ABSTRACT

The oxygen affinity of red cells increases stepwise with temperature reductions below $37^\circ C$. In vitro studies demonstrated that biochemically modified red cells with increased 2,3-diphosphoglycerate (2,3 DPG) (150% and 250% of normal) exhibited significantly less oxygen affinity at $24^\circ C$ than did unmodified cells. At $15^\circ C$, significant attenuation of affinity was observed with 250%, but not 150%, of normal 2,3 DPG cells.

Measurements made of isolated fibrillating dog hearts during perfusion at $24^\circ C$ alternately with unmodified (80% of normal 2,3 DPG) and modified (300% of normal 2,3 DPG) red cells demonstrated significantly greater oxygen consumption, higher coronary sinus partial pressures of oxygen and carbon dioxide, higher in vitro $P_a$ values, and lower arterial and coronary sinus lactate levels during perfusion with modified as compared with unmodified cells. This evidence, indicating improved oxygen delivery to hypothermic dog hearts by red cells with 300% of normal 2,3 DPG activity, suggests that high 2,3 DPG cells might protect myocardial tissue in patients undergoing hypothermic cardiac operation.

Hypothermia and potassium-containing cardioplegic solutions with or without blood have been used to protect the myocardium during open-heart operations [1, 8–23, 26–33, 36, 37, 42–45]. Hypothermia protects the heart during cardiac operation by reducing myocardial oxygen demand. On the other hand, hypothermia also reduces the availability of oxygen to the myocardium by increasing the oxygen affinity of the red blood cells. This affinity for oxygen is increased further by increases in blood pH and reductions in partial pressure of carbon dioxide (PCO$_2$) during hypothermia [35]. What has been regarded in general as a reduction in oxygen consumption during hypothermia may actually be a reduction in oxygen availability.

Studies have shown that increasing the levels of 2,3 diphosphoglycerate (2,3 DPG) and adenosine triphosphate (ATP), especially 2,3 DPG, attenuates the increased affinity of red blood cells for oxygen at low temperatures [2, 24]. The initial experiments in the present study were designed to evaluate the relation between 2,3 DPG level and red cell oxygen affinity at various temperatures. In vitro studies were carried out on human red blood cells with 2,3 DPG levels approximately 70%, 150%, and 250% of normal at temperatures of $37^\circ C$, $24^\circ C$, $15^\circ C$, and $10^\circ C$. In addition, isolated hypothermic dog hearts at $24^\circ C$ were perfused with red blood cells with 80% of normal (nonrejuvenated) and 300% of normal (rejuvenated) 2,3 DPG levels, in the course of which arterial blood and coronary sinus blood were serially tested for partial pressure of oxygen (PO$_2$), PCO$_2$, pH, lactate concentration, oxygen content, and oxygen consumption.
Materials and Methods

In Vitro Studies of Oxygen Affinity

The oxygen affinity of human red cells with 70%, 150%, and 250% of normal 2,3 DPG was measured at different temperatures. Three units of O-positive citrate-phosphate-dextrose (CPD) anticoagulated blood were obtained from healthy human volunteers. The red blood cells were concentrated to a hematocrit of 75% vol per 100 ml and stored at 4°C for 6 to 8 days. The 3 units were then pooled and immediately separated into three equal portions. One of the three portions was incubated at 37°C for 1 hour with 50 ml of PIGPA Solution A, a rejuvenating solution containing 50 mmole/L of pyruvate, 50 mmole/L of inosine, 100 mmole/L of glucose, 50 mmole/L of disodium phosphate, 5 mmole/L of adenine, and 9 gm/L of sodium chloride, pH 7.2, producing an increase in 2,3 DPG to approximately 150% of normal. The second portion, similarly treated with 50 ml of PIGPA Solution B, containing 100 mmole/L of pyruvate, 100 mmole/L of inosine, 100 mmole/L of glucose, 200 mmole/L of disodium phosphate, 5 mmole/L of adenine, and 5 gm/L of sodium chloride, pH 7.2, had a 2,3 DPG level approximately 250% of normal. The third portion, serving as control, biochemically unmodified, had a 2,3 DPG level approximately 70% of normal.

Each of the 3 units of red cell concentrate was glycerolized to a final concentration of 40% w/v and then divided into three further 130 ml aliquots, making a total of nine aliquots. Each aliquot was frozen and stored at -80°C in a mechanical refrigerator, storage periods ranging from a few days to 6 months.

