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## Perfusion vs. oxygen delivery in transfusion with “fresh” and “old” red blood cells: The experimental evidence

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### Abstract

We review the experimental evidence showing systemic and microvascular effects of blood transfusions instituted to support the organism in extreme hemodilution and hemorrhagic shock, focusing on the use of fresh vs. stored blood as a variable. The question: “What does a blood transfusion remedy?” was analyzed in experimental models addressing systemic and microvascular effects showing that oxygen delivery is not the only function that must be addressed. In extreme hemodilution and hemorrhagic shock blood transfusions simultaneously restore blood viscosity and oxygen carrying capacity, the former being critically needed for reestablishing a functional mechanical environment of the microcirculation, necessary for obtaining adequate capillary blood perfusion. Increased oxygen affinity due to 2,3 DPG depletion is shown to have either no effect or a positive oxygenation effect, when the transfused red blood cells (RBCs) do not cause additional flow impairment due to structural malfunctions including increased rigidity and release of hemoglobin. It is concluded that fresh RBCs are shown to be superior to stored RBCs in transfusion, however increased oxygen affinity may be a positive factor in hemorrhagic shock resuscitation. Although experimental studies seldom reproduce emergency and clinical conditions, nonetheless they serve to explore fundamental physiological mechanisms in the microcirculation that cannot be directly studied in humans.

### Indexable words

Red blood cell storage; microcirculation; capillary perfusion; blood viscosity; blood transfusion

### Introduction

Blood transfusions are used in multiple scenarios associated with acute blood loss that leads to the impairment of oxygen delivery to the tissue. The principal problem being treated by transfusion is insufficient oxygen delivery due to the deficit of oxygen carrying capacity

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arising from decreased hemoglobin (Hb) concentration, persisting after volume resuscitation by plasma expansion restores the major transport functions to the circulation by increasing blood pressure and cardiac output. In this scenario, blood transfusion is used primarily to improve oxygen transport and secondarily to treat hypotension. A blood transfusion intrinsically restores oxygen transport capacity, since blood collection, storage and transfusion do not affect the inherent biochemical properties of hemoglobin. However, while oxygen transport capacity is invariably restored by a blood transfusion, this is not the only critical function of blood, a realization that has only recently emerged.

Blood and in particular blood flow, sets the mechanical environment of the circulation, a multi-level phenomena that modulates conditions from major blood vessels to the microcirculation. The mechanical properties of flowing blood significantly affect its biochemistry as well as the coagulation inflammatory cascades. A blood transfusion is an acute intervention, implemented to solve life- and health-threatening conditions on a short term basis, and in general its long term effects tend to be of secondary importance. However, blood transfusion does not always accomplish the intended clinical goal. Indeed, there is a well-known association between multiple transfusions and mortality, although whether this is a causal relationship, or transfusion is a surrogate indicator of mortality risk has not been established<sup>1-4</sup>. These poor outcomes are probably not directly related to the immediate goal of blood transfusion of restoring oxygen transport capacity, but are likely due to the failure to adequately maintain microvascular function, certainly in the short term, but which conceivably could cause damage in the long term.

A critical question is whether the short and long term effects of blood transfusion are intrinsic to the process, or are exaggerated by blood storage. The answer to these questions is difficult and would require objective clinical trials based on a material, blood, which is non-uniform in source and composition. A less satisfactory alternative is resorting to experimental studies in animal species, which can be configured to create a supply of relatively homogeneous blood, but do not fully reproduce clinical conditions or replicate human physiology. Regardless of these obstacles, experimental studies remain at present a valid methodology for analyzing fundamental physiological and biophysical mechanisms that should be operational in most mammalian species, particularly in acute conditions. In what follows, we analyze the available experimental evidence that deals with the effects of transfusion in relation to the time of storage of the transfused blood.

## What does a blood transfusion remedy?

The organism receiving a blood transfusion is usually in hemodynamic and metabolic state different from normal. Two conditions that have been analyzed experimentally are acute isovolemic anemia (hemodilution) and hemorrhagic shock. In principle a blood transfusion in extreme anemia addresses the deficit of oxygen carrying capacity, while the treatment of hemorrhagic shock implies the additional restoration of blood volume. Analysis of these conditions shows a perspective on functional requirements for blood transfusions that can be discerned from experimental studies.

