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Dedicated to Professor Zeno Simon
on the occasion of his 80th anniversary

MODULATION OF OXYGEN RELEASE BY RED BLOOD CELLS: PHYSIOLOGICAL AND THERAPEUTIC PERSPECTIVES

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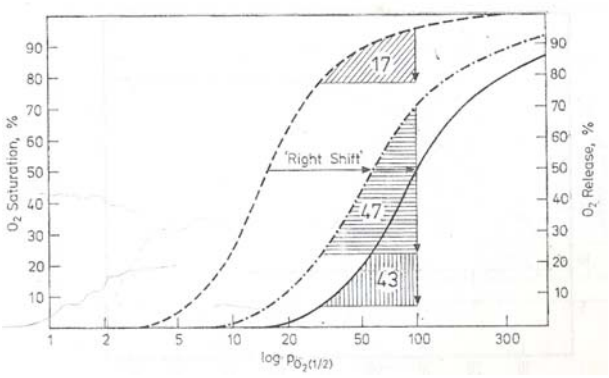
Oxygen delivery by Red Blood Cells to tissues depends upon several factors, such as blood flow, number of Red Blood Cells (RBC), concentration of hemoglobin, O_2 affinity etc. The O_2 release capacity of the RBCs is controlled by the microcirculation as well as by molecular characteristics of intracellular hemoglobin; that is cooperativity and O_2 half saturation pressure (P_{50}). The O_2 release capacity can be enhanced by an increase in the cooperativity of the hemoglobin molecule and/ or by shifting of the entire HB- O_2 dissociation curve toward higher O_2 pressures. In human RBC the shift to the "right" (*i.e.* the higher PO_2) is controlled by several allosteric mechanisms. Both effects, the HB-allosteric effect of 2, 3 bis-phosphogylcerate (BPG) and CO_2binding play a major role. As improved O_2 delivery could have therapeutic implications in cases of hypoxemia/ischemia, we have devised methods to improve O_2 delivery through the use of powerful allosteric effect of hemoglobin. The present reviews describes these methods and some of their physiological and therapeutic results.



INTRODUCTION

The work described in this review, extending from 1979 to today is the direct outcome of an observation which I made as a child, watching the sky in spring and in autumn. I saw large numbers of birds – swallows and others, flying in spring northwards and in autumn southwards. My grandmother explained to me that these were migrating birds which spent winter in Africa, where it was warm and in spring came back. I was fascinated by the fact that such small creatures could fly so far, so fast and so high. I asked my teacher, my father and others how come that the small birds could successfully make such efforts, while big animals, humans included were unable to cross even the English Channel (37 km). No one could give me a satisfactory answer. Much later, I read the papers by Benesch & Benesch[1] about the avian allosteric effect of hemoglobin Inositol Penta-phosphate, present in the birds' red blood cells, lowering hemoglobin's affinity for oxygen

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much more than does 2, 3, bisphosphoglycerate (2, 3-BPG) the allosteric effect of hemoglobin present in mammalian red blood cells, humans included. It is this reading that awoke my life-long interest in engineering human red blood cell by encapsulating in them allosteric effects of hemoglobin, more powerful that 2, 3-BPG in order to improve oxygen delivery by the RBCs, which, I thought, could have significant physiological and therapeutic effects.

Life as we know it depends on the presence of oxygen in the environment. In particular, oxygen is transported from the ambient environment to various intracellular sites of O_2 utilization. This is accomplished by a complex of semi-independent, but linked structural and functional mechanisms.[2] O_2 transport in the blood is an important segment of this overall complex (2). Red blood cells are the carriers of O_2 to the tissues and the delivery of O_2 to the tissues involves (*i*)) O_2 transport from the lungs to the microvasculature, (*ii*) oxygen flux out of the RBCs to the surrounding plasma and (*iii*) O_2 diffusion from the vessels to the cells.[3]

The oxygen flux out of the RBCs which regulates the critical O_2 tension in the capillary system interconnects the two processes mentioned above.[3] The O_2 flux is influenced by the O_2 release capacity of the hemoglobin in the RBCs, which release is facilitated in two ways: (a) increase of cooperativity between the $4O_2$-binding sites in HB leads to a steeper slope of the sigmoid O_2 binding curve in the region of 30-70 % saturation and (b) the "right shifting" of the entire oxy-hemoglobin dissociation curve towards higher O_2 pressures.