On three separate occasions during storage, one aliquot from each unit was removed from the refrigerator for evaluation. Each aliquot was thawed and was washed sequentially with 12% sodium chloride, 1.6% sodium chloride, and a solution containing 0.9% sodium chloride, 0.2% glucose, and 25 mEq/L of disodium phosphate. A modification of the washing procedure for the IBM Blood Processor 2991-1, as previously described [38], was used.

The in vitro oxygen tension at which 50% of the hemoglobin was saturated was determined in triplicate at 37°C, 24°C, 15°C, and 10°C by modifications of the spectrophotometric technique of Bellingham and Huehns [3]. This value is referred to as the in vitro P50. The temperatures were controlled both in the water bathing the tonometer for the washed red blood cells and in the cuvette holder of the spectrophotometer. The P50 in the tonometer at the time of measurement was calculated from the standard gas law. The 2,3 DPG and ATP levels were determined as previously described [38].

Perfusion of Isolated Hypothermic Dog Hearts with Human Red Cells Containing 80% and 300% of Normal 2,3 DPG

Human red blood cells were used to perfuse the dog hearts in this study. This is an acceptable procedure as long as the dog plasma is first removed to avoid heterophile erythroagglutination.

Approximately 450 ml of blood was collected from each healthy O-positive volunteer into 63 ml of CPD anticoagulant in a triple plastic-bag collection system,* then centrifuged in an RC-3 centrifuge† at 3,700 revolutions per minute (3,550 × g) for 10 minutes. The red blood cells were concentrated to a hematocrit value of 75% vol per 100 ml and stored for 3 to 5 days at 4°C. The stored red cell concentrates were divided into two equal portions. One portion was untreated and served as control. The other half was biochemically modified with PIGPA Solution B [39]. Modification of red cell concentrates was similar to that of the in vitro studies except that the pretreatment storage period was 3 to 5 days instead of 6 to 8 days. Both the modified and control preparations were frozen with 40% w/v glycerol and stored at -80°C for periods of at least a year. Prior to the perfusion studies, the red cells were thawed, washed, and suspended in an albumin-crystalloid solution. Thawing took about 10 minutes at 37°C, and sodium chloride solutions were used for washing the red cells in the Haemonetics blood processor with the original disposable polycarbonate bowl [38]. The washed unit was concentrated by centrifugation, and all the supernatant was removed. The

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†Dupont Instruments, Newtown, CT.
red blood cell concentrate was diluted with the albumin-crystalloid solution used to flush the dog blood from the coronary circulation, and the hematocrit of the red blood cell–albumin–crystalloid solution was adjusted to 30 vol per 100 ml.

Each mongrel dog was sedated with 30 mg of phencyclidine hydrochloride (Sernylan) and 130 mg of pentobarbital. Following endotracheal intubation for mechanical ventilation, the heart was exposed through a median sternotomy. The brachiocephalic artery was cannulated. The heart was isolated by dividing the superior vena cava, inferior vena cava, azygos vein, pulmonary artery, and the thoracic aorta distal to the brachiocephalic trunk. In order to prevent agglutination of the human red blood cells by canine plasma, the dog blood was washed from the coronary circulation by perfusion of the aortic root with an isotonic solution of 50 gm/L of albumin, 2.1 gm/L of sodium bicarbonate, 5 gm/L of sodium chloride, 300 mg/L of potassium chloride, 1 gm/L of glucose, 11.2 mg/L of monobasic potassium phosphate, 48 mg/L of dibasic sodium phosphate, 100 mg/L of calcium chloride, maintained at 5°C at 100 ml per minute for 15 minutes. The heart was excised and placed in an extracorporeal circuit (Fig 1) consisting of a Buckner reservoir, a calibrated roller pump, a Travenol disposable heat exchanger, and a Travenol membrane oxygenator (model 5M-0936). Three gas tanks were connected to the circuit and, through adjustable flow valves, delivered 100% oxygen, 5% carbon dioxide–20% oxygen–75% nitrogen, and 3% carbon dioxide–20% oxygen–77% nitrogen. The circuit was completed by Tygon tubing. The left and right ventricles were vented by stab wounds, and a needle thermistor was inserted into the interventricular septum to monitor myocardial temperature. Aortic root perfusion pressure was displayed continuously.

After the canine blood was washed out, the mechanical pump was primed with 2 units of blood, and each isolated dog heart was perfused at 100 ml per minute for 1 hour with 2 units of nonrejuvenated or rejuvenated red blood cells. The system was then flushed for an hour through a new, separate circuit with 700 ml of the other type of cell suspension (rejuvenated or nonrejuvenated). This was followed by a third hour of perfusion with the second blood prime. In 2 of the fibrillating hearts, the nonrejuvenated red blood cells (80% of normal 2,3 DPG) were used during the first hour, followed by rejuvenated cells (300% of normal 2,3 DPG). In the other 2 fibrillating hearts, rejuvenated
red blood cells were perfused first, followed by nonrejuvenated blood. The $P_{O_2}$, $P_{CO_2}$, and pH of the perfusate were controlled at physiological levels by the introduction of appropriate gas mixtures and addition of sodium bicarbonate as required. The temperature was maintained at 24°C during the entire experiment.