### a) Critical functions of blood transfusion in extreme anemia

Blood transfusions are instituted upon the appearance of clinical signs of hypoxia or hypovolemia and reaching the “transfusion trigger” level of Hb concentration. In the setting of acute blood loss, this point is usually reached after the deployment of plasma expanders to maintain blood volume has reduced blood Hb concentration to a point that there is an increased risk of tissue damage and eventually mortality. The decision is usually supported by evaluation of additional parameters, such as base excess and lactate concentration. In this scenario the actual level of tissue oxygenation is only known through indirect

measurements. Furthermore the physiological condition of the tissue is significantly different from normal, because as Hb is reduced by hemodilution, microvascular function is progressively impaired, jeopardizing tissue survival due to the local microscopic maldistribution of blood flow as shown experimentally in the hamster window chamber model by Tsai *et al.*<sup>5</sup>. These effects take place at Hb concentrations greater than those defining the oxygen supply limitation.

Experimental studies show that microvascular function can be maintained in extreme hemodilution by increasing either blood or plasma viscosity<sup>5-7</sup>. This effect can be achieved experimentally using non-conventional, high viscosity plasma expanders<sup>8</sup>. Restoration of blood viscosity during hemodilution and hemorrhage is desirable, because it maintains functional capillary density (FCD), defined as the number of capillaries with passage of RBCs per unit surface of the field of view of a microscopically observed tissue. This microvascular parameter was found to be critical in sustaining tissue survival by Kerger *et al.*<sup>9</sup>, who showed the direct correlation between maintenance of FCD above a specific threshold and survival in extended hemorrhagic shock. FCD is also determined by the maintenance of capillary pressure, which in extreme hemodilution can be obtained using high viscosity plasma expanders<sup>5</sup>.

The blood viscosity threshold that causes the decrease in FCD appears to coincide with the critical value of Hb concentration below which oxygen consumption becomes supply limited. Thus the “transfusion trigger” may also be a “viscosity trigger”, and some of the results obtained with a blood transfusion may also be achieved by increasing plasma viscosity.

#### **b) Critical functions of blood transfusion in shock resuscitation**

The hamster chamber window model has provided unique opportunities to simultaneously study systemic and microvascular effects associated with the effects of blood transfusion and resuscitation, and comparing different strategies, including the effects of blood storage.

Baseline studies related to transfusions in shock resuscitation compared effects of using Ringer’s lactate, 70 kDa dextran and blood.<sup>10</sup> These studies employed a standard shock model induced by stepwise hemorrhage of the hamsters of 50% of their blood volume, which lowered the mean arterial blood pressure (MAP) to 40 mmHg. Resuscitation was implemented with 50% volume restoration with shed blood or dextran, or 100% volume restoration with Ringer’s lactate. As expected, fresh blood provided a significantly better initial recovery of all parameters, particularly MAP and FCD which were restored immediately. All fluids led to full hemodynamic restoration at 24 hr; however 70 kDa dextran and Ringer’s lactate caused prolonged flow impairment and tissue hypoxia.

The role of restoration of blood viscosity by blood transfusion, independently from the restitution of oxygen carrying capacity and volume, was analyzed by using RBCs whose oxygen carrying capacity was eliminated, either by saturating Hb with carbon monoxide (CO)<sup>11</sup> or by converting it to metHb<sup>12</sup>. Using the hamster model system described above, 50% of the animals’ blood volumes were removed and resuscitation was carried out 1 hr after hemorrhage with a single infusion of 25% of the blood volume with untreated, fresh RBCs, or RBCs treated with CO suspended in human serum albumin. Systemic and microcirculatory recovery were identical for resuscitation with native RBCs or RBCs which could not carry oxygen both initially (5 – 10 min) and up to 90 min after resuscitation. CO concentration decreased over 90 min, increasing O<sub>2</sub> carrying capacity and gradually re-oxygenating the tissue<sup>11</sup>.

The oxygen carrying capacity of RBCs was also inactivated by converting their Hb to metHb by exposure to nitrate<sup>12</sup>. The same hemorrhage-resuscitation experiment as described above was performed using fresh RBCs and hydroxyethyl starch (10% HES) as controls. Resuscitation with RBCs with or without oxygen carrying capacity both resulted in a greater MAP than in the starch resuscitation group. FCD was substantially higher for RBC transfusions ( $56 \pm 7\%$  of baseline) vs. starch ( $46 \pm 7\%$  of baseline), and the metHb RBCs had the same effect on FCD and microvascular hemodynamics as untreated RBCs. As expected, oxygen delivery and extraction were significantly lower for resuscitation with hydroxyethyl starch and metRBCs compared to oxygen carrying RBCs. Systemic and microvascular conditions after volume restitution with starch were notably worse than with RBC related recovery<sup>13</sup>. There was a minimal difference in MAP between the two types of RBCs: MAP was about 10 mm Hg higher with normal RBCs.