Normally in the lungs, with a O_2 pressure of 100 mm Hg, almost 98% of the circulating HB is saturated with O_2, which represents the total O_2 transport capacity of the blood. Only about 25% of this transport capacity is used as oxygen tension falls from 95% in arterial blood to 40 mm Hg in venous blood.[4] "Right shifts" of the O_2 binding curve lead to an optimum O_2 release, if the steepest part of the O_2 binding curve (30-70% saturation) corresponds to the O_2 pressure region comprised between 100 and 40 mm Hg. An abnormally high affinity of hemoglobin for oxygen shifts the O_2 binding curve to the left and the P_{50} (O_2 partial pressure at which 50% saturation of hemoglobins occurs) decreases. This causes a lower oxygen release to the tissues. In human RBC, the "right shift" is controlled by several allosteric mechanisms:[5] Bohr-effect, 2, 3- bis-phosphoglycerate (BPG) and CO_2 binding. Improved O_2 delivery has therapeutic implications for the treatment of hypoxemia / ischemia, but up to now, relatively small right shifts of the O_2 binding curves were obtained by incubating human RBC with glycolytic intermediates followed by an accumulation of the non-permanent erythrocyte 2,3-BPG in response to specific stimulation of glycolysis.[6] The O_2 release capacity of RBC can be drastically enhanced by uptake of the non-permanent inositol hexaphosphate (IHP) , which has a 1000- fold higher affinity for Hb,[1] replaces 2,3-BPG at its binding site[6b] and increases the P_{50} of Hb up to values of 96.4 mm Hg at pH 7.4 and 37 ºC.[6c]

The major difficulty of encapsulating IHP on RBC was overcome by using lipid vesicles loaded with IHP in hypertonic IHP solution.[3,7-9,11] The interactions of the vesicles with separated red blood cells (RBC) lead to the penetration of IHP into the RBC[7-11] but the mechanisms of this process were not correctly understood.[8] Nonetheless, on a laboratory scale, loading of the RBC with IHP was achieved and significant "right- shifts" in the Hb- O_2 dissociation curve were obtained.

Fig. 1 shows 41-day-old RBC characterized by a P_{50} value of 15.5 mm Hg (measured at pH and 37 ºC) were incubated with different amounts of IHP loaded in liposomes. After IHP incorporation the P_{50} values are 58 and 98 mm Hg, respectively. The "right shifts" of the two types of IHP-RBC correspond to 56 and 100% IHP-Hb in the treated RBC. The O_2 release of the non-treated RBC is 17% of the total O_2 transport capacity, whereas the IHP-RBC are characterized by an O_2 release of 47% (56% IHP-Hb) and 43% (100% IHP-Hb), respectively. The O_2 tension ranges from 100 mm Hg in the lungs to an O_2 tension of 30 mm Hg in the brain. Thus at 37 ºC a right shift up to P_{50}-58 mm Hg leads to an optimum increase of the O_2 release in the periphery by about 280%.[3]

In Fig. 2, O_2 binding curves of RBC having incorporated increasing amounts of IHP are shown at 25 °C and pH 7.4 in the absence of CO_2. It becomes obvious that only the O_2 binding curve of non-treated RBC and that with 100% IHP-Hb are symmetrical with Hill coefficients of 3.2 and 2.7, respectively. Curves which correspond to RBC with fractions of IHP-HB <100% are non-symmetrical and biphasic. These binding curves of treated RBC are in agreement with those calculated on the basis of a mixture of two HBs, namely BPG-HB with P_{50}=11-6 mm Hg and n=3.2 and IHP-HB with P_{50} 40.9mm Hg and n=2.7. Therefore, the characteristic shape of the experimental O_2-binding curves of the RBC can be used to determine the amount of IHP-HB which is formed in the cells after IHP incorporation.

