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REVIEW

Oxygenating the microcirculation: the perspective from blood transfusion and blood storage

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Tissue oxygen delivery depends on red blood cell (RBC) content and RBC flow regulation in the microcirculation. The important role of the RBC in tissue oxygenation is clear from anaemia and the use of RBC transfusion which has saved many lives. Whether RBC transfusion actually restores tissue oxygenation is difficult to determine due to the lack of appropriate clinical monitoring techniques. Some patients with restored haemoglobin levels and stable haemodynamics still develop tissue hypoxia, emphasizing that, in addition to global parameters, local microcirculatory control mechanisms are also important in the restoration of tissue oxygenation. Both clinical and animal experimental studies have indicated that storage of RBC diminishes their ability to oxygenate the tissue. Several intrinsic RBC parameters that change during storage and might influence tissue oxygenation will be mentioned. The release of vasodilators from RBC that will alter blood flow during hypoxia, mediated by haemoglobin in the RBC that functions as an oxygen sensor, could be impaired during storage. A better understanding of hypoxia-induced vasodilator release from RBC might become a potential target for drug development and improve tissue oxygenation after transfusion.

Key words: blood storage, blood transfusion, microcirculation, oxygenation.

Introduction

Tissue oxygen delivery depends on red blood cell (RBC) content and RBC flow regulation in the microcirculation. The importance of RBC concentration in tissue oxygenation is clear from the ill effects of massive blood loss in trauma, surgery, or haemolytic disease. Both the development of transfusion medicine and the immediate availability of stored RBC concentrates enable the direct restoration of blood oxygen content during anaemia and have saved many lives. At what haemoglobin (Hb) level a patient is considered anaemic and needs a RBC transfusion, although still debated [1], largely depends on the physical condition of the patient. Critical care patients tolerate anaemia to a lesser extent. But a liberal transfusion strategy in critical care patients (except for cardiac patients) correlates with increased mortality, which has resulted in a redefinition of the lower transfusion limit from 9 to 7 g/dl of Hb [1]. To explain the negative effect of liberal transfusion, several studies have looked snto the role of RBC storage on the efficacy of RBC transfusion [2–5].

But even with restored Hb levels and stable haemodynamics, some patients, e.g. those in intensive care with sepsis, will still develop tissue hypoxia. When unobserved, this can lead to organ dysfunction [6,7]. Thus, besides global parameters like total Hb concentration and Hb saturation, local microcirculatory control mechanisms are important in the restoration of tissue oxygenation. Recent studies show a role for the RBC beyond that of an oxygen carrier. Haemoglobin in the red cell can act as an oxygen sensor during hypoxia, enabling the RBC to regulate blood flow releasing the vasodilators nitric oxide (NO) and adenosine triphosphate (ATP) [8–10]. These new findings can be important in transfusion medicine and blood banking, as storage of RBC could impair their hypoxic vasodilator release. This review will focus on oxygen delivery

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in the microcirculation after transfusion of stored RBCs. After a general introduction on the acute regulation of oxygen delivery in the microcirculation, storage-induced changes in RBC function and their possible influence on post-transfusion microcirculatory control and tissue oxygenation will be discussed in reference to clinical and animal experimental studies.

Oxygenating the microcirculation

The microcirculation usually is defined as that part of the vascular tree comprising blood vessels smaller than 100 µm, including arterioles, capillaries, and venules [11]. Its many branches, which expand the oxygen exchange area, and its close proximity to the tissue cells make the microcirculation ideal for oxygen exchange with surrounding tissue. Variation of arteriolar resistance (autoregulation) and of the number of open capillaries (functional capillary density) assure a continuous supply of oxygen to meet tissue metabolic demands [12]. The mathematical cylinder model developed by Krogh [13] in 1919 has long dominated the view of how oxygen is transported to the tissue. This model assumes that the capillaries in the tissue are equally spaced in uniform arrays with forward flow, and are the only vascular segment from which oxygen can diffuse into a surrounding cylinder of tissue. The Krogh model therefore predicts that the amount of oxygen diffusing across the capillary wall correlates directly with blood flow, and that venous partial oxygen pressure (PO_2) directly reflects capillary and thus tissue PO_2.

More than 80 years have gone by, and new measurement techniques are extending our understanding of the mechanisms of tissue oxygenation. These are described in more detail in reviews by Tsai *et al.* [14] and Siegemund *et al.* [15]. The oxygen micro-electrode for the first time allowed the quantification of tissue oxygen concentration [16]. Intravital microscopy made it possible non-invasively to measure microcirculatory blood flow, Hb concentration and Hb saturation in animals [16]. This technique also provides a detailed description of the architecture of capillary networks, showing that overlying and adjacent capillary networks may have reverse or forward flow due to the presence of cross capillaries [17]. Recently developed handheld microscope devices using surface reflection filtering techniques such as orthogonal polarization spectral (OPS) imaging [18] and sidestream dark field (SDF) [19] allow the study of the microcirculation through mucus membranes and on the surface of organs in humans.

