# Purinergic control of red blood cell metabolism: novel strategies to improve red cell storage quality

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## Abstract

Transfusion of stored blood is regarded as one of the great advances in modern medicine. However, during storage in the blood bank, red blood cells (RBCs) undergo a series of biochemical and biomechanical changes that affect cell morphology and physiology and potentially impair transfusion safety and efficacy. Despite reassuring evidence from clinical trials, it is universally accepted that the storage lesion(s) results in the altered physiology of long-stored RBCs and helps explain the rapid clearance of up to one-fourth of long-stored RBCs from the recipient's bloodstream at 24 hours after administration. These considerations explain the importance of understanding and mitigating the storage lesion. With the emergence of new technologies that have enabled large-scale and in-depth screening of the RBC metabolome and proteome, recent studies have provided novel insights into the molecule-level metabolic changes underpinning the accumulation of storage lesions to RBCs in the blood bank and alternative storage strategies to mitigate such lesion(s). These approaches borrow from recent insights on the biochemistry of RBC adaptation to high altitude hypoxia. We recently conducted investigations in genetically modified mice and revealed novel insights into the role of adenosine signalling in response to hypoxia as a previously unrecognised cascade regulating RBC glucose metabolism and increasing $O_2$ release, while decreasing inflammation and tissue injuries in animal models. Here, we will discuss the molecular mechanisms underlying the role of purinergic molecules, including adenosine and adenosine triphosphate in manipulating RBCs and blood vessels in response to hypoxia. We will also speculate about new therapeutic possibilities to improve the quality of stored RBCs and the prognosis after transfusion.

**Keywords:** adenosine, metabolomics, transfusion medicine, additive solution, packed red blood cells.

## Introduction

Transfusion of packed red blood cells (RBCs) is one of the most effective life-saving and condition-improving treatments for several categories of recipients with different clinical backgrounds, including, but not limited to, trauma and haemorrhagic shock, haemolytic anemia and major surgical operations. The first transfusions of preserved blood in the Rous-Turner solution, a combination of citrate and dextrose, was conducted by Oswald Robertson during World War I[1,2]. After that, sustained efforts to maintain corpuscular integrity and post-transfusion survival have extended the storage period to 42 days by the addition of phosphate, adenine, and various nutrient solutions[1,3]. Over the last century of clinical transfusion practice, RBC transfusion has been accepted as a key element of modern medical care. It supports all forms of surgery in the treatment of heart diseases, cancer, and trauma and intensive care. Despite declining trends in the last decade, storage of packed RBCs in the United States today meets the need for approximately 15 million units of blood for over 4-5 million massively or chronically transfused American recipients every year (http://www.hhs.gov/ash/bloodsafety/nbcus/). While the current standard of practice is generally safe and effective[4], transfusion of packed RBCs has, in the past, been retrospectively associated with potential untoward consequences, such as increased morbidity and mortality, in certain categories of recipients[5]. Recent retrospective clinical studies have indicated that transfusion of RBCs stored longer than 14 days may correlate with poor prognosis in certain traumatised, critically ill or perioperative patients[6,7]. On the other hand, reassuring clinical evidence has been reported by several independent randomised clinical trials about the substantial non-inferiority of the current standard of practice when compared to the preferential transfusion of the freshest units available[6,8-11].

Despite the disconnection between retrospective and prospective clinical evidence, biochemical and morphological changes during RBC storage have been recognised for decades and recent studies using mass spectrometry-based metabolomics, proteomics, and lipidomics have revealed detrimental changes in RBCs after 14 days on the shelf[12-17]. Among all these changes, energy and redox metabolic reprogramming stand out and likely play important roles in mediating RBC haemolysis and post-transfusion survival and performances[18-20]. Recent studies have indicated that

inheritable and processing-incurred proteome and metabolome differences explain, to a large extent, the variations observed among the storage quality of blood coming from different donors[21-25]. Understanding and harnessing the metabolism of RBCs for the purpose of improving the quality of stored blood seems plausible and promising. Here in this review, we will summarise recent novel understandings of the regulations of RBCs metabolism by extracellular signallings and the potential therapeutic implications.