These studies show that resuscitation from hemorrhagic shock can be achieved by volume restoration with a fluid having rheological properties similar to those of blood, independent of its oxygen carrying capacity. This is relevant to the transfusion of stored RBCs, which only moderately raised oxygen delivery capacity upon transfusion due to the Hb oxygen dissociation curve being left shifted. However, it is a clinical observation that blood transfusions often produce an immediate sensation of well-being and beneficial effects on patient energy level, exercise tolerance, etc. It may be that this is due to increased blood viscosity, improving perfusion and FCD, allowing oxygen delivery by the remaining RBC and flushing out metabolites produced during shock or chronic hypo-perfusion.

Therefore a critical function of transfused blood would appear to be restoration of microvascular function, independently of the repletion of oxygen carrying capacity, leading to the hypothesis that restoration of blood rheological properties improves resuscitation independently of the restitution of oxygen carrying capacity. A blood transfusion using stored blood may not fully restore oxygen carrying capacity in acute conditions; however, it functions as a restorer of blood volume and blood viscosity, provided that this process leads to the restoration of FCD. As a corollary, using RBCs for the purpose of increasing blood viscosity may be unnecessary if a material is introduced that increases plasma viscosity in the circulation. However, as one of the primary goals of blood transfusion is the restoration of oxygen carrying capacity, only RBCs can fill this need.

## Paradigms of oxygen transport related to blood transfusion

Oxygen delivery to tissue depends on the oxygen carrying capacity of blood, its convection from the lung to the tissue and the mechanisms that control its uptake and release from blood. This process is defined by the intrinsic oxygen carrying capacity of Hb and its affinity for oxygen, conventionally defined by  $pO_2$  at which it is 50% saturated ( $p50$ ). In the circulation, there is no specific barrier to oxygen diffusion in the blood vessels that prevents oxygen exit. As a consequence, oxygen continuously diffuses out of the blood vessels, and its residence (dwell time) within the circulatory system is determined by its transit velocity, in the same way that the delivery of fluid transported by a leaky container is a function of the container's velocity of translation. The competition between blood flow velocity and outward diffusional exit from the circulation determines a circulatory longitudinal  $pO_2$  and oxygen saturation gradient, and the location in the circulation where blood  $pO_2$  equals RBC  $p50$ .

The oxygen dissociation curve of RBCs has the steepest slope at  $pO_2 = p50$ . At the location in the microvasculature where the  $pO_2 = p50$ , small changes in  $pO_2$  produce the offloading of large amounts of oxygen. The sensitivity of oxygen off-loading from Hb to small changes in  $pO_2$  suggests that the blood vessels may be a part of an oxygen regulatory mechanism.

This model is in part supported by the observation that in some tissues  $pO_2 = p50$  in 3rd order arterioles, which have been shown to possess the highest density of adrenergic innervation<sup>14,15</sup>, microvessels that in many tissues exhibit the maximal rate of oxygen exit. However, while  $p50$  has a unique value for blood throughout the circulation, the location of  $pO_2 = p50$  may vary among different tissues and organs, depending on the level of tissue metabolism.

$P50$  is affected by blood storage, during which levels of intraerythrocytic 2,3 DPG drop. The lack of this allosteric factor increases the affinity of Hb for oxygen (lowers  $p50$ ) to about 20 mmHg from the level of 28 – 32 mmHg seen in RBCs in vivo. This effect has been mostly interpreted as a potentially negative outcome of blood storage; however, recent experimental findings report conditions in which the increase of oxygen affinity of blood may be beneficial. In the following we review the experimental evidence in support of either outcome.

### Increased oxygen affinity during storage

Impairment of oxygen transport of stored RBCs was first reported when measurement of the corresponding oxygen dissociation curves showed an immediate and significant increase in the oxygen affinity during the initial week of storage at 4 °C. This resulted in a decrease of oxygen delivery during transfusion of stored RBCs by comparison to normal RBCs, the difference being proportional to the volume transfused and storage time<sup>16</sup>.

### Is 2,3 DPG a problem?

The possibility of controlling oxygen release by in vitro manipulation of RBC 2,3 DPG levels led to the concept of targeting oxygen delivery by right shifting the oxygen dissociation curve. This process can take days to fully engage while transfusion with 2,3 DPG loaded RBCs can produce a more rapid effect<sup>17</sup>. Thus the need of evoking compensatory mechanism such as increased cardiac output in critically ill patients could be avoided by lowering blood oxygen affinity with a transfusion of RBCs with increased levels of 2,3 DPG<sup>18,19</sup>. RBCs were “rejuvenated” by incubation with a combination of inosine, pyruvate and inorganic phosphate to produce hyperconcentrated 2,3 DPG RBCs. Studies in rabbits transfused with incubated RBCs showed sustained increase in 2,3 DPG for over 2 weeks, however, direct intravenous infusion into mildly hypoxic patients by the same investigators did not support the usefulness of increased 2,3 DPG since it did not lead to improved tissue oxygenation. Their conclusion was that regulation of oxygen delivery was primarily determined by cardiac output rather than concentration of 2,3 DPG.