Studies [20–22] using these techniques could not confirm a direct relation between blood flow and oxygenation as predicted by the Krogh model, and showed that capillaries are not the only vascular segment where oxygen diffusion takes place. Both pre-capillary arterioles and post-capillary venules contribute to tissue oxygen delivery and supplement RBC capillary oxygen delivery. Thus, they can be additional sites of regulation [14,20–22]. Going down the vascular tree a longitudinal gradient was found for both PO_2 and Hb saturation [16]. The direction and magnitude of the oxygen diffusion into the tissue are mainly determined by the PO_2 gradient [16]. Wieringa *et al.* [23] showed in a mathematical model that intercapillary oxygen exchange combined with a heterogeneous flow distribution promotes a more homogeneous tissue oxygen environment.

In addition, the use of the albumin-coupled oxygen-quenched phosphorescent dye Pd-porphyrin in animals allowed quantification of microcirculatory and tissue oxygen levels [14,24,25]. The main advantages of this technique are that it is non-invasive and becomes more sensitive at lower PO_2. Also, fitting of the phosphorescent decay signal improves the signal to noise ratio and dramatically reduces the drift in signal observed with oxygen electrodes [14,24].

Based on their earlier studies using Pd-porphyrin to measure regional microcirculatory oxygen levels, Ince and Sinaasappel [26] described four principal mechanisms that could shunt the microcirculation in pathologic conditions and cause local hypoxia. Oxygen delivery can bypass the microcirculation by shunting blood from arterioles to venules (arteriovenous shunting) or oxygen can directly diffuse from arterioles to venules that lie close to each other (diffusive shunting) [27]. Shunting can also result from increased heterogeneity in microvascular perfusion due to vasoconstriction or vasodilation [28], or at low saturation levels when the oxygen off-load time of RBC exceeds its passage time through the microcirculation and oxygen release does not occur until the venules. Due to shunting, the microvascular and tissue oxygen concentration (PO_2) can become lower than the venous PO_2, a phenomena referred to as the PO_2 gap [29]. This has mainly been seen in the vascular dysfunction caused by sepsis. Shunting can also make parts of the microcirculation more vulnerable to hypoxia because of their vessel architecture [30].

The regulatory mechanisms for maintaining adequate tissue oxygen concentration are still not well understood. To match local blood flow to tissue oxygen consumption and avoid tissue hypoxia, an oxygen sensor probably exists in the tissue [8]. Either the arterioles themselves sense blood or tissue oxygen levels, or the tissue releases a metabolite that affects arteriolar tone. The latter mechanism is probably too slow and less anticipatory. An oxygen sensor at a specific site within the tissue is also less probable considering that arterioles and venules are sites of oxygen transfer as well as capillaries [21]. The RBC can use Hb not only as an oxygen carrier but also as an oxygen sensor, which during hypoxia can modulate flow by the release of the vasodilators NO [9,10,31] or ATP [8,32]. This recent finding seems an attractive alternative regulation mechanism. The cystic fibrosis transmembrane conductance regulator (CFTR) is involved in ATP release [33], but the mechanism for release of NO from the RBC is currently unclear. Deoxyhaemoglobin has nitrite

reductase properties [9] converting nitrite to NO and therefore RBC nitrite influx could also regulate NO release from the RBC. A G protein is involved in the signal transduction pathway for ATP release [34]. Also, the finding by Kleinbongard *et al.* [35] of the production of NO by eNOS in the RBC means the RBC oxygen sensing and flow modulating properties could be directly influenced by vasoactive drugs or other agents affecting these signalling systems.

These new findings indicate that RBC function is more complex than previously assumed and make the RBC also a potential target for future drug therapy. An important advantage of oxygen sensing by the RBC is that it allows an immediate feedback during oxygen off-loading as hypoxic vasodilator release can directly alter local vascular tone and hence adapt tissue perfusion and oxygenation according to local needs.

Clinical benefits of transfusion with stored RBC

The primary goal of RBC transfusion is to increase tissue oxygen concentration and improve tissue oxygen consumption. In severe blood loss and large decreases in tissue oxygen concentration, RBC transfusion is a rapid and immediate way to restore oxygen content explaining the undeniable positive effects on mortality in such cases [36]. Recent large cohort studies have shown that 40% to 80% of red cell transfusions in the intensive care unit (ICU) are not administered for haemorrhage, but for low Hb levels, a decrease in physiological reserve or alterations in tissue perfusion [37,38].

