to document this regeneration of their cellular metabolism. It was possible to show a significantly accelerated synthesis of DPG (10 - 30 %) in ozone-pretreated RBCs compared with untreated preserves.

Introduction

After transfusion, the ability of hamoglobin to pass oxygen on to the tissue from the lungs decreases all the more rapidly the longer it has been stored. This is because the oxygen affinity of haemoglobin increases to values above the physiological level, primarily due to the rapid loss of an instable allosteric effector responsible for oxygen bonding in the red blood cells (RBCs), i.e. *2,3-Diphosphoglyceric acid* (DPG) (Chanutin and Curnish, 1967; Akerblom et al, 1968; Bunn et al, 1969). Under physiological conditions, DPG is present in bonded form between the β-polypeptide chains of the haemoglobin molecule. With the binding of DPG, the deoxy configuration of the haemoglobins with its low affinity is stabilised, thus facilitating oxygen release from the red blood cells at the periphery (Benesch et al., 1968).

As a restoration of the normal DPG content, i.e. approx. 4 mmol/l red blood cells, via the resynthesis process resumed after transfusion can take up to 24 hours (Beutler and Wood,

1969), the transfused red blood cells are only able to release a limited amount of oxygen to the emergency patient's tissue. We have no detailed information as to the extent to which red blood cells stored for a long time are at all capable of restoring the DPG level or of normalising oxygen affinity. The loss of DPG - which increases with storage - must be viewed as a loss in quality of blood preserves. A massive transfusion from old preserves can attain a pathophysiological importance, especially in patients with reduced compensatory functions such as, for example, increased heart rate, too high a haemoglobin content or an intraerythrocytic contraregulation (Hess, 1987).

This is why interest has focussed on developing processes making it possible for stored blood cells to maintain or regain their full functional ability, an important parameter in supplying oxygen properly to the tissue. The aim is to reduce an increased oxygen affinity of the red blood cells by suitable treatment of the preserves. This can only be achieved by accelerating a return to the normal DPG content in the transfused cells.
In a patent by Viebahn (1992), a process is described for maintaining or reestablishing the function of red blood cells in blood preserves by which the DPG content in RBCs is demonstrably increased after 1 to 2 days by treating preserves under long-term storage with an O_3/O_2 gas mixture at room temperature. It is conjectured that ozone produces chemical changes in the lipids of the erythrocytic membrane to result in an increased synthesis of 2,3-diphosphoglycerate by the cells concerned. Under the condtions selected, the DPG content of totally depleted cells increased to a maximum of 10% of the normal content.

Using the description given in the patent as a basis, we undertook to produce preconditions for a practical method of refunctionalising stored blood preserves in the context of their oxygen transport function.

Materials and Methods

Materials

Blood preserves

CPD human blood preserve, consisting of approx. 70 ml CPD (citrate phosphate dextrose) stabilizer and 450 - 550 ml human blood (prod.: German Red Cross (*Deutsches Rotes Kreuz, DRK*) Blood Donor Service (*Blood spendedienst*) Thüringen GmbH

Red blood cell concentrate, buffy coat free, consisting of approx. 210 ml RBC concentrate obtained from a single whole blood donation by centrifuging at 4000xg and vacuum decantation of the buffy coat, and suspended in approx. 110 ml SAGM stabilizer (prod.: DRK Blood spendedienst Thüringen GmbH

To investigate storage-dependent changes in blood conserve parameters, the material was supplied in separate baby bags.

Ozone generator: Ozonosan, Type: „photonik" (Manuf. *Dr. J. Hänsler GmbH*)

Gas treatment incubator: BB6220 (Manuf.: *Heraeus Instruments*)

Low profile roller: Type: M 1241-6001 (Manuf.:*New Brunswick Scientific Co, Inc*.)

Gas infusion flasks: 250 ml vacuum flask with microbubble system, sterile (*Dr. J. Hänsler GmbH*)

Transfusion unit: in accordance with German Industrial Standard (*Deutsche IndustrieNorm, DIN*) No. 58360 TG-G (*Clinico GmbH*)

Transfusion set: „Valu-set"19 G 3/4 butterfly cannula with Luer Lock connection (Manuf.: *Becton Dickinson*)

Ozone transfusion set: with bacterial filter and cylinThe clamp (*Clinico GmbH*)

Disposable syringe: 50 ml, pyrogen-free (*Transcoject*)

Tyrode's solution: for diluting blood preserves: protein-free artificial blood liquid maintaining the pH value of the diluted blood in combination with the CO_2 content of the gas mixture used in the incubator within the physiological range at $37^{\circ C}$. Modified composition: 140 mmol NaCl/L; 2,7 mmol KCl/L; 1,8 mmol $CaCl_2$/L; 1,05 mmol $MgCl_2$/L; 60 mmol $NaHCO_3$/L; 0,42 mmol Na_2HPO_4/L; and 5,05 mmol Dextrose/L.