Samples were obtained every 15 minutes during the first and third hour of the experiment, but not during the washout period, from the aortic root and from the coronary sinus blood as it exited from the right ventricular vent. Supernatant hemoglobin, extracellular potassium ion, and red blood cell 2,3 DPG and ATP were measured as previously described [38]. Arterial and coronary sinus $P_{O_2}$, $P_{CO_2}$, and pH were measured at 37°C in the IL-313 Blood Gas Analyzer* and the values were corrected to 24°C using the Severinghaus nomogram [35]. Total hemoglobin was measured by the cyanmethemoglobin procedure in a Coleman spectrophotometer. The percent oxyhemoglobin and percent carboxyhemoglobin were measured in the IL-182 Co-Oximeter.* The oxygen content in arterial and coronary sinus blood was measured in the Lex-O2-CON fuel cell† and calculated from the total hemoglobin, the percent oxygen saturation, and the dissolved oxygen [41]. The dissolved oxygen was calculated from the $P_{O_2}$ at 24°C and the solubility constant of 0.0042 at 24°C [34].

Upon completion of perfusion, the isolated heart was weighed, and oxygen consumption was calculated as ml/min/100 gm of myocardial tissue from the arteriovenous difference in oxygen content multiplied by coronary blood flow, which was controlled by the calibrated roller pump. Blood lactate was measured in the arterial blood and coronary sinus blood [25]. The $P_{O_2}$, measured at 37°C and corrected to 24°C, and the percent saturation of available hemoglobin were used to calculate the in vitro $P_{50}$ value by the Hill equation, assuming an $n$ value of the oxyhemoglobin dissociation curve of 2.7 [39, 40]. Lactate production by the red blood cells with 80% of normal 2,3 DPG and with 300% of normal 2,3 DPG was measured after 3 hours of incubation at 37°C in an albumin-crystalloid solution.

**Results**

Baseline measurements made on washed fresh heparinized red blood cells indicated the $P_{50}$ value of the oxyhemoglobin dissociation curve to be $27.0 \pm 1.0$ mm Hg (mean ± standard deviation at pH 7.2, $P_{CO_2} = 0$, and temperature 37°C); carboxyhemoglobin level, 1 to 2%; red blood cell ATP level, $4.5 \pm 1.0$ μmole per gram of hemoglobin; and red blood cell 2,3 DPG level, $13.5 \pm 1.5$ μmole per gram of hemoglobin.

In the in vitro study, red blood cells with 150% of normal 2,3 DPG and 125% of normal ATP and red blood cells with 250% of normal 2,3 DPG and 175% of normal ATP had significantly lower oxygen affinity at 24°C and 37°C than did red blood cells with 70% of normal 2,3 DPG and 90% of normal ATP (Figs 2, 3). At 15°C, red blood cells with 250% of normal 2,3 DPG had significantly lower oxygen affinity than did red blood cells with 70% of normal 2,3 DPG or 150% of normal 2,3 DPG. At 10°C, oxygen affinity was not significantly different among the three groups.

Each of 4 isolated fibrillating dog hearts was perfused with red blood cells with 80% of normal 2,3 DPG (nonrejuvenated) and red blood cells with 300% of normal 2,3 DPG (rejuvenated) (Table 1). After 30 minutes of perfusion, the nonrejuvenated red blood cells had 2,3 DPG levels of 80% of normal and the rejuvenated red blood cells had about 300% of normal 2,3 DPG levels. In all 4 isolated hearts, $P_{O_2}$ and $P_{CO_2}$ were decreased and pH was increased in the arterial and coronary sinus blood when the measurements made at 37°C were corrected to 24°C, the temperature of the isolated heart. Data from the four samplings were combined to calculate mean values for analysis: measurements of arterial $P_{O_2}$, $P_{CO_2}$, and pH and of coronary sinus pH were not significantly different whether nonrejuvenated or rejuvenated red blood cells were perfused (Table 2); measurements of coronary sinus $P_{O_2}$ and $P_{CO_2}$, however, were significantly higher (by about 5 mm Hg) after perfusion with rejuvenated red blood cells.