## Metabolic disruptions during RBC storage

### Glucose metabolism alterations

Glucose metabolism plays pivotal roles in RBC functions in three aspects: 1) RBCs rely solely on glycolysis to generate adenosine triphosphate (ATP); 2) approximately 25% of glucose in RBCs is used to produce the RBC specific metabolite 2,3-bisphosphoglycerate (2,3-BPG) for haemoglobin $O_2$ affinity modulation[26]; 3) RBCs depend on the oxidative branch of the pentose phosphate pathway (PPP) to generate reducing equivalents (NADPH) to preserve glutathione homeostasis and counteract oxidative stress. In the last few years, a series of observational studies have been published by us and others addressing the metabolic adaptation of packed RBCs stored at 4 ºC for up to 42 days under blood bank conditions in different additive solutions, including SAGM, AS-1, AS-3, AS-5 and AS-7. These studies reported that routine storage results in the rapid loss of ATP and 2,3-BPG, which leads to detrimental changes in haemoglobin $O_2$ binding/off-loading kinetics[15,27-31]. Because of the negative influence of hypothermia on enzyme activities, RBC storage causes progressive depletion of ATP reservoirs to only approximately 40% by the 42-day expiry date, accompanied by the decrease of 2,3-BPG as well[32]. For decades, a close association between the ATP content of stored red cells and their increased rigidity has been shown from the observation that restoring the ATP content of the RBCs before transfusion restores their normal flexibility and greatly enhances viability[33-35]. It is presumed that when ATP content is below a certain critical level, RBCs lose their capacity to phosphorylate and metabolise glucose[36], thereby generating a vicious cycle that further deteriorates the integrity and function of RBCs. Indeed, ATP-synthesis capacity decreases as intracellular pH falls owing to ongoing glycolysis in the closed system of storage bags, a phenomenon that is further exacerbated by ATP-dependency of some cation pumps or inhibition of others at 4 °C. Energy and redox metabolism are connected in RBCs. Storage of RBCs also results in the progressive deregulation of the redox balance maintained by the levels of GSH and GSSG, owing to impaired synthesis and turnover[37], as gleaned through tracing experiments[12], a phenomenon that is in part donor dependent[24]. Studies have shown that reactive oxygen species (ROS), generated through Haber-Weiss and Fenton reactions from haeme iron, reach a maximum within the first two weeks of storage[17].

### Metabolic modulation

A progressive loss of $O_2$-dependent metabolic modulation of glucose metabolism was also observed in stored RBCs[13,38]. In oxygenated RBCs *in vivo*, the glycolytic enzymes are inhibited by binding to the cytoplasmic domain of the most abundant RBC membrane protein band 3 (1 million copies per cell; abbreviated as cdb3)[39]. This allows a finely-regulated shift of glucose metabolism between glycolysis and PPP depending on the availability of $O_2$. The cdb3 protein is also directly involved in redox homeostasis as it interacts with peroxiredoxin 2, a scavenger of hydrogen peroxide that has been found to be progressively oxidised and translocate to the RBC membrane[40,41] and supernatants[42] during storage[43]. It has been suggested that the oligomerisation of cdb3[44], which affects its function in modulating metabolism, can be promoted by excessive ROS generated in stored RBCs. ROS or enzymatic digestion can also tackle cdb3 and impair band 3-dependent metabolic modulation in stored RBCs[45,46].

## Purinergic signalling in blood transfusion

Besides the recent omics studies that revealed these changes, research conducted on RBCs from human and genetically modified animal models also add tremendous insights into revealing the mechanisms behind such changes[47,48]. Moreover, alterations in cellular metabolism and integrity that correlate with *in vivo* performances in mice have also been revealed[18]. Recently, it has been appreciated how alkaline additives or anaerobic/hypoxic storage of RBCs offer the opportunity to boost energy and redox metabolism of RBCs in the blood bank[13,31,46,49-51]. Of note, these storage strategies exploit RBC capacity to reprogramme metabolism to physiologically adapt to (high altitude) hypoxia[52], a phenomenon we recently explored mechanistically in mice and humans[53,54]. Understanding these mechanisms will pave the way for the designing and testing of novel storage additives or the optmisation of alternative strategies such as hypoxic storage of packed RBCs.