Incubator gas mixture
Composition: 9% O_2; 6% CO_2; N_2, to make up 100% (Manuf.: *Messer Griesheim GmbH*)

Test kits for analysis

For 2,3-diphosphoglycerate and adenosin triphosphate (ATP);
Quantitative enzymatic assay (Manuf.: *Sigma Diagnostics*) For glutathion (GSH);
Quantitative colorimetric assay (Manuf.: *Calbiochem- Novabiochem Corporation*) For plasma haemoglobin (Hb); Quantitative colorimetric assay (Manuf.: *Sigma Diagnostics*)
For lactate in plasma; Quantitative enzymatic assay (Manuf.: *Boehringer Mannheim*)

Methods

Determination of blood parameters

Determination of the parameters DPG, ATP, and GSH in RBCs as well as Hb and lactate in plasma is performed using commercial test kits in accordance with manufacturers' instructions. Optical density is measured using a Typ V 550 UV-VIS spectrophotometer (*Jasco Labor- and Datentechnik GmbH*).

Blood gas and/or oximetric parameters pO_2, pCO_2, sO_2, bicarbonate and K^+ content as well as the pH value in the preserve medium are measured using a Type ABL 520-B blood gas/oximetry unit (Manuf.: *Radiometer GmbH*).
Red blood cells are counted using a Cell-Dyn 1600 Type analyzer (*Abbot*).

Standard protocol on the investigation of ozone effects

a) Filling of gas infusion flasks

For resuspension of their content, the blood preserve bags are slowly shaken on a rocking table for 20 minutes at 4 °C . Following this, 35 g blood - corresponding to a volume of 33 ml - are transferred according to a standardized procedure to the cooled gas infusion flask and kept at 5 °C prior to gas application.
To determine, in the form of a „cold sample check", the prevailing parameters of the preserves, an additional 2500 µl sample of CPD preserves or 1600 µl RBC preserves, filled up with 900 µl of Tyrode's solution (ice-cooled) are transferred to plastic screw-cap tubes, and stored at 4 °C prior to determination.

b) Gaseous treatment of blood samples in the infusion flasks

O_2 and O_3 gas is passed through the samples , slowly swirling the flasks in titlted position.
Gas application is always performed - as specified for ozonized blood reinfusion (autohaemotherapy: Beck and Viebahn-Hänsler, 1995) - with a gas volume corresponding to the volume of treated blood, over a period of 35 seconds.

c) Incubation conditions

To investigate and validate the effect of ozone, the treated bottles are stored at room temperature, samples being taken at different times (see below).
To investigate metabolic changes during simulated transfusion: after gas treatment, 10 ml CPD preserve or 6,4 ml RBC concentrate and 3,6 ml cold Tyrode's solution are transferred into 2 precooled cylindrical glasses (plastic 50 ml *Corex* centrifuging beakers) and, to start simulation, placed in the gas treatment incubator on a slowly rotating (1 rpm) cylindrical mixer. The temperature is kept at 37 °C , the relative air humidity at 100%. A special gas mixture corresponding to a pO_2 of 64 mm Hg and a pCO_2 of 43 mm Hg is used for treatment. During simulation, the pH values of the prepared samples are principally kept within a physiological range (pH 7,2 - 7,4) via the pCO_2 in conjunction with the bicarbonate contained in the Tyrode's solution.

d) Sample taking

Each sample amounts to 2,5 ml and is taken at different times. In the simulation tests, the samples (ca. 125 µl) are drawn directly from the cylindrical glasses by the blood gas/oxymetry unit. Immediately following this, a further 2,2 ml are drawn from the preparation, and placed in an ice bath for later aliquot sampling. To avoid a change in medium when taking out the cylindrical glasses, the incubator door was only kept open for as short a time as absolutely possible. After taking it out, the glass is immediately placed in a Styropor insulator and sealed with a rubber disc, which is only removed on taking samples.

Results

For our investigations, 2 types of human blood preserve principally apply. The first is a preserve prepared from the venous blood of a single whole blood donation collected in CPD stabilizer. At 4 - 6 °C , these preserves may be transfused up to 21 days after preparation. These preserves are used in the patent. For quite some time now, however, such CPD preserves are no longer used beyond the 5^{th} day after donation, but are then used for blood products, such as processing into a RBC concentrate.

The second type, RBC concentrate, contains the total quantity of red blood cells found in a CPD preserve and, in place of the removed plasma, an additive solution, in this case SAGM. Nowadays, this RBC preserve is only used to substitute the red blood cells in cases of acute blood loss and transfused in chronic anaemia. Its period of utility is 35 days at the most. Consequently, according to the present state of the art, the development of a process for refunctionalising the oxygen transfer ability can only be of therapeutical interest where RBC concentrate is concerned.

The behaviour of some important red blood cell parameters during storage without ozonization

At the start of our investigation, it was necessary to obtain information on the changes over time in the main parameters measured in the preserves during storage. Apart from the main parameter, DPG, the ATP (adenosine triphosphate) content was also determined as a marker for energy metabolism, and the glutathion content as marker for the RBC reduction potential. The Hb content in the plasma/stabilizer medium was determined to assess the integrity of the RBC membrane. More detailed investigations included further parameters necessary to characterize the metabolic status of the preserves.

CPD (citrate phosphate dextrose) blood preserves

The serial tests on metabolite content first took place with 2 full blood preserves prepared using CPD stabilizer under usual storage conditions (in a refrigerator at 4 - 6 °C). Samples were drawn from the preserves under sterile conditions on different days after preparation, and their contents determined (see below under Methods). As, for technical reasons, the initial concentrations in the preserves could not be determined the first day after withdrawal, normal values taken from literature were entered in the results (Fig. 1) for better orientation (CIBA-GEIGY AG, 1979).