Anemic baboons were given stored RBCs with higher or lower oxygen affinity which was achieved by no treatment or in vitro incubation with a rejuvenation solution to elevate their 2,3 DPG levels. Increases in cardiac output were to a much lesser degree than the increase in cerebral blood flow obtained with the higher oxygen affinity-low 2,3 DPG group. Additionally they had a decrease in mixed venous  $pO_2$ <sup>20, 21</sup>. Lower oxygen affinity-high 2,3 DPG group had improved oxygen delivery to tissue under conditions of low arterial  $pO_2$  but their ability to load oxygen in the lungs was impaired. It was concluded that the demand for increased blood flow needed for the higher oxygen affinity-low 2,3 DPG groups should be avoided because it would likely not be achievable in the critically ill patient.

Spector *et al.*, 1977<sup>22</sup> analyzed the effect of supranormal high levels of blood 2,3 DPG on oxygen delivery in hypoxic anemia. Fresh RBCs (< 1hr) incubated with a rejuvenation solution were infused into baboons so that after transfusion the resultant 2,3 DPG levels in blood were 125% of normal. During the hypoxic state systemic oxygen extraction was similar in the two groups, however oxygen saturation was lower in the high 2,3 DPG groups

than in the control animals. Cardiac output was significantly reduced 30 min after the arterial pO<sub>2</sub> was restored to normal. These data suggest that RBCs with decreased affinity maintained satisfactory oxygen delivery to tissue during hypoxia.

The importance of 2,3 DPG depletion was analyzed by Collins and Stechenberg<sup>23</sup> who studied the effect of the exchange transfusion of 90% of the original RBC mass in rats with blood stored 1 day (p50 = 35 mmHg) or 14 – 20 days (p50 24 mmHg) to final Hct of 36% (normal), 28% (moderate anemia) and 17% (severe anemia). Animals were then subjected to a severe hemorrhage (removal of 3.2 ml of blood per 100 g of body weight over 50 min) and resuscitated with the shed blood from the same animal. A separate group was handled similarly but resuscitated using 1.15 times the volume of shed volume. Survival was the same through the range of Hcts when fresh blood was used, but was lower in rats receiving old blood at low Hct. Animals exchange transfused with old RBCs and hemorrhaged were resuscitated with fresh and old RBCs at low and high Hct, with survival being lower only for the animals transfused with old RBCs at low Hct. The conclusion of this study was that survival after hemorrhage is impaired if oxygen delivery capacity is reduced by a combination of anemia and increased oxygen affinity.

A critique to the conclusions of this study on the significance of oxygen delivery impairment is that increased oxygen affinity was obtained with “old blood”, i.e., blood stored for about 2 weeks (acid citrate dextrose, NIH solution A, Fenwall, 4 °C), thus testing the combination of increased affinity and RBC damage induced by storage. As shown by Tsai *et al.*<sup>24</sup> RBC damage due prolonged storage results in lowering capillary perfusion and FCD upon retransfusion, which is a significant factor in survival. In fact it cannot be excluded that increased oxygen affinity of stored blood may in fact be neutral or beneficial, as shown by experiments of Cabrales *et al.*<sup>25</sup>. Conversely Vilella *et al.* using the same model, showed that resuscitation from hemorrhagic shock was significantly improved using RBCs with low p50<sup>26</sup>.

The reason why lower p50 can be beneficial at times relates to the previously described longitudinal oxygen gradient in the microcirculation, which causes high p50 blood to deliver oxygen in oxygenated regions, while the low p50 blood delivers oxygen primarily in deoxygenated regions, providing a more uniform oxygen distribution. In man p50 (= 27 mmHg) is lower than in rat and hamsters and depletion of 2,3 DPG lowers p50 to 18 mmHg. Therefore the increase in oxygen affinity within the range seen in stored RBC because of loss of 2,3 DPG would appear to be a problem only because it is associated with RBCs with additional functional defects acquired during storage that affect primarily the maintenance of FCD. These considerations highlight again the significant role played by FCD in tissue survival in comparing fresh and old RBCs.

## Determinants of functional capillary density

Functional capillary density is variable in both normal and diseased tissue. When this parameter is defined as the number of capillaries that possess transiting RBC, changes in FCD reflect mechanisms that modulate the entrance of RBCs in capillaries. These mechanisms have an anatomical origin due to changes in capillary diameter, and can also be due to hydrodynamic effects that control the entrance of RBCs into capillaries. Capillary lumen diameter is determined by a composite of mechanical and cellular factors, intravascular pressure being one of the principal determinants due to the elastic properties of the capillary/tissue system<sup>27</sup>. The degree of hydration of the surrounding tissue and cell volume regulation of the endothelium are additional factors<sup>28</sup>. Furthermore, there is increasing evidence that capillaries possess contractility<sup>29</sup> and that this phenomenon has

spontaneous components<sup>30</sup>. Consequently, FCD is the result of both passive and active processes in single vessels.