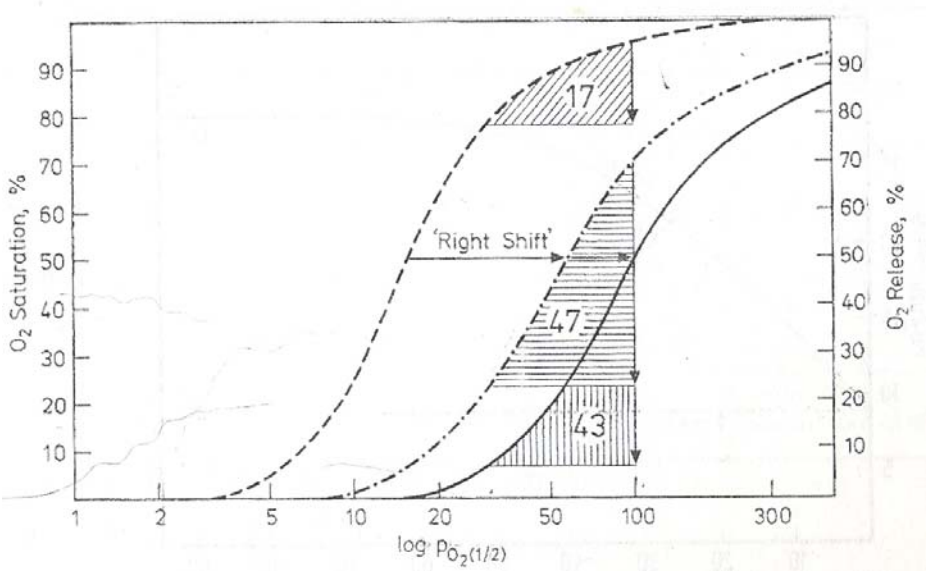


Fig. 1 – "Right-shift" of the O_2–binding curve or RBC after incorporation of IHP. P_{50} was measured at 37 ºC and pH 7.4 in the absence of CO_2. O_2 release: 41-day-old RBC (----), 17%; IHP-RBC with 56% low-affinity Hb(-.-), 47%; IHP-RBC with 100% low affinity (Hb(-----), 43%.[3]

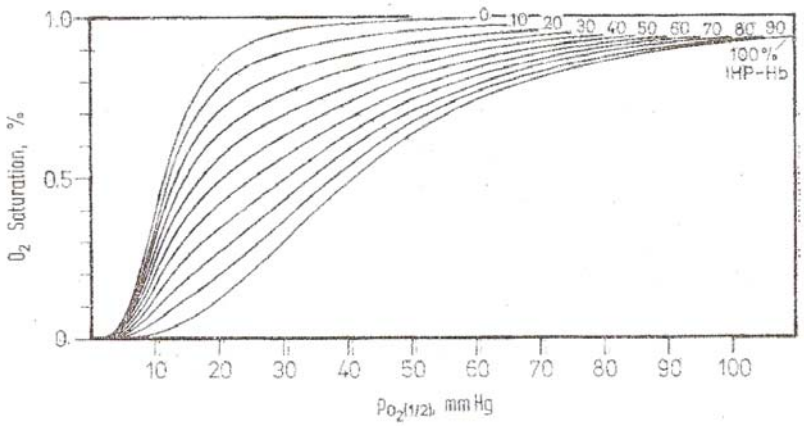


Fig. 2 – Right-shifts of the O_2 binding curves of human RBC after incorporation of increasing amount of IHP measured at 25 degree C and pH 7.4 without CO_2 (5).

IHP-HB enhances the Bohr proton release during O_2 binding. Loading of RBC with IHP up to 77% leads to an approximately threefold increase of the Bohr proton release ($\Delta P_{50}/\Delta pH$=1.20 protons/mole O_2).[5] The increase of the Bohr proton uptake by deoxygenated IHP-HB in RBC is identical with that measured for free IHP-HB in solutions.[9] This result further indicated that the O_2 transport via bicarbonate formation is also enhanced.[5]

Despite these results the incorporation of IHP into red blood cells via interaction with liposomes loaded with IHP, suspended in IHP solutions has many drawbacks like:

1. Poor reproducibility of the IHP concentrations incorporated in RBC
2. Significant hemolysis of the red blood cells
3. A tedious and complicated procedure

Together with C. Ropars and M. Chassaigne[12] we have devised and realized a procedure of IHP incorporation into RBC by controlled lysis and resealing, which avoids the RBC-liposomes interactions, yields high encapsulation levels, is exactly reproducible and easy to perform. The procedure is describ in detail by Ropars *et al.*[13]