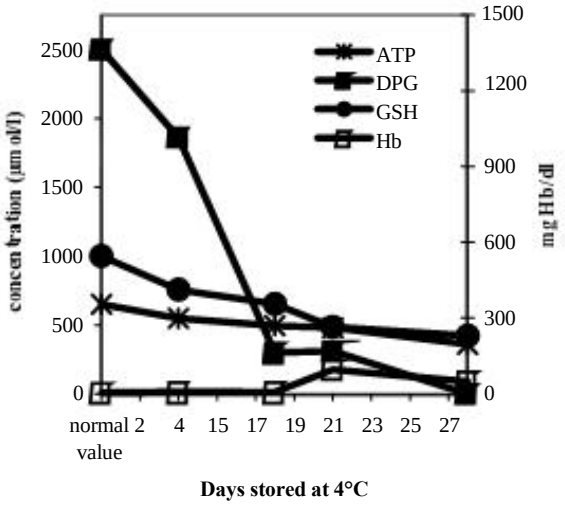

Figure 1 Level of ATP, DPG and GSH in RBC and Hb in plasma during storage at 4°C in CPD-stored blood. Mean from 2 preserves.

As Fig. 1 shows, the DPG content had already dropped markedly on the 4th day after taking blood from donor. This drop continued consistently. After approx. 11 days, the content had reached 50% of the initial value. attaining a level of 10% after 18 - 20 days. After about 28 days, no DPG can be found any more in the CPD preserves involved.

The ATP content of the cells stayed relatively stable, only dropping to approx. 75% of the initial value after 18 days. Even after 28 days, a content of approx. 55% was still found. The GSH content of the cells dropped to approx. 65% after about 18 days; and approx. 40% of the original GSH content was still found after 28 days. As criterium for the integrity of the cell membrane, the plasma-Hb content stayed for the most part stable throughout the entire storage period.

RBC concentrate

The serial tests on the metabolite content of RBC concentrate were carried out in a total of 5 preserves for a wider range of parameters. The number of RBCs in the sample were adjusted to the original number of cells (approx. 4,5 $x10^{12}$ /L) via dilution with Tyrode's solution (i.e. a complete physiological saline solution[1]), so that the given contents for both preserves are comparable. The values obtained for the individual metabolites are presented after formation of the mean value curve in each case.

As expected, the average results for DPG, ATP, glutathion, and haemoglobin agreed for the most part re changes in content with those found in the CPD-preserves. The stability of the 4 parameters, particularly that of DPG is, however, clearly lower in the RBC

1 A modified Locke's solution; containing 8 g of NaCl, 0.2 g of KCl, 0.2 g of CaCl2, 0.1 g of MgCl2, 0.05 g of NaH2PO4, 1 g of NaHCO3, 1 g of D-glucose, and water to make 1000 ml; used to irrigate the peritoneal cavity, and in laboratory work (STEDMAN'S Medical Dictionary).

concentrate than in the CPD whole blood preserve. As a rule, already after 13 days of storage, no DPG can be found in the cells any more (Fig.2).

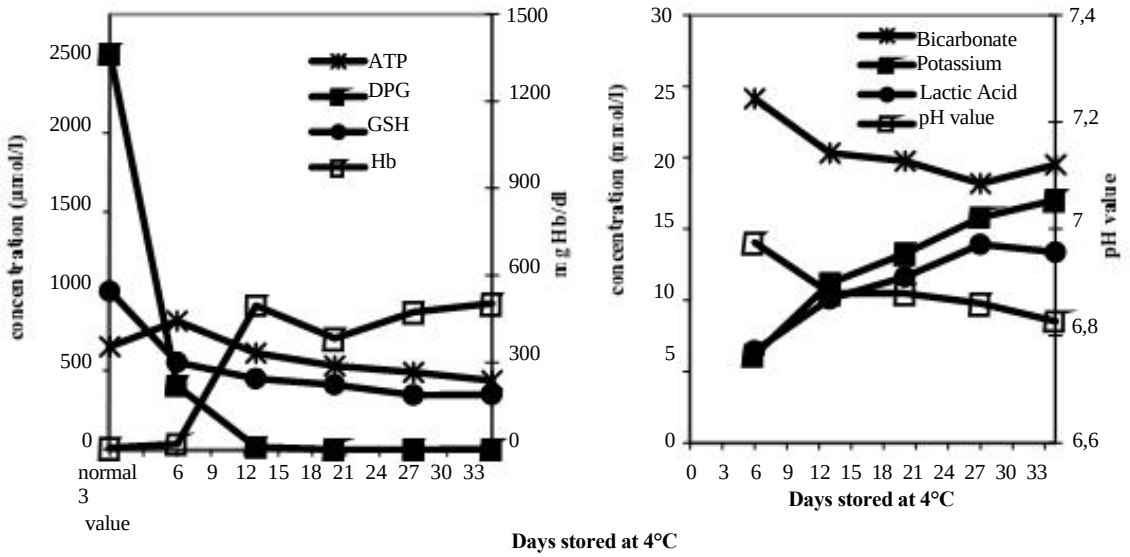

Fig. 2 Level of ATP, DPG, and GSH in RBC and of haemoglobin, bicarbonate, potassium, lactic acid, and pH value in the preservative solution during storage at 4°C in RBC concentrates. Mean from 5 preserves.

At the end of the permissible time of usage, i.e. after 35 days, the ATP and GSH contents dropped to approx. 50% of their initial value. The Hb content in this solution is, compared with the CPD preserve, a number of times greater, and also shows a rising tendency with storage time.