ICU patients with anaemia are given RBC transfusion with the assumption that low blood cell concentration causes tissue damage by hypoxia and in the hope that increasing the low Hb levels will restore tissue oxygenation. But in such pathological conditions as sepsis, systemic oxygenation parameters do not reflect local tissue oxygen levels, because of mechanisms like shunting or vascular dysregulation [19]. As RBC transfusion also entails risks, e.g. transmission of infective diseases and immunodepression, its beneficial effects need to be objectively established. Hebert *et al.* [1], in a randomized, controlled trial comparing transfusion thresholds, suggested that transfusion of stored blood to Hb levels higher than 100 g/l could even be harmful.

Few clinical techniques can directly monitor tissue oxygen levels or quantify tissue oxygen consumption. So it is still hard to determine when a patient has tissue hypoxia, where exactly in the body, and how many red cells would be necessary to restore the oxygen deficit. It is even difficult to say whether RBC transfusion really improves tissue oxygenation. Clinical studies on the efficacy of blood transfusion use indirect or surrogate end-points for tissue oxygenation, such as systemic oxygen consumption, tonometry, blood lactate, and base excess levels [2,39]. More general and indirect survival measurements, such as mortality, morbidity, or length of hospital stay [3,5,37,40] are prone to confounding and difficult to correct [3].

Marik *et al.* [39] were among the first to show that RBC transfusion failed to improve systemic oxygen uptake in septic patients, and that patients receiving blood stored for more than 15 days developed splanchnic ischaemia as measured by tonometry. Purdy *et al.* [40] found an association between the age of transfused RBC and patient mortality in a retrospective study in septic patients. Vamvakas *et al.* [3] studied the effect of RBC storage on post-operative morbidity in 268 patients receiving transfusion for coronary artery bypass graft surgery. They found no deleterious effect of stored blood after adjustment for confounding factors. The above studies were not randomized and not always prospective. A protocol randomizing patients to receive fresh or stored RBCs has been difficult to accomplish and only recently has such a study been done on critical ill patients. With a study design similar to that of Marik and colleagues [39], but using leucodepleted RBCs, Walsh *et al.* [2] could not show clinically worsening of tissue hypoxia after transfusion with RBCs stored for more than 3 weeks compared with fresh cells. Like Marik *et al.* [39], they could not show beneficial effects of transfusions on tissue hypoxia even when very fresh RBC was used. Possible explanations for the differences in results are that the patients in the Marik study were at an earlier stage of sepsis and more oxygen supply dependent, or that the Walsh group used leucodepleted RBC and transfused only two RBC units, whereas Marik used three [2].

A large prospective observational study by Vincent *et al.* [37] showed that transfused patients had a longer ICU stay, more severe organ failure and higher mortality rates, all related to the number of RBC units a patient received. To what extent their results depend on the underlying condition of the patients is difficult to determine, as tissue oxygenation was not measured. A pilot trial by Hebert *et al.* [5] found no differences in clinical outcome between fresh and stored RBC. The disappointing effects of RBC transfusion during sepsis may be because oxygen uptake is not supply dependent, or because the oxygen is not distributed to the tissues that need it.

Oxygenation of the microcirculation comes from a close interplay between RBCs and microcirculatory vessels. If transfusion does not restore tissue oxygen levels, this could relate to the condition of the microcirculation in the patient or the RBC itself. Reperfusion injury after ischemia, or inflammatory processes occurring during sepsis could cause endothelial dysfunction or decreased vascular contractility. In trauma, the release of cytokines that interfere with vasoregulation [41] and oxygen consumption could compromise the efficacy of transfusion. In addition, bacterial contamination and growth in stored RBC, the release of cytokines by primed leucocytes and storage-induced lesions of the RBCs themselves could explain the observation that their storage affects tissue oxygen variables, patient morbidity and mortality [4,42–44].

Intrinsic RBC factors that could alter tissue oxygenation

Current guidelines prescribe at least 75% 24 h post-transfusion survival of red cells in the circulation [45] and are therefore based on RBC integrity and viability rather than oxygen distribution to the tissues. Even in modern improved storage solutions, changes in biomechanical and biochemical properties of the RBC [46–48] may interfere with their oxygen distributing function.