HIGH P_{50} RBC

While 2-3 bisphosphoglycerate (BPG) is the normal physiological effector in mammalian

erythrocytes, phosphorylated inositols are found in the erythrocytes of birds and reptiles. Since IHP is unable to pass through the erythrocyte membrane we have introduced here a continuous method for incorporating IHP into human or pig RBC through a reversed osmotic lysis process.[12] When compared to liposomal incorporation the main advantages of the present continuous flow procedure are: larger scale transformation, control of sterility and pyrogen, reproducibility, and the use of readily available commercial equipment and reagents. Warner[10] and Franco *et al.*[14] described a method for incorporating this polyphosphate by using dimethylsulfoxide (DMSO) as a lysing agent. This procedure obtained high P_{50} values (45 mm Hg instead of 23mm Hg for basal P_{50} value), but was accompanied by the loss of 43% hemoglobin from the cells, which decreased the performance in terms of oxygen binding and oxygen transport. The toxicity of DMSO has still to be considered. As mean cell hemoglobin content and oxygen binding capacity were found unchanged when resealed IHP- RBC were compared to the control RBC, no hemoglobin was apparently lost from the individually released RBC. With P_{50} measured in the range 35 to 80 mm Hg (under standard conditions) the method appeared efficient and reproducible in human and pig blood. As already shown by Benesch *et al.*[1] in hemoglobin solution, the IHP fixation on hemoglobin increased the fixed acid Bohr effect.

LYSIS AND RESEALING TECHNIQUE

Lysis and resealing of RBC proved superior to the liposomal methods of IHP (Inositol Hexaphosphate) encapsulation in red blood cells. I present here the method in some detail, as it was used to prepare high – P_{50} RBCs for physiological studies:

Human blood or pig blood, collected on acid-citrate-dextrose solution, was stored at 4 degrees C for no longer than 5 days. After centrifugation of one blood unit (1,000 g for 10 min), the plasma was collected and the erythrocytes were washed with 0.15 M NaCl solution at 4 ºC. The supernatant and buffy coat were discarded. Preliminary experiments have shown that it is of value to eliminate the polymorphonuclear cells to avoid microaggregate formation during massive blood transfusion in the pig. This was obtained through absorbent-cotton filtration before the washing steps (erypur system, Organon West Orange, NJ). The polymophonuclear content was thus lowered to <5% of the basal value. The erythrocyte suspension was then washed twice more with chilled 0.15 M NaC1 solution. For the treatment of human erythrocytes two further Washington solutions were alternatively used. In the first one, the sodium salt of inositol hexaphosphate (IHP-12Na; Sigma) was neutralized to pH 7.40 using 1 M hydrochloric acid and finally adjusted to 260 mosmol/L. This solution (vol/vol) was used to wash the human erythrocytes before the following lysing and resealing steps. To obtain a very large Increase of the P_{50} (>50 Torr), it was necessary to increase the IHP concentration in the last washing step. This was performed by neutralizing the IHP-12 Na solution at pH 7.4 using an IHP acid solution instead of HC1. This IHP acid solution was prepared through ion exchange chromatography on a Dowex 50-W column (H+form). Similarly, the final osmolarity was adjusted to 260 mosmol.1-1. By adjusting the IHP concentration, it was thus possible to vary the IHP encapsulation in the human erythrocytes and obtain P_{50} values between 30 and 80 Torr. Pig erythrocytes were similarly modified using the same IHP solutions; however, the final osmolarity was adjusted to 330 mosmol. After washing the erythrocyte suspension with one of the previous IHP solutions, spinning erythrocytes at 1,000 g for 10 min, decanting the supernatant, and adjusting the final hematocrit to 70%, the lysis of the erythrocytes was performed as follows.[15] The erythrocyte suspension, cooled to 4 ºC, flowed continuously into the blood compartment of a hemodialyzer (Gamro, Minor; dialysis surface, 0.41 m2; membrane thickness, 13.5 um) with the use of a peristaltic pump. The erythrocyte flow rate was adjusted to 20-60 mL/min. The hemodialyzer was fed at a 500 mL/min. flow rate using a low ionic strength buffer (pH 7.4) at 4 ºC containing (in mol.1-1): Phosphate, 0.01; sodium bicarbonate, 0.01; glucose, 0.002. The erythrocytes were carefully lysed during this dialysis step and collected at 37 ºC before being resealed through the addition (1:10 vol/vol) of a hypertonic solution containing (per liter) 1 M chloride salt with a K-toNa-ration of 8.3 to maintain a high ATP content in the resealed erythrocytes.