As expected, the pH value in the solution decreased during storage, with a simultaneous drop in glucose and bicarbonate levels and a rise in lactate content. Due to a marked inhibition of the membrane-bound, Na^+- and K^+-dependent ATPase resulting from the low storage temperature, a slow equalisation of the intracellular Na^+ /K^+-gradient built up by this enzyme occurs. The diffusion of K^+ from the erythrocytes is reflected by the rise in potassium content in the suspension solution.

Verification of ozone effect on DPG synthesis at room temperature

In an initial test - as described in the patent - a blood preserve at its expiry date (35 Days after preparation) was used. For the reasons described above, this and later tests were all carried out using the red blood cell concentrate as test product.

In this assay, the preserve is adjusted by adding Tyrode's solution under sterile conditions to the cell content of CPD preserves. From these, specific quantities of aliquot samples are transferred to gas infusion bottles, immediately after which either equal volumes of air and/or oxygen are passed through, or a gas containing 20, 30, 40 or 50 µg ozone per ml oxygen. These preparations are incubated at room temperature for 52 hours. At intervals of several hours, samples are drawn from these for DPG analysis. As Fig. 3

shows, no DPG can be found after storage for 35 days in the preserve, which was expected.

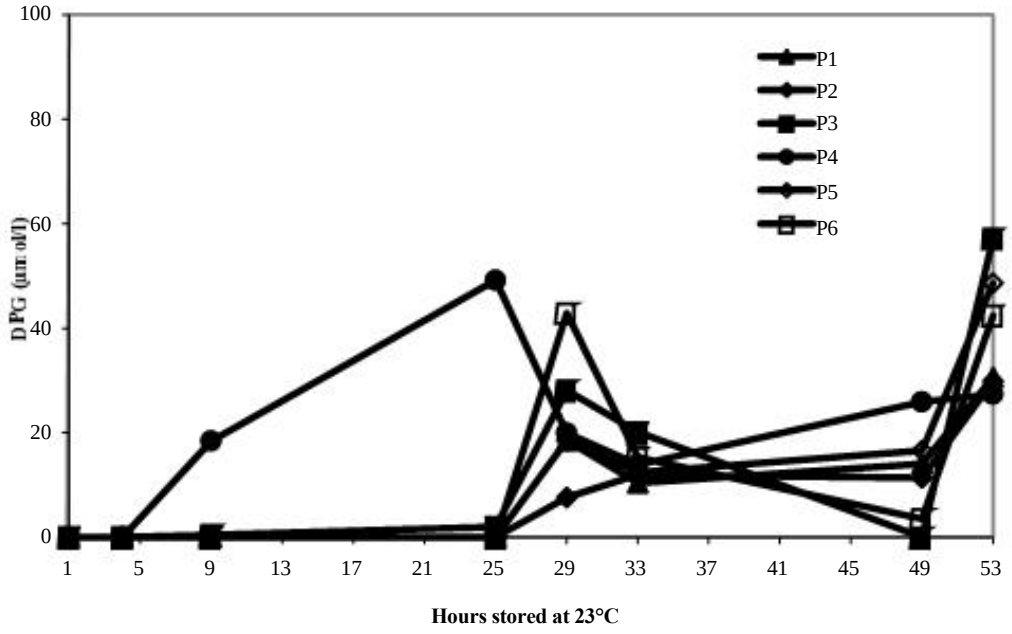

Fig. 3 Influence of Ozone on the DPG level in RBC during storage in RBC concentrates at 23°C after storage for 35 days at 4°C.
Application of 20 µg O_3 (P1) resp. 30 µg O_3 (P2) resp. 40 µg (P3) O_3 resp. 50 µg O3 (P4) resp./ml O_2 at 1 ml preserve. Controls: Application of 1 ml air (P5) resp. 1 ml O2 (P6) at 1 ml preserve. For application of gas see text. Mean from 2 preserves.

During the incubation at 23 °C, a slight increase in DPG content was observed in all samples. The increase in the control sample (treated with air and oxygen only) and in the sample treated with 20 - 40µg O_3/ml oxygen reached a maximum after approx. 28 hours, only to decrease again afterwards. A renewed increase in DPG content was observed after 52 hours. In the sample treated with 50µg O_3/ml the start of a marked DPG synthesis could already be observed after 8 hours. The maximum is here at a content of of 50 µmol DPG/L, corresponding to a mass concentration of 13 mg/L. After a further 24 hours, the content was at approx. 7 mg/L. Both in its magnitude and in its progress, this effect of ozone agrees with that described in the patent: in overstored CPD preserves, DPG contents of 47 - 78 mg/l were measured after an incubation period of approx. 24 hours, and contents of 31 - 59 mg/l after 48 hours. This effect was only observed in cells with depleted DPG. With an increase in content from 0 to approx. 10% of the normal content, this is slight.

In vitro *simulation of transfusion conditions*

The transfusion of preserves which have exceeded their date of expiry or which have been subjected to temperatures above the required storage level of 4 - 6 °C for longer than 6 hours, is obsolete. Thus, to characterize the effect of ozone more closely, we looked for conditions which apply to the handling of blood preserves. In addition, it was necessary to be able to assess the effects of ozone treatment on renewed erythrocytic metabolism following subsequent transfusion.