Capillary oxygen delivery is determined by their large area/volume ratio and low oxygen gradients. Because of their small intrinsic oxygen-carrying capacity and low intravascular pO<sub>2</sub> (hence low pO<sub>2</sub> gradient into tissue), each capillary supplies oxygen to a very limited tissue volume. Some, but not all, tissue may also be within the diffusion field of an arteriole. Consequently, there is a portion of the tissue supplied only by capillaries which is at risk for hypoxia when RBC flow ceases and FCD is reduced, and possibly irreversibly damaged.

Specific mechanism affecting the number of capillaries with RBC flow comprise: (i) capillary lumen narrowing beyond the point where capillary pressure can provide the energy needed for RBC deformation adequate for passage; (ii) capillary luminal obstruction by leukocytes, microthrombi, and rigid RBCs; and (iii) hydrodynamic effects at capillary bifurcations which direct RBCs to the stream with the greater flow. Capillary diameter variability underlies these scenarios, although it is generally assumed that the capillary lumen is mostly invariable and independent of transmural pressure<sup>31</sup>.

It is generally assumed that if perfusion pressure decreases, flow rate decreases throughout the microvascular network; however, this is not the case as capillary flow does not stop (i.e., there is no change in FCD) associated with lowered perfusion pressure. Studies in skeletal muscle microcirculation<sup>32</sup> show that FCD changes reversibly when perfusion pressure varies in a normal organism. Pressure flow studies in isolated organs show that flow hindrance increases as perfusion pressure decreases, a behavior attributed to the shear dependence of blood viscosity and diameter changes in the distensible segments of the vasculature. Decreased FCD density has been observed in low-flow conditions associated with ischemia-reperfusion injury which has the complication of oxidative stress and leukocyte activation<sup>33</sup>.

Capillary diameter is also a function of effects in the glycocalyx as well as endothelial cell volume. Expansion of endothelial cells can only be accommodated by intrusion into the luminal compartment and therefore may have a profound effect on FCD. The situations associated with malfunctions in cell volume regulation may present with different patterns. In ischemia, the process is heterogeneous with swollen cells interdispersed with normal cells<sup>34</sup> while in shock there is a more uniform thickening of the endothelial cells<sup>28</sup>.

In the absence of leukocyte plugging<sup>35</sup> or microthrombi, the lack of RBCs in capillaries arises from filtering effects due either to a decrease in the luminal diameter below the threshold even for deformed RBCs, or hydrodynamic effects in the network. The existence of spontaneous variability in the capillary lumen caliber indicates the possible existence of individual capillary flow regulation through a mechanism intrinsic to endothelial contractility. Pathophysiological conditions such as ischemia, oxidative stress, hemorrhagic shock) may impair endothelial function interfering with local capillary flow regulation, causing endothelial swelling, increased endothelial tone, and increased capillary permeability promoting pericapillary edema. It should be noted that the whole capillary need not necessarily be involved, since only one or a few cells along each capillary intruding into the lumen to the extent that RBCs cannot pass cause the whole capillary to cease functioning. Capillary perfusion pressure would appear to be the primary factor in determining the extent of FCD<sup>7</sup>. Lowered perfusion pressure coupled to endothelial dysfunction gives rise to the potential for pathological capillary flow hindrance as three mechanisms converge to promote this phenomenon, namely tissue edema, endothelial edema<sup>28</sup> and the mechanical elastic contraction potential of endothelial cells<sup>36</sup>.

## Red blood cell storage lesion

Changes in RBC functionality and integrity during storage are commonly referred to as the storage lesion that may hinder their function during transfusion<sup>37</sup>, particularly microvascular perfusion. During storage, ATP decreases in time leading to energetic compromise, loss of membrane stability, and morphological and rheological changes including RBC adhesion to the endothelium<sup>38-42</sup>. In addition, during storage pH drops, lactate is produced, glucose is consumed, potassium levels increase, iron and free hemoglobin (Hb) are released upon hemolysis, and membrane vesicles are formed<sup>39, 41, 43-46</sup>.

Changes of oxygen Hb affinity during storage are also due to reduced concentrations of ATP within the cell. The fall in pH during storage lowers oxygen Hb affinity however this factor could be considered a minor complications as upon transfusion the buffering system of blood should be able to negate the acidic pH except in the case of massive transfusions where the red cell system of buffering becomes exhausted<sup>47</sup>.