Total intraerythrocytic phosphate concentration was determined before and after the IHP incorporation procedure. The method used was that of Ames and Dubin[9] with perchloric acid deproteinization and ashing to mineralize the organic phosphates. The difference between the

two phosphate concentrations was considered to be the intracellular IHP concentration.

Hematological parameters were measured for the cells subjected to IHP encapsulation procedure and for the control cells; *i.e.*, fresh RBCs without any treatment[15] as shown in Table 1.

Fig. 3 illustrates the changes in P_{50} obtained by trapping IHP in resealed human erythrocytes. From a value of P_{50} = 20.6 torr (3 day old blood), this procedure allowed to increase of P_{50} within a large range (36.8-77 torr (mmHg) at pH = 7.4, 37 ºC).[15]

Despite the improvement of IHP encapsulation brought about by lysis and resealing of RBCs in the presence of IHP solutions, serious drawbacks affected the methods. 1) Loss of significant volume of blood during the procedure. 2) Elaborate and tedious techniques, sterility control, etc. 3) Relatively modest final volumes of IHP-RBC could be processed.

For these reasons, we developed a continuous-flow electroporation technique which could process a unit of blood in 90 minutes, with very minimal loss of blood.[16]

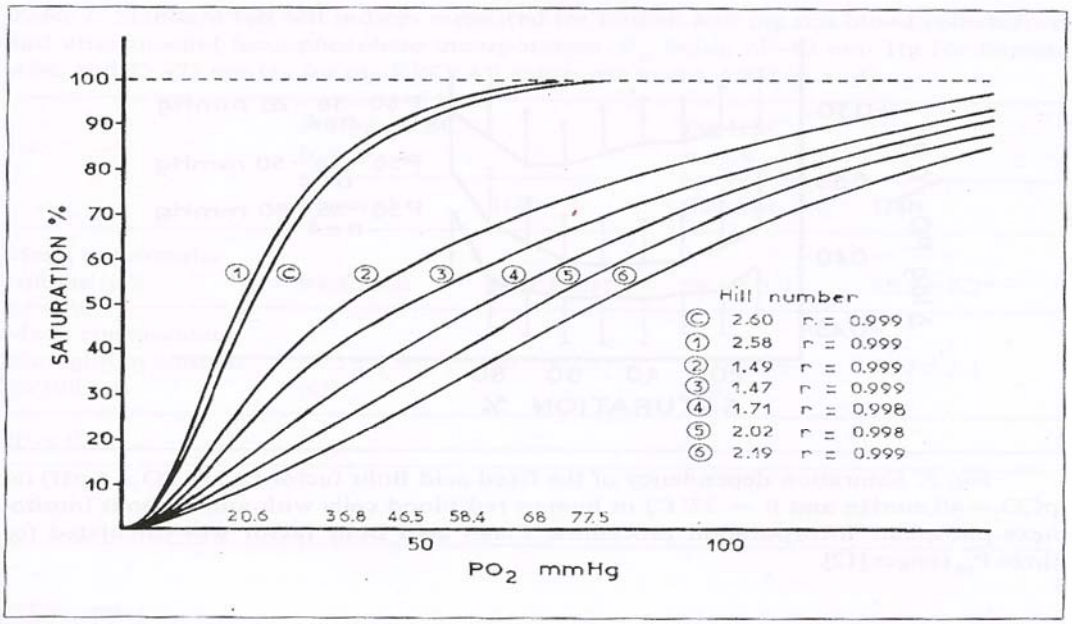
Levels of ATP and cellular energy metabolism were unchanged in the resealed cells using glucose (2 mm) in the resealing/washing solution.

The encapsulation of IHP into RBC by different variations of static electroporation has been reported.[17] Whereas the incorporation of IHP and the stability of the RBC was satisfactory, the volumes processed remained quite small.

Therefore, we designed an electroporation chamber shown in Fig. 4, for an electroporation device, meant to process relative large volumes of blood.