It seemed promising to test whether, during subsequent transfusion, the RBCs - in a blood preserve with an already depleted DPG content and after ozone treatment performed at storage temperature - would show any considerable increase in DPG synthesis by comparison with an untreated control sample. For a number of reasons, *in vivo* investigations of this kind are at present not feasible or only with a high outlay (Valeri and Hirsch, 1969; Beutler and Wood, 1969). This is why incubation conditions were developed which are able to simulate the environment present within the circulatory system in vitro for a limited period of time (simulated transfusion).

For our investigations, Tyrode's solution at 5 °C was added to the blood samples cooled at the same temperature before and/or after ozone treatment, whose composition had been determined in preliminary tests for their suitability and the age of the preserves. These had been mixed in such a way that the most important parameters for RBC environment such as pH value, glucose- and bicarbonate content are approximately normalized, and metabolic products such as lactate are diluted. The RBC count gave approx. 4,5 x 10^{12}/L. The blood samples transferred to *Oak Ridge* centrifuging glasses were placed on a slowly rotating cylindrical mixer in a gas incubator as 37 °C. Simulation of the pO_2 and pCO_2 pressures present in the tissue was obtained in an atmosphere saturated with water vapour via a special gas mixture. To avoid any change in the medium, great care was taken that withdrawing a blood sample be carried out as quickly as possible. During the almost 3 hours of incubation, samples were withdrawn at 4 different times to determine metabolic parameters (see under Methods for further details).

When stored, untreated blood preserves are subjected to simulated transfusion, their metabolism adjusts to approximate physiological conditions after an adaption phase lasting about 60 minutes (Fig. 4). This condition, characterized by a static continuation of the parameters, remains constant for approx. 1 to $1^1/_2$ hours. We here compared the parameters to find changes between control and ozone sample (within the given time frame), to assess those effects of O_3 on RBC metabolism which would have been expected after a genuine transfusion.
The DPG level of the red blood cells rises spontaneously to approx. 170 µmol/l within almost 3 hours.

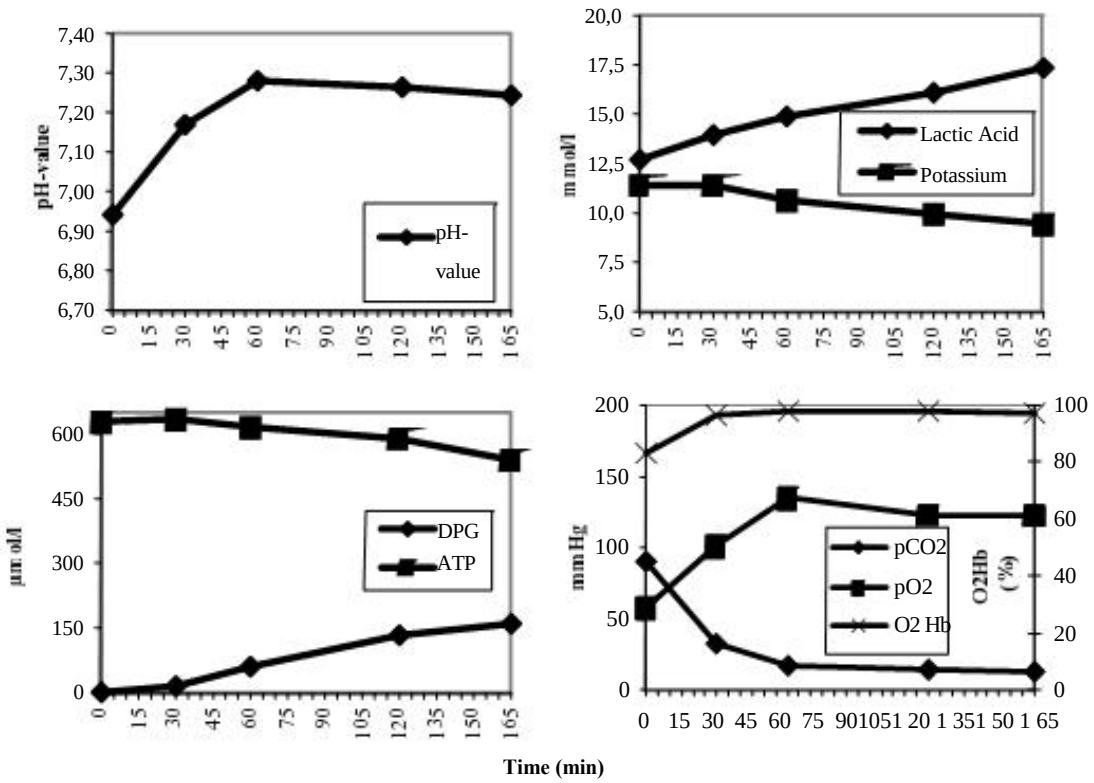

Fig. 4 Restoration of some metabolic arameters after start of transfusion simulation (see text) with 20 days stored RBC. Mean from experiments with 2 RBC preserves.

Investigating the effect of ozone on DPG metabolism in stored RBC concentrates

Once we had established the modalities for simulating transfusion as a prerequirement for a standard assessment of ozone effects, we investigated the influence of different dosages of ozone (10 - 60 µg O_3/ml O_2 per ml preserve) - in addition to other parameters - on the DPG content of red blood cells in RBC preserves stored over different times. Here, the results obtained with 50 µg O_3/ml O_2 per ml preserve in relation to the increase of DPG concentration showed an optimum (not presented) with a slightly haemolytic effect. Each test was carried out at different storage times (after 13, 20, 27 and 34 days) in four RBC preserves.