The ability of fresh and stored RBCs to maintain microvascular perfusion and oxygen delivery to the tissue was studied in the hamster window chamber model<sup>24</sup>. The animals were hemodiluted to decrease the oxygen reserve so that the difference between transfusing fresh and stored cells can be discerned. Stored (28 days at 4°C in CDPA-1) and fresh (< 1 hr) were exchange transfused until 25% of the circulating RBC were study cells. The most salient finding was that there were no differences in outcome at the systemic level but were dramatically different at the local level. Stored RBCs significantly reduced perfusion as compared to the fresh RBCs, FCD and microvascular blood flow being reduced by 63% and 54%, relative to the level achieved by fresh cells. Additionally oxygen extraction and tissue pO<sub>2</sub> in the stored cell group was greatly reduced relative to the fresh cell group suggesting the potential development of focal ischemia. This study clearly showed that while oxygen carrying capacity of blood may be the same, the loss in microvascular perfusion dramatically affects oxygen delivery, and that systemic parameters may not always reflect local conditions especially the case of acute anemia.

Studies were performed to evaluate storage lesions in rats with septicemia induced by cecal ligation and perforation<sup>48</sup>. Septic animals were reduced to an oxygen supply dependency state by hemodilution with plasma and then transfused with stored (28 days, CPDA-1) or fresh (< 3 days, CPDA-1) blood. Results showed no immediate improvement of systemic oxygen uptake (VO<sub>2</sub>) up to 2 hrs after the transfusion of old RBC while the fresh cells acutely increased VO<sub>2</sub> from conditions following hemodilution.

In a hemorrhagic shock model, rat intestinal micro-hemodynamics and oxygen tension were found to be significantly decreased with stored cells (28 days, CPDA-1). Intestinal microvascular pO<sub>2</sub> improved only with the transfusion of fresh RBCs, however storage induced changes were not sufficient to impair intestinal oxygen consumption<sup>49</sup>.

In an attempt to translate results in rats to human more directly, a detailed biochemical and functional alterations of rat and human RBC stored in CPDA-1 was undertaken<sup>50</sup>. When rat RBCs were stored in a solution which has been designed for humans cells, they were found to be much more fragile after 29 days in storage. Rat RBCs were able to regenerate ATP but not 2,3 DPG in vivo. The viability of rat RBCs was reduced to 79% after 7 days of storage and to 5% after 4 weeks of storage. This result led subsequent studies in rats to be performed with RBCs stored for 7 days. Recently, a similar examination was performed for mice and similar findings were obtained showing that murine cells had accelerated aging with standard storage conditions<sup>51</sup>. These results of viability generally parallel the average RBC



circulation lifetime for each species: human, 120 days; rat, 60 days; hamster, 50 days<sup>52</sup>; and, mouse 40 days.

These studies show that experimental investigations must specifically consider species differences in cell structure and metabolism. Additionally, the specifics of the animal in terms of physiology and its adaptive physiological differences should be kept in mind.

## Structural RBC changes due to storage

### a) Membrane deformability

The first group of changes in RBC properties is membrane alteration<sup>53</sup>. The structure of the RBC is complex, and membrane phospholipids and proteins, cytoskeletal proteins and cytoplasmic components are all related to each other<sup>54</sup>. Hemorheological alterations – such RBC shape changes, decreased membrane deformability and surface/volume ratio, increased mean cell Hb concentration and osmotic fragility, increased aggregability and intracellular viscosity can occur during storage and may possibly disturb the flow of RBCs through the microcirculation and influence RBC transport of oxygen to the tissues<sup>55,56</sup>. During storage, RBCs undergo progressive morphological changes, from deformable biconcave disks to echinocytes with protrusions, and finally to echinocytes<sup>54,57,58</sup>. In parallel with these changes, redistribution and loss of phospholipids in the red-cell membrane by the formation of microvesicles are seen both in storage and in RBC aging<sup>59–61</sup>.

The storage-related decrease in RBC membrane deformability is a crucial change in RBC properties associated with post-transfusional 24-hour survival<sup>62, 63</sup>. The decreased deformability was thought to be associated with reduced ATP levels. While ATP depletion as seen during storage can reproduce many shape changes, a reduction in surface/volume ratio and increases in intracellular viscosity and post-transfusion 24-hour survival of RBCs precede the decreases in ATP concentration<sup>62</sup>. Only decreases beyond 50% of the ATP concentration can be shown to be associated with increased mortality, suggesting that the role of ATP depletion in storage-related damage may be limited. Nevertheless, restoring ATP levels in RBCs appears to correct membrane alterations to some degree. It is probable that a basal ATP level is necessary for the survival of RBCs, and therefore the adenine pool (AMP, ADP, and ATP) has more effect on cellular changes than ATP alone.