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†Lexington Instruments, Waltham, MA.
Oxygen consumption was higher after perfusion with rejuvenated red blood cells (see Table 2). Mean values from four samplings indicated that the in vitro $P_{50}$ was significantly higher, arteriovenous oxygen content differences significantly greater, and oxygen consumption significantly higher after perfusion with rejuvenated red blood cells (see Table 2).

At each of four sampling periods, arterial and coronary sinus blood lactate levels were higher after perfusion with nonrejuvenated red blood cells, and when the data from the four sampling periods were combined, the increases were significant (see Table 2). Lactate levels were higher in coronary sinus blood than in arterial blood samples after perfusion with red blood cells containing 80% of normal 2,3 DPG levels. During perfusion with red blood cells containing 300% of normal 2,3 DPG levels, the lactate levels were higher in the arterial blood than in the coronary sinus blood (see Table 2). After incubation of the red blood cells in an albumin-electrolyte solution at 37°C for 3 hours, lactate production was not significantly different between the nonrejuvenated and rejuvenated red blood cells, suggesting that it was the dog's heart that was responsible for the production or utilization of lactate and not the donor red blood cells.

Hemoglobin concentrations in arterial and coronary sinus blood were significantly higher after perfusion with rejuvenated red blood cells because recovery of the red blood cells after the freeze-thaw-wash process was higher in these
Table 1. 2,3 DPG Levels 30 Minutes after Initiation of Perfusion

<table>
<thead>
<tr>
<th>Variable</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perfusion hour</td>
<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2 3</td>
<td>1 2 3</td>
</tr>
<tr>
<td>Perfusate</td>
<td>NR WO REJ</td>
<td>REJ WO NR</td>
<td>NR WO REJ</td>
<td>REJ WO NR</td>
</tr>
<tr>
<td>2,3 DPG (mm/gm Hb)</td>
<td>10.1 ... 38.2</td>
<td>38.0 ... 11.8</td>
<td>10.2 ... 38.1</td>
<td>40.8 ... 12.0</td>
</tr>
</tbody>
</table>

NR = nonrejuvenated red blood cells with 80% of normal 2,3 DPG; REJ = rejuvenated red blood cells with 300% of normal 2,3 DPG; WO = washout between different perfusates.

Table 2. Paired t Test Combining Four Time Periods for the Four Experiments (n = 16)

<table>
<thead>
<tr>
<th>Measurements</th>
<th>300% of Normal 2,3 DPG RBC (Rej)</th>
<th>80% of Normal 2,3 DPG RBC (NR)</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>O$_2$ consumption (ml/min/100 gm heart tissue)</td>
<td>0.96 ± 0.16</td>
<td>0.79 ± 0.2</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AV O$_2$ difference (vol/100 ml)</td>
<td>3.48 ± 0.60</td>
<td>2.87 ± 0.87</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Lactate, arterial (µmole/ml blood)</td>
<td>3.51 ± 1.58</td>
<td>4.85 ± 1.34</td>
<td>&lt;0.03</td>
</tr>
<tr>
<td>Lactate, coronary sinus (µmole/ml blood)</td>
<td>3.40 ± 1.62</td>
<td>5.44 ± 1.46</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Hemoglobin, arterial (gm/dl)</td>
<td>9.96 ± 0.54</td>
<td>9.18 ± 0.55</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Hemoglobin, coronary sinus (gm/dl)</td>
<td>9.92 ± 0.56</td>
<td>9.21 ± 0.52</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Po$_2$, arterial, 24°C (mm Hg)</td>
<td>96.4 ± 29.4</td>
<td>80.9 ± 54.0</td>
<td>NS</td>
</tr>
<tr>
<td>Pco$_2$, arterial, 24°C (mm Hg)</td>
<td>25.5 ± 6.26</td>
<td>22.8 ± 3.53</td>
<td>NS</td>
</tr>
<tr>
<td>pH, arterial, 24°C</td>
<td>7.59 ± 0.09</td>
<td>7.59 ± 0.05</td>
<td>NS</td>
</tr>
<tr>
<td>Po$_2$, coronary sinus, 24°C (mm Hg)</td>
<td>22.94 ± 2.32</td>
<td>17.87 ± 3.64</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Pco$_2$, coronary sinus, 24°C (mm Hg)</td>
<td>22.75 ± 2.18</td>
<td>18.07 ± 3.41</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>pH, coronary sinus, 24°C</td>
<td>7.54 ± 0.16</td>
<td>7.56 ± 0.15</td>
<td>NS</td>
</tr>
<tr>
<td>In vitro Po$_2$, 24°C (mm Hg)</td>
<td>14.08 ± 0.94</td>
<td>9.89 ± 1.56</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

RBC = red blood cells; Rej = rejuvenated; NR = nonrejuvenated; AV = arteriovenous; Po$_2$ = partial pressure of oxygen; Pco$_2$ = partial pressure of carbon dioxide.