For this purpose, on test day, 35 ml RBC concentrate were drawn into two cooled gas transfusion flasks and - without previously adding Tyrode's solution - treated with oxygen or the optimally effective ozone dosage (50 µg O_3/ml O_2). After this, half of the contents of each were distributed into two cylindrical glasses (twin preparations) now containing cooled Tyrode's solution. The suspensions were subjected to simulated transfusion immediately afterwards. The gas infusion flask with the other half portion was stored for a further 5 hours at 5 °C before also being diluted with Tyrode's solution in two cylindrical glasses and transferred to the medium optimized for cellular metabolism. Samples were taken at different times and their metabolic parameters determined. The RBC concentration was adjusted to 4,5 x 10^{12}/L in the prepared samples

and determined in all of them at each time of withdrawal. Where cell concentrations were changed, the contents found for the DPG were adapted to the initial cell concentration via calculation.

The results obtained from the four independent tests were determined and are presented separately according to age per preserve. The results obtained in DPG-determination after treatment with oxygen or with ozone were compared and significance caluculations performed using the one-sided t-Test. Results with a probability of error significantly different by less than 5% have been marked with an asterisk (*).

The test results for DPG resynthesis show that, even under the modified, practically relevant conditions, the desired ozone effects are still obtained (Fig. 5).

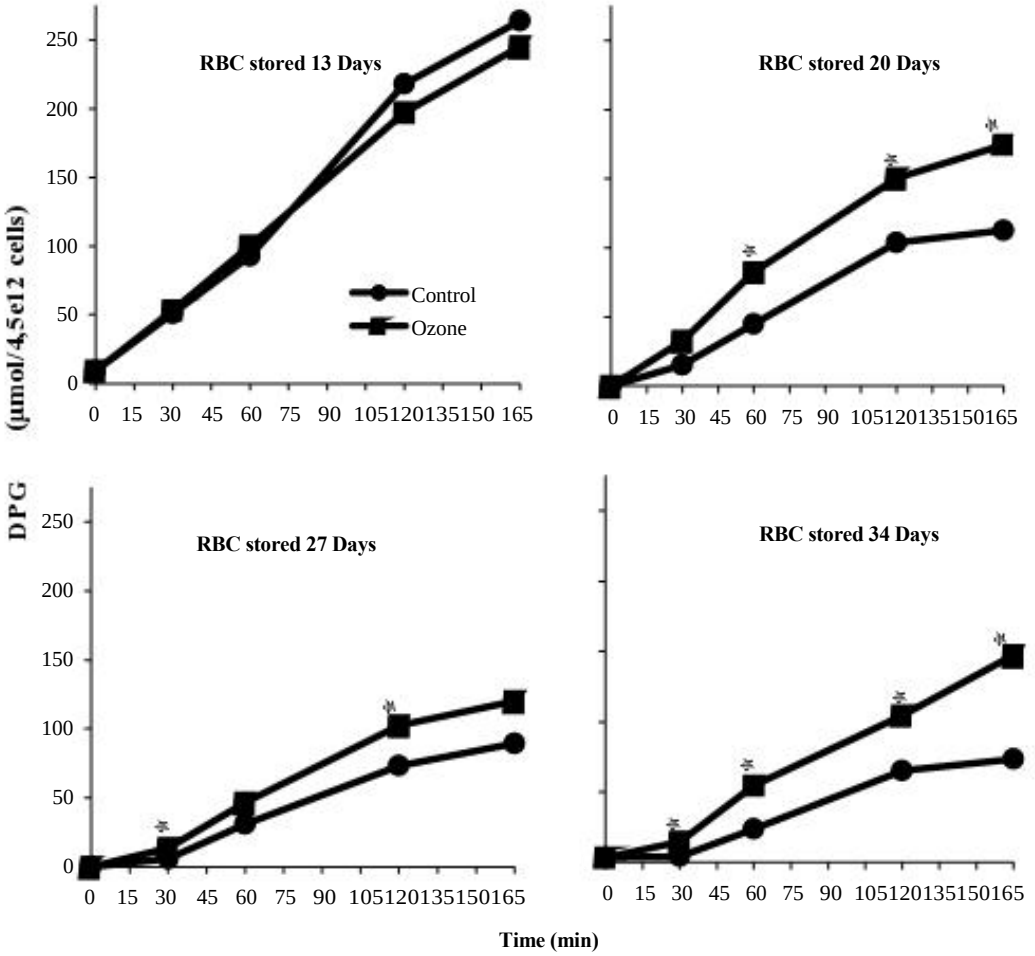

Fig. 5 Influence of Ozone (50µg O_3/ml O_2 at 1ml preserves) on the increase of DPG in RBC induced by transfusion simulation (see text) immediately after Ozone application.
Mean from experiments with 4 preserves each stored for 13, 20, 27, or 34 days.
*) $p < 0,05$

In ozone-treated preserves stored for 20 days and longer, synthesis is significantly accelerated by 10 - 30 %. And the fact that the performance of DPG in producing this synthesis in the RBC´s decreases with age here becomes quite clear. Whereas the content in 13-days‘ old, DPG-depleted preserves is increased to as much as 250 µmol/4,5e12 cells by simulated transfusion, a content of only 60 µmol/4,5e12 cells is obtained after a 34-day storage period.