Mechanistically, these storage lesions also impair oxygen delivery to tissues by decreasing microvascular perfusion and reducing the amount of oxygen released from Hb. The deformability of RBCs due to their membrane flexibility is a factor in maintaining normal blood flow in the microcirculation, allowing their transit through capillaries whose lumen is narrower than the cell diameter<sup>64</sup>. The major determinants of RBC deformability are cell geometry, intracellular fluid viscosity, and the viscoelastic properties of the cell membrane<sup>64,65</sup>. Several methods for studying RBC deformability *in vitro* have been reported in the literature<sup>64,66</sup>, however, the role of RBC deformability in the maintenance of capillary perfusion is not well established<sup>67–69</sup>.

The effect of changes in RBC deformability on *in vivo* FCD during acute anemia was studied in the microcirculation using the hamster window chamber model. After anemia (18% Hct), animals were exchange transfused with fresh RBCs or RBCs whose flexibility was reduced by glutaraldehyde incubation. Animals that received the RBCs with reduced flexibility had compromised FCD, microvascular flows and oxygenation<sup>69</sup>, the deficit in oxygen delivery being more pronounced than at the extreme anemic state (11% Hct). Systemic hemodynamic parameters with fresh RBC were different compared to RBCs with reduced flexibility, independent of the maintenance in oxygen carrying capacity. RBC deformability alone has a significant effect on microvascular function, although blood viscosity was not different between RBC and RBCs with reduced flexibility groups at high

shear rates ( $> 150 \text{ s}^{-1}$ ). Storage-related loss of deformability or increased aggregation may account for impaired microvascular oxygenation following transfusion, an effect reported in several preclinical studies <sup>54, 56,66,67</sup>

The deformability of RBCs decreases progressively during storage as shown by studies based on micropipette elongation, filtration, and laser-assisted ektacytometry <sup>70-72</sup>. Results indicate that membrane loss is the likely cause for decreased deformability as a function of time during storage, leading to the conclusion that membrane deformability correlates with RBC viability after transfusion. However, these conclusions are tentative since data of RBC mechanical fragility in vivo as a function of storage period is not available and quantitation of the degree of hemolysis and resulting plasma Hb levels after transfusion are not available <sup>73</sup>.

## b) Plasma hemoglobin and nitric oxide bioavailability

It is well established that NO plays several major roles in human physiology <sup>74,75</sup>. It functions as a neurotransmitter and a macrophage-derived host-defense molecule, inhibits platelet aggregation and endothelium adhesion molecule expression, is an antioxidant, and is a potent vasodilator <sup>74-76</sup>. The balance of NO in the circulation is affected by hemolysis, which is linked to NOS uncoupling <sup>77</sup>, and is generally associated with increased oxidative damage due to heme-based Fenton and auto-oxidation chemistry and reduced nitric oxide-availability due to heme scavenging of NO <sup>77,78</sup>.

Nitric oxide is the most important relaxation factor synthesized by endothelial cells <sup>74,75</sup>. To elicit its vasodilatory activity, NO must diffuse to the smooth muscle cells. Theoretical models suggest that the endothelium's proximity to millimolar concentrations of hemoglobin would severely compromise the efficiency of the NO vaso-relaxation pathway <sup>79</sup>. Hemoglobin scavenges NO primarily through a classic dioxygenation reaction, in which NO reacts with oxyhemoglobin to form metHb and nitrate. Thus, NO bioavailability decreases upon hemolysis and endothelial vasomotor function, blood cell adhesion, and homeostasis are all adversely affected. The enhanced ability of acellular Hb to scavenge NO has been widely attributed to the hypertension, increased systemic and pulmonary vascular resistance, and morbidity and mortality associated with administration of hemoglobin-based oxygen carriers (HBOCs) <sup>80-82</sup>.

Currently, HBOC have been tested in clinical trials, having mostly met with unfortunate failures that have been principally attributed to their vasoactivity. An important consequence of vasoactivity is the decrease of tissue perfusion, capillary pressure, and, consequently FCD. HBOC carriers developed as blood substitutes have been problematic, due to the fact that these hemoglobin molecules scavenge nitric oxide more than RBC encapsulated hemoglobin <sup>83</sup>. We have established that even small amounts of acellular Hb as found in several hemolytic diseases and in stored blood can decrease perivascular NO bioavailability <sup>83</sup>. In addition, we suggest that aged, stored blood has reduced ability to produce nitric oxide from the newly discovered blood nitric oxide synthase. Pathological consequences of reduced NO bioavailability result in microvascular vasoconstriction, platelet activation and pro-oxidant and proinflammatory effects. These pathways may be extremely relevant to observed cardiovascular risk of aged blood transfusion, much in the same way that low NO bioavailability has been shown to lead to general cardiovascular risk.