One of the characteristic alterations in stored RBC is a change in shape from a normal biconcave to a spindly cell (echinocyte) with protrusions that can be shed as lipid vesicles. This results in lower surface to volume ratio, spherocytosis, increased cell Hb concentration and viscosity, increased osmotic fragility [49] and loss of deformability [46,47]. Osmotic fragility will cause release of Hb, long known as a potent scavenger of the vasodilator NO, but only recently shown to constrict vessel and therefore reduce blood flow at levels as low as 0·01 g/dl [50]. Those effects depend on free haptoglobin, therefore patients with chronic haemolysis are likely to be more susceptible.

Until now, most studies have reported a decrease in red cell deformability during storage [46,47]. But this research was done with whole blood, although at the moment in Europe, we use RBC concentrates. We recently measured the deformability in leucodepleted red cell concentrates using ektacytometry and found no significant decrease in the elongation index after 6 weeks of storage [51]. This result supports earlier suggestions that white blood cells in stored whole blood secrete cytokines and other substances that worsen the storage lesion of RBC [42]. Do these less deformable cells impair blood flow and tissue oxygenation during or after blood transfusion? This is hard to answer and will depend on the reversibility of the RBC deformity and the body's ability to remove such cells from the circulation. In whole animals, treating RBC with heat or formaldehyde to mimic the deformability of old RBC showed a drastic decrease in tissue flow. However, these results might have exaggerated the effects of transfusion of old RBCs. Cells treated that way have such poor deformability [52] that they probably do not mimic physiological or clinical conditions. Using fluorescent RBC labelling, Parthasarathi *et al.* [53] showed that reduced RBC deformability leads to a shunting of RBCs through larger-diameter vessels in the microcirculation, suggesting that such stiff RBC are not as likely to obstruct vessel flow as previously thought.

During the first 2 weeks of storage all 2,3-diphosphoglycerate (DPG), an intermediate metabolite and allosteric modifier of Hb, is lost from the RBC [51]. This causes an increase in the oxygen affinity of Hb, measurable by a leftwards shift in P_{50} from 29 to around 20 mmHg. Although the increase in oxygen affinity of Hb is often quoted as one of the explanations why stored blood impairs tissue oxygenation, recent studies in experimental animals do not support this hypothesis [51,54,55]. D'almeida *et al.* showed in an anaemic rat model that storage decreased 2,3-DPG levels by 50% and dropped P_{50} by 5 mmHg, but that critical oxygen delivery, cardiac index, and oxygen extraction were not altered, and there was only a minimal effect on oxygen reserve. Increasing the P_{50} by 12 mmHg using the allosteric Hb modifier RSR13, Eichelbronner *et al.* [55] could neither demonstrate an increase in systemic oxygen uptake nor an increase in the threshold for cricitcal oxygen delivery in an isovolaemic haemodiluted rat model. This might mean that at low Hb concentrations other microvascular mechanisms are activated, e.g. an increase in perfusion heterogenetity, that counteract the theorectical effect on tissue oxygenation of changes in P_{50}.

During RBC storage, ATP levels drop to about 60% of initial levels after 5 weeks [51]. Initial studies suggested a correlation between ATP levels and reduction in surface-to-volume ratio and increase in cytoplasmic viscosity [56], but later it was found that these alteration precede the reduction in ATP [48]. There seems to be no clear correlation between ATP levels in the RBC and their post-transfusion survival except when levels are below 50% of initial levels. This corresponds to a storage time of more than the currently allowed 5–6 weeks [43]. ATP depletion alone does not seem to explain the RBC storage lesion or determine post-transfusion viability, although secondary effects that alter cell calcium or phosphorylation may affect cell integrity [48]. We recently developed a rat haemodilution and isovolaemic exchange model to test the oxygenation capacity of stored human leucocyte-depleted RBC concentrates [51]. Increasing intracellular ATP levels in stored RBC by either a rejuvenation solution or an ATP-maintaining storage medium improved their oxygenation capacity. On the other hand, decreasing ATP levels in fresh cells by incubation with deoxyglucose reduced their ability to maintain microcirculatory oxygen levels after exchange towards that of stored cells (unpublished data). Future studies will have to answer whether the reduced ATP levels in the RBC after 5–6 weeks storage also prevent their hypoxic ATP release. Although ATP levels in the RBC restore within hours after transfusion by uptake of adenosine from the plasma (as has been shown in *in vitro* experiments [57]), a blood transfusion is usually given with the goal of immediately increasing oxygen availability. This could explain the variable patient outcome after transfusion, as the direct need for tissue oxygenation might have been different for individual patients.