The time between ozonization and simulated transfusion: its influence on DPG synthesis

As already described above, to test the lastingness of the stimulus produced by ozone, portions of the ozonized RBC concentrate were stored for a further 5 hours at 5 °C before

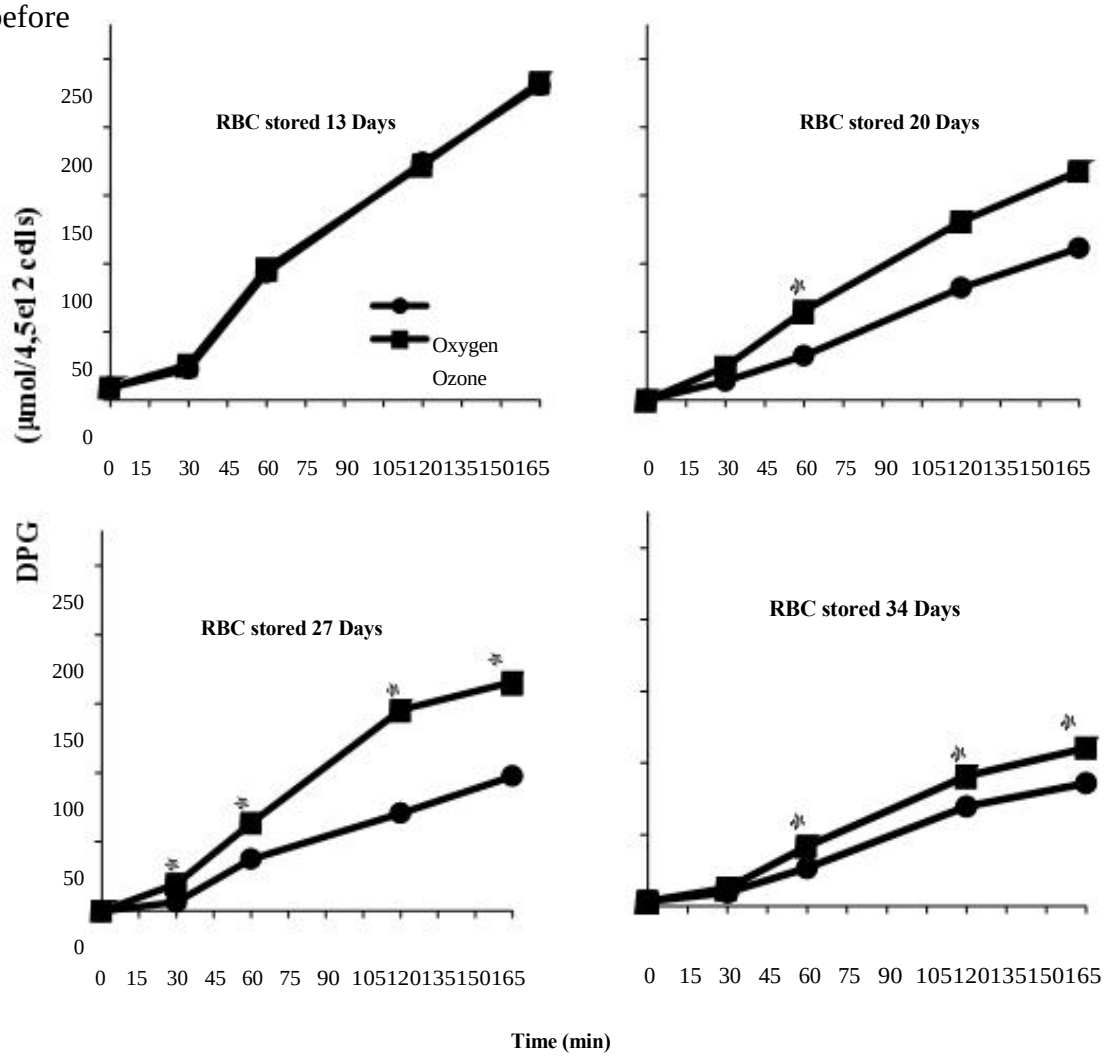

Fig. 6 Influence of Ozone (50µg O_3/ml O_2 at 1ml preserve) on the increase of DPG in RBC induced by transfusion simulation 5 h after Ozone application.
Mean from experiments with 4 preserves each stored for 13, 20, 27, or 34 days.
*) $p < 0,05$

being subjected to simulated transfusion. The results on the progress of DPG resynthesis are shown in Fig. 6. They agree to a marked extent with the results obtained with the immediate simulation. As the comparison of results in Tab. 1 shows, the increase in DPG synthesis remained unchanged even 5 hours after ozone treatment .