Table 1

	Human (4)		Pig (2)	
	Control	Resealed	Control	Resealed
Mean All Volume (3)	94.3+4	84+/- 3.1	59	52.2
Mean all HB (g/100 mL)	32.2+/- 1.9	30+/-2.5	31	32.2
Red distribution width (%)	15.4 +/-2.8	23.1 +/-7.2	21.3	29.2



Changes in affinity obtained in human red blood cells. Curves 2 to 6 represent affinity curves drawn from RBC which have been subjected to the inositol hexaphosphate (IHP) incorporation procedure [11]. Curve 1 represents RBC subjected to the same procedure but without IHP incorporation. Curve C is the control curve. Oxygen dissociation curves were drawn in standard conditions, (pH 7.40, PCO_2 = 40 mm Hg, θ = 37°C). The corresponding Hill number was calculated from the slope of a straight line between 30 and 70% saturation on log Sat/100 sat versus log PO_2 relationship [12].

Fig. 3

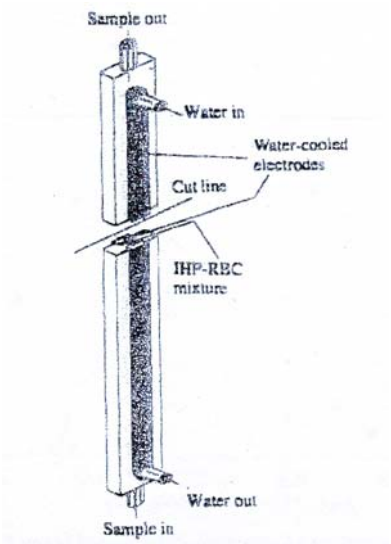
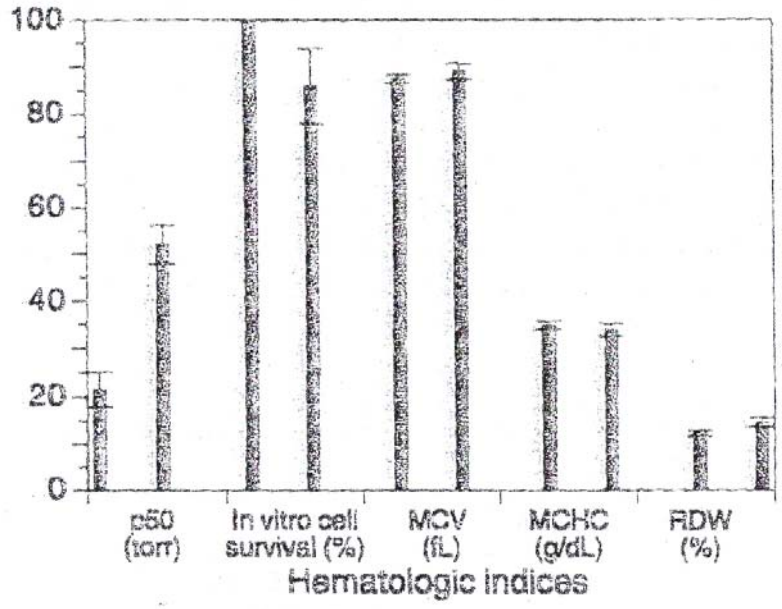


Fig. 4 – Flow electroporation chamber for the encapsulation of IHP to RBC.[16]

This chamber was developed in our laboratory for the purpose of incorporating IHP into RBCs. It consists of two parallel electrodes made of gold-plated, hollow brass tubes with a rectangular cross-section. The electrodes are set in a plastic block with a central channel, open at both ends, for the passage of the cellular mixture in contact with the electrode surfaces. The internal volume of the chamber is 1.8 mL and the interelectrode gap is 3 mm. Cold water (2-5 ºC) from a circulating water bath (RTE-210 Neslab, Newington NH) is pumped through each of the hollow electrodes to cool them and the cellular mixture. The chamber is held in a vertical position and the IHP and RBC mixture is pumped in the bottom and out of the top of the central channel to allow easy passage of any bubbles in the mixture out of the chamber. The cooling water is pumped through the electrodes in the direction opposite that o f the cell suspension, so as to provide an efficient countercurrent heat exchange.

The electrodes are connected to a repetitive-pulse, exponential-decay pulse generator (T720rx, modified with a 300-uF [microfarad] capacitance, BTX Electronics, San Diego CA). An in-line timer switch (CKK-30-461, National Controls Corp) controls the relays between pulses.

The hematological indices are nearly identical, between electroporated RBC and untreated controls,[16] as seen in the figure below.