The hypoxic release of NO is another potential mechanism that could be compromised after RBC storage. Either the substrate influx mechanism of nitrite, the NO efflux pathway, or the nitrite reductase activity of deoxyhaemoglobin could be affected. Other potential mechanisms contributing to the RBC storage lesion are oxidative injury, protein oxidation, and lipid peroxidation [43].

Effect of stored RBC on tissue oxygenation in animal studies

The question whether storage of RBC impairs their ability to transport oxygen to the tissue can be studied in animals under better-controlled physiological conditions than in patients. Use of animals has the additional advantage that the Pd-porphyrin technique can be used to measure microvascular and tissue oxygen, as its use in humans is limited by renal toxicity.

Fitzgerald *et al.* [58] showed that storage of rat RBCs for 28 days in citrate–phosphate–dextrose (CPD) adenine-1 impaired their ability to improve tissue oxygenation when transfused into either control or septic rats with supply limited systemic oxygen consumption. A later study in the rat by van Bommel *et al.* [59] supported these results showing that the transfusion of rat RBCs stored for 28 days did not restore the microcirculatory oxygenation. But except for CPD-stored RBCs, the storage induced changes were not enough to impair intestinal oxygen consumption and mesenteric venous PO_2.

Can these results be extrapolated to the human situation? Observations by d'Almeida *et al.* [60] and our own unpublished results indicate that this may not be the case. Not only do rat RBCs age about four times more rapidly during storage than human RBCs, but unlike human RBCs, rat RBCs fail to regenerate 2,3-DPG when treated with a rejuvenation solution [60]. We therefore developed a haemodiluted rat model (haematocrit 15%) that allowed isovolaemic exchange transfusion with fresh and stored human leucodepleted and washed RBCs to determine whether intestinal microvascular oxygenation would be equally maintained [51]. Three storage-age groups were studied: 2–6 days (indicated as fresh), 2–3 weeks (indicated as intermediate), and 5–6 weeks (indicated as old). Isovolaemic exchange transfusion with old stored human RBCs decreased intestinal microvascular oxygen levels by about 25% compared with fresh or intermediate cells, both of which did not produce significant changes. But in the more clinically relevant procedure to double the haematocrit to levels above 30%, the increase in microvascular oxygen was comparable for fresh and stored RBC. We found similar effects in the kidney in recent experiments (unpublished data). Thus, only during limited tissue oxygen delivery, stored RBCs are less able to oxygenate the tissue than fresh cells. This supports the hypothesis that the release of vasodilators from RBCs during hypoxia would be deteriorated in RBC stored for 5–6 weeks. A disturbance of hypoxia-induced blood flow regulation in stored RBC may explain the variable clinical results seen after transfusion in patients whose tissue oxygenation response may vary because of their physical condition.

Tsai *et al.* [61] found that hamster RBC stored for 28 days reduced microvascular flow, functional capillary density. and oxygen extraction compared with fresh RBCs. They concluded that transfusion of stored hamster RBCs in a haemodiluted hamster resulted in significantly malperfused and under-oxygenated microvasculature that was not detectable at the systemic level. Whether hamster RBC, like those of the rat, age four times more quickly in storage is unknown, but important for a correct interpretation of this study. In contrast, Torres-Filho *et al.* [62] recently investigated the systemic effects of a 50% exchange with fresh or 10-day-stored rat blood and a subsequent isovolaemic haemodilution and showed that the tolerance to critical oxygen delivery was comparable between the two groups. According to D'Almeida *et al.* [60] the 10-day-old rat RBC are biochemically (ATP and 2,3-DPG levels) comparable to human RBC stored for 6 weeks, although it is not clear how they compare for other biochemical or morphological functions. The current animal models all have their drawbacks, but as long as we lack clinical techniques to measure tissue oxygenation they remain the only way to increase knowledge about the effect of stored RBCs on tissue oxygenation. For future animal studies it will be important to examine the effects of RBC storage in the setting of an impaired microcirculatory function with lower tissue PO_2, because our own results suggest that differences in tissue oxygenation between fresh and stored RBC will be more pronounced.

Conclusion

Clinical and animal studies have reported contradictory findings about the oxygenation capacity of stored RBCs. Studies in healthy animals under oxygen-restricted conditions suggest impaired oxygenation by stored RBCs, but it is difficult to extrapolate these results to clinical outcome. In clinical studies, different physical conditions in patients might explain contradictory results, emphasizing the need for clinical devices to measure local tissue oxygen needs. RBC regulate blood flow during hypoxia by releasing vasodilators. A better understanding of this could generate new ways of improving tissue oxygenation by RBCs during and after transfusion. Such studies could answer the fundamental question whether improvement of local blood flow and tissue oxygenation bring about a beneficial clinical outcome.

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