Comment

Although the relative merits of cold and potassium during ischemic arrest are debated, it is almost universally recognized that the combination of these two modalities in the form of hypothermic cardioplegia provides the best protection for the myocardium. The most commonly employed method of producing cold cardioplegia is through the use of a cold asanguineous crystalloid solution containing potassium. Recently, however, a blood vehicle has been suggested in order to meet the residual oxygen requirements of the relaxed cold heart. According to Buckberg [9], oxygenated blood has several advantages: (1) it prevents the depletion of high-energy phosphate stores during the short period of electromechanical activity in the fibrillating heart before asystole is achieved; (2) it replenishes ATP stores during the period of ischemic arrest; (3) it obviates the need to add substrate for prolonged anaerobic metabolism; and (4) it provides the oncotic component, thereby eliminating the need for the addition of plasma, mannitol, or dextran to the cardioplegic solution.

The major advantage of a blood vehicle is, however, essentially predicated on the delivery of oxygen to the myocardium. Although hypothermia reduces myocardial oxygen requirements, the cold temperature also decreases oxygen availability to the tissues by raising the oxygen–hemoglobin affinity state. This bond is further enhanced by an increase of blood pH and a reduction of Pco$_2$ during hypothermia. It
is probable that the observed decrease in oxygen utilization during hypothermia may be in part due to reduced oxygen availability secondary to the enhanced affinity state. It is also likely that if the hemoglobin-oxygen bond were weakened, the myocardium would consume more substrate. Thus, the attenuation of the hemoglobin-oxygen affinity state may facilitate higher oxygen utilization and thereby improve myocardial protection during ischemic arrest of the heart.

The increase of red cell 2,3 DPG levels attenuates oxygen affinity and increases oxygen availability to the tissues. The present experiment was designed to study the strength of the bond at various temperatures and to observe the influence of various levels of blood 2,3 DPG on this relationship. Additionally, we also investigated the impact of elevated 2,3 DPG levels on myocardial metabolism in an isolated heart preparation.

The in vitro studies reaffirmed the fact that with decreasing temperatures, the hemoglobin-oxygen bond is strengthened and consequently oxygen availability to the tissues decreases. At 24°C, oxygen availability can be increased by the use of blood containing 150% and 250% of normal 2,3 DPG while at 15°C, only blood with the higher level of 2,3 DPG is effective. At 10°C, however, raising the level of 2,3 DPG in the blood even to the higher levels employed in this study has no beneficial influence on oxygen availability to tissues.

Perfusion of isolated dog hearts with human blood appears to be an acceptable experimental model as long as the dog blood is adequately washed out and heterophile antibody agglutination thereby prevented. Our isolated canine heart experiments demonstrate that, at low temperatures, an increase in 2,3 DPG blood levels facilitates oxygen release to the tissues and the myocardium takes advantage of the improved oxygen availability. Transmyocardial oxygen extraction and oxygen consumption increase. Additional evidence of aerobic metabolism is provided by data suggesting a reduction in arterial and coronary sinus lactate levels and transmyocardial lactate extraction instead of production. It is evident from the present observations that raising the level of 2,3 DPG in cold blood improves oxygen availability to the tissues and that the resultant aerobic metabolism enhances myocardial protection.

Oxygen in physical solution in plasma increases with lowering of temperature, an increase that may offset in small part the stronger hemoglobin-oxygen bond and may improve to some degree the quality of myocardial protection. Hyperbaric oxygen was once, but is no longer, used during surgical repair of cyanotic congenital heart defects to increase the amount of oxygen dissolved in blood and increase oxygen availability [4-7].

The perfusion studies reported in this paper indicate that elevated 2,3 DPG levels exert additive protective influence by further facilitating aerobic metabolism as evidenced by increased oxygen consumption. Our previous studies in man have indicated that the use of high 2,3 DPG blood for transfusion during open-heart operations improved myocardial performance as documented by a favorable shift in left ventricular volume loading function curves [13]. The present experiment in the isolated heart provides more direct evidence for the beneficial influence of red cells with high 2,3 DPG levels in coronary perfusates. Based on this information, we think that increasing 2,3 DPG levels in the blood used as a vehicle for delivery of cardioplegia enhances myocardial preservation, and therefore we have instituted a clinical study using a blood cardioplegic agent with a high 2,3 DPG content.

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