Table 1. Acceleration of DPG synthesis by ozone during simulated transfusion started after ozone application in RBC stored over various times

Start of transfusion simulation after Ozone application	**Increase of DPG level in RBC in %* after storage of preserve at 4°C for**			
	13 days	**20 days**	**27 days**	**34 days**
Immediately	77,6	115,3	131,2	120,0
5 hour	93,9	131,0	247,7	114,7

*) Increase of DPG level in the control each from the first to the second hour after start of transfusion simulation = 100%. Values taken from Figures 5 and 6

Discussion

The initial investigations carried out on the behaviour of characterized RBC parameters during the storage of blood preserves confirm in all points the details supplied by other authors (survey in: Beutler, 1989). The metabolite of principal interest in our study, i.e. DPG, here attracts our attention due to its extraordinary instability. This applies above all where RBC concentrate is concerned, in which no DPG can be detected any more already after a storage period of 14 days. This DPG deficiency is - as initially mentioned - known to be the cause of an inadequate function of the red blood cells in conveying oxygen to the tissue. The continuous drop in pH value is primarily responsible for the rapid decrease in the DPG level in stored blood preserves. As demonstrated by Kuchel and coworkers (MULQUINEY et al., 1999a, 1999b, 1999c) in a comprehensive investigation on regulation of the DPG metabolism in human red blood cells, both biphosphoglycerate mutase itself as well as the glycolytic enzymes hexokinase and phosphopyruvate kinase are inhibited due to the rise in the concentration of protons in the preserve. By a simultaneously increase in the inorganic phosphate content, the enzyme responsible for breaking DPG down, i.e. DPG phosphatase is activated in addition.
As with glutathion (to a lesser extent), the breakdown of DPG particularly in fresh preserves is additionally accelerated by adverse mechanical influences or chemicals acting on the cells. Thus, the influence of gas alone, and more so of the ozonization process on preserves with a still relatively high DPG content also produces an

acceleration in DPG breakdown during simulated transfusion. On the other hand, when the metabolism is activated by simulated transfusion in older, DPG-depleted preserves, relatively high spontaneous DPG synthesis rates have been observed. This was also found by Valeri and Hirsch (1969), Beutler and coworkers (1969) as well as Beutler and Wood (1969) on investigating the development of DPG resynthesis in red blood cells. The latter investigated the resynthesis of DPG after the transfusion of red blood cells stored for 20 days. Their investigations showed that, approx. 3 hours after transfusion, the DPGdepleted cells had approx. 25% and after 24 hours more than 50% of the original DPG content available to them again.

Under the transfusion conditions simulated to study the effect of ozone, spontaneous resynthesis in the cells stored as RBC concentrate after 3 hours in dependence on age of the preserves amounted to approx. 15% of the normal value. Nevertheless, the results also showed that, with increasing storage duration, at least *in vitro*, the ability of the cells to synthesize higher DPG levels is reduced.

The results of our investigations confirm the results obtained by Viebahn-Hänsler(1992), according to whom an increase in the DPG content in the DPG-depleted red blood cells occurs as the result of the action of ozone on preserved blood. The DPG content is increased to approx. 110 - 130% of the value found in the controls.
According to the active mechanism proposed (Viebahn-Hänsler, 1995) in its reaction with unsaturated fatty acids of the erythrocytic membrane, ozone forms hydroperoxides which are in turn inactivated by the large amount of intracellular glutathion . Due to the increased conversion of glutathion via the rise in the $NADP^+$ level of the pentose phosphate pathway and the preconnected glycolysis chain, activation continues until the former NADPH/ $NADP^+$ quotient is reestablished. During this time, DPG synthesis is also activated via rechanneling of a side reaction of the glycolytic pathway.

Accordingly, an acceleration in DPG synthesis could also only be observed in ozonized erythrocytes at the beginning of simulated transfusion. Simultaneously, an accelerated increase in pH value versus control was measured (not presented). Due to the enzyme inhibition through protons mentioned above, we can assume that the increase in DPG synthesis with observed drop in protons are connected with each other.
As a response to the stimulus produced by ozone, a series of cellular metabolic reactions occur as a result of which there is an increase in DPG synthesis. Whereas the powerfully exergonic trigger reactions of the ozone or the hydroperoxides take place very rapidly, even at temperatures around 5 $^{\circ}$C, the enzyme catalysed metabolic reactions are almost completely blocked under these conditions. It is thus possible that the ozone effect occurring at storage temperature is retained for a certain time. In this way, it is possible to break the reaction down into two temporally separate processes:

a) stimulation of the red blood cells via the ozone reaction at 5 °C , i.e. without an interruption of the storage temperature;

b) the cellular response reaction of the red blood cells including DPG synthesis at a desired point in time due to introduction into a physiological medium, such as can be found following transfusion.

As the optimum dosage for setting off an increased DPG synthesis in cooled RBC concentrate - using the microbubble flasks - we applied 1 ml of a mixture containing 50 µg O_3/ml oxygen gas per 1ml preserve within 35 seconds. Lower ozone concentrations had an uncertain effect. At higher concentrations, there is a danger of extensive cellular damage. A slight, but marked Hb release can already be observed at a concentration of 50 µg O_3/ml. It is easy to see that the transition from an optimum dosage only slightly disturbing the cell membrane up to a dosage damaging it to the extent of haemolysis occurring is a flowing one. The level of distribution of the gas mixture applied is of decisive importance. By means of a very fine distribution of the gas flow, such as that obtained with microbubble gas infusion flasks, it is possible to activate a large number of cells with a low ozone dosage in a reproducible way (Viebahn and Busch, 1994). The development of a correspondingly suitable microbubble system for applying gas in blood bags will be important in future for the application of this procedure.

Conclusion

Conditons for a refunctionalisation of haemoglobin in its ability to transport oxygen to the tissue in RBC concentrates stored over extensive periods were developed and tested. By means of the process used, erythrocytes with a resting metabolism were changed by ozone in such a way that a transfusion of metabolically reactivated cells carried out within approx. 5 hours after treatment produced an accelerated reestablishment of the oxygen transport function.

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