The levels of RBC NO drop within hours of storage, however they are restored quickly in vivo after transfusion in both "young" and "old" banked RBC. Furthermore free hemoglobin from lysed stored RBC is rapidly bound by haptoglobin and filtered out by the kidney so effects on NO would tend to be transient. Therefore the intrinsic restoration of NO

bioavailability that is lost upon transfusion of stored blood may in part mitigate complications the storage lesion.

## Compensating for acute anemia

An important concept emerging from the material presented is that adequate tissue oxygenation does not depend solely on a normal Hb concentration. Oxygen delivery to the tissues is determined by the product of flow (cardiac output) and arterial oxygen content, therefore compensatory mechanisms for acute anemia include increasing cardiac output and arterio-venous oxygen extraction<sup>84,85</sup>. The anemia-related decrease of Hct produces a proportional reduction of blood viscosity. As a consequence, venous return to the heart and left ventricular preload increase, while systemic vascular resistance and thus left ventricular afterload decrease<sup>86,87</sup>.

At the microcirculatory level, the decrease of blood viscosity entails a redistribution and homogenization of regional blood flow, which enables oxygen extraction<sup>8,88</sup>, reflected by the decrease of microvascular venous oxygen saturation<sup>8,88-90</sup>. Oxygen delivery to the tissues begins to decrease at Hcts lower than 20% (corresponding to an Hb concentration of ~ 6g/dL). At this Hct, compensation for acute anemia via an increase in cardiac output becomes exhausted and oxygen delivery starts to decrease. However, since oxygen delivery exceeds oxygen demand under physiological conditions by a factor of 3 to 4, the organism's oxygen demand can be met over a large range despite a decreasing oxygen supply<sup>91,92</sup>.

A transfusion trigger based on a pre-defined hemoglobin concentration is usually met before the individual anemia tolerance is completely exhausted and patients are usually transfused before the point that they are O<sub>2</sub> supply limited. In this situation, the transfusion of RBC is intended to increase oxygen delivery and hence tissue oxygenation. To what extent oxygen delivery is actually enhanced by a RBC transfusion depends mainly on the degree of anemia at the initiation of transfusion. Besides increasing arterial oxygen content, the restoration of Hct and hence blood viscosity, counteracts the effects of acute anemia on left ventricular preand afterload and thereby the major compensatory mechanisms of acute anemia<sup>8</sup>. As a consequence, cardiac output may decrease so that oxygen delivery may not necessarily increase following transfusion of RBCs. Whether an RBC transfusion increases oxygen delivery depends on how severely tissue oxygenation is impaired, i.e., on the existence of tissue hypoxia and oxygen debt. Typically, a supply-dependency of oxygen extraction is observed in shock and in critical normovolemic anemia<sup>8, 25, 93-95</sup>. Van der Linden *et al.* demonstrated that in anaesthetized dogs subjected to cardiopulmonary bypass with critically decreased pump flow, oxygen supply dependency could be reversed by the transfusion of fresh RBC as effectively as by providing appropriate pump flow<sup>96,97</sup>.

## Conclusions

Experimental studies permit exploring a number of variables related to blood transfusion that cannot be tested in the human population. In this context, these studies are essential for understanding underlying, general mechanisms; however, they seldom reproduce emergency and clinical conditions. Accepting these limitations, we conclude that fresh blood is intrinsically a better resuscitation fluid than older, stored blood in the animal model systems in which it has been carefully studied. It is also apparent that blood transfusions are called for to restore oxygen carrying capacity, while one of the major problems is deficient microvascular perfusion and function. In fact it is questionable whether a blood transfusion is the optimal remedy for reestablishing tissue perfusion in an organism subjected to hemodilution and hemorrhage, or both.

A precise understanding of the mechanisms involved in blood transfusion may be intrinsically impossible, because blood composition *per se* is a moving target. Distinguishing between “fresh” and “old” RBCs overlooks that the distribution of RBC age in the circulation is presumed to be approximately Gaussian, with an average centered at their circulatory half life, therefore even “fresh” blood has a fraction of RBCs at the end of their cycle.

Experimental studies in transfusion studies have presently addressed the question and provide preliminary answer to: What is the problem that must be remedied when a blood transfusion is called for. We propose that oxygen is only one part of the problem, and that microscopic perfusion is the other component. Remarkably their relative importance is not yet established, however there is evidence that adequate restoration of microvascular perfusion deficiency in anemia and hemorrhagic resuscitation may be as efficacious as restoring oxygen carrying capacity, therefore potentially significantly reducing the use of blood.

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