Hematology and p50 values for IHP-loaded human RBCs (▩) and control RBCs (▨) after 24 hours' incubation at 37°C in autologous plasma. The electroporation was done in a static system with three pulses per sample applied with a delay of 3.4 seconds. The average field strength was 2.76 ± 0.04 kV per cm, and the average pulse length was 1.77 ± 0.13 msec. MCV = mean corpuscular volume; MCHC = mean corpuscular hemoglobin concentration; RDW = RBC size distribution width.

Fig. 5

This new technique provided reproducible, efficient results free of the shortcomings of the lysis and resealing method. However, it was a complex, ex vivo method and as such subject to possible contaminations, etc.

The RBCs loaded with IHP showed significant physiological effects.[15,18]

Isovolumic exchange transfusion of high P_{50} RBCs in anesthetized and ambient air-ventilate piglets led to substantial in vivo P_{50} increases. The "right-shift" of the oxyhemoglobin dissociation curve occurred simultaneously with an increase in the arterial PO_2 and of the arteriovenous content difference, 19 and 59 % respectively above their control value. The mixed venous PO_2 (PvO_2) remained unchanged. The cardiac output was shown to be inversely related to the P_{50} value. In spite of the O_2-transport reduction (37%) O_2 consumption was maintained due to enhanced O_2 extraction.[15]

Similar observations were made over a period of 30 days.[18] The life span of the loaded RBC equaled that of control RBCs. The long-term physiological effects of IHP=RBC in piglets were increased O_2 – release and reduced O_2-affinity of the RBCs which were still effective 20 days after transfusion.

These observations strongly suggested that modifying the O_2-affinity of hemoglobin might constitute a future treatment for congestive heart failure, etc. However, the medical community was reluctant to use the complicated incorporation technology of IHP in RBC, where the ex vivo aspects played an important role.

In an attempt to find a similar allosteric effector of hemoglobin, but capable to cross the RBCs' plasma membrane and be non-toxic, we started a collaboration with Professor Jean-Marie Lehn at the University of Strasbourg.

The collaboration proved very fruitful: short time after its inception, J-M Lehn and K Fylaktakidou in Lehn's laboratory synthesized myo-inositol-tris-pyrophosphate[19] which proved to be a strong allosteric effector of hemoglobin. In reaction with free hemoglobin the compound (ITPP) shifted to higher O_2 pressure the value of P_{50} by 225+/-19 %.[19] The very important observation made was that ITPP could cross the plasma membrane of RBC in vitro and was completely non-toxic, in vivo. It binds, with a relatively high affinity to Band 3 on the RBC plasma membrane, with significant consequences.[23]

We conducted extensive biological studies with the new molecule. Results – similar to those obtained with IHP loaded in vivo were obtained with ITPP in congestive heart failure.[20]

It could be demonstrated that ITPP via its effects regarding P02 was an inhibitor of angiogenesis[21,22] and, most importantly, in animal models it proved to be a strong anti-cancer agent.[24-27] At present, a Phase II Clinical Trial of ITPP as an anti-cancer drug is underway at the University Hospital, University of Zurich, Switzerland.

This, however, is another story!

CONCLUSIONS

Several methods and techniques have been devised in order to encapsulate Inositol Hexaphospate – a strong allosteric effector of hemoglobin, in red blood cells.

Several of the methods reviewed yielded RBCs, loaded with IHP having unchanged life spans and hematological indices, despite the high P_{50} achieved.

Red Blood Cells loaded with IHP showed strong beneficial physiological effects in animal (pig) models *e.g.*, reduction of the cardiac output at normal oxygen consumption

A novel allosteric effector of HB,myo- inositol-tris-pyrophrophate has been identified. It shows strong anticancer activity in animal models and is now in a Phase II clinical study as anticancer agents in patients with cancers of the biliary tract.

Abbreviations:

Bohr Effect: Hemoglobin's oxygen binding affinity is inversely related both to acidity and to CO_2 concentration.
BPG: 2,3-bis phosphoglycerate
HB: Hemoglobin
IHP: myo-Inositol Hexabphosphate
ITPP: myo-Inositol Trispyrophosphate
ODC: oxyhemoglobin dissociation curve
P_{50}: half saturation oxygen pressure
RBC: red blood cell.

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