### Purinergic signalling

In 1972, extracellular signalling mediated by purine nucleotides and nucleosides such as adenosine and ATP was first proposed[55]. Although purine nucleotides and nucleosides have long been recognised as key components in energy metabolism and cell proliferation, their functions as signalling molecules were accepted only after separate families of receptors for adenosine

(P1) and ATP and ADP (P2) were recognised in 1978, and receptors for purines and pyrimidines were cloned and characterised in the early 1990s[56,57]. So far, four P1 receptor subtypes (ADORA1, ADORA2A, ADORA2B and ADORA3), seven P2X ion channel receptor subtypes (P2X1-7) and eight P2Y G protein-coupled receptors (P2Y1, 2, 4, 6, 11, 12, 13 and 14) have been identified[58]. Using RT-PCR of red blood cell progenitor cells, messenger RNA (mRNA) expression of P2X1, P2X4 and P2X7, as well as P2Y1 receptors (but not for P2Y2, P2Y4 or P2Y6), were reported[59]. Recently, studies have shown that blood cells, especially RBCs, have emerged as one of the most interesting targets of purinergic signalling. RBCs contain high concentration of ATP (approximately 1 mM)[60] and adenosine that can be released when haemolysis happens and when reacting to mechanical deformation, β-adrenoceptor agonists, prostacyclin analogues, reduced $O_2$ tension, acidosis or swelling[61]. The non-lytic ATP and adenosine release involves cystic fibrosis transmembrane conductance regulator (CFTR), hemichannel pannexin 1, and equilibrium nucleoside transporters (ENTs). More importantly, because of the widely expressed purinergic receptors in almost every organ and cell type, changes in ATP and adenosine release can directly signal through their receptors and regulate responses of other tissues and cell-types to transfusion.

## ATP metabolism and signalling in RBCs

### ATP release from RBCs increases blood flow

Due to lack of mitochondria, mature RBCs rely completely on the glycolytic pathway for the generation of ATP. With one glucose molecule metabolised, two ATP molecules are generated. It is important to keep in mind that intracellular ATP concentrations are very high[60]. As such, it is not surprising that many forms of "cellular stressors" will cause the release of ATP from intracellular storage pools into the extracellular compartment. In 1951, release of ATP from RBCs exposed to hypertonic solutions was first reported[62]. Later on, the release of ATP from human RBCs was shown to occur in response to hypoxia[63,64] and to mechanical deformation as might occur when red cells are squeezed through small vessels or deformed in areas of high velocity[65]. ATP release in these situations requires signal transduction pathways involving activation of pathway-specific membrane-bound adenylyl cyclase, cyclic adenosine monophosphate (cAMP), protein kinase (PK) A and CFTR; the direct stimulation of the G protein Gi can lead to the release of ATP as well[66,67]. Moreover, the release of ATP from RBCs in response to reduced $O_2$ tension is linked to the oxygenation state of the haemoglobin molecule[68], which also regulates the metabolic flux of glucose to either glycolysis or the PPP in RBCs[69]. A number of other factors can also affect RBC ATP release. Prostacyclin analogues and β-adrenergic agonists increase ATP release[70,71] while nitric oxide (NO) inhibits the signal transduction pathway for ATP release from RBCs *via* its action on heteromeric G protein, Gi[72]. Statins can increase RBC deformability and reduce low $O_2$-induced ATP release[73] while insulin inhibits it[74]. Caffeine enhances ATP release from RBCs, most likely due to its effect on levels of cAMP[75], while lactate, in the absence of changes in pH, interferes with ATP release[76]. RBC ATP release is also sensitive to physiological increases in temperature, possibly *via* activation of CFTR channels[77]. Treatment of RBCs with diamide, a compound that decreases RBC deformity, inhibits low $O_2$ tension-induced ATP release[78]. Hydroxyurea, a substance that affects RBC deformability, stimulates the release of ATP from RBCs through an increase in calcium and NO production[79]. It has been well-characterised that free ATP in circulation causes vasodilation and increases blood flow in organs such as skeletal muscle and heart[80-82]. This release of ATP in response to changes in oxygen saturation and other environmental conditions happens within minutes, indicating a complementary mechanism to the NO-based vascular toning.

### Decreased ATP release in stored RBCs after transfusion induces lung injury and pulmonary hypertension

In many instances, purinergic signalling events are initiated by the release of ATP from the intracellular toward the extracellular compartment. Previous studies have demonstrated that ATP release from RBCs is increased in response to low $O_2$ tension and low pH, which serves as an important mechanism for hypoxia response because ATP can signal through P2Y receptors on the vascular endothelium and induces vasodilation[83]. Regulated release of ATP from RBCs in response to mechanical deformation or haemoglobin desaturation is a critical physiological process to supply enough $O_2$ to systemic tissues. Furthermore, disruption in ATP release has been found in diabetic and pulmonary hypertensive patients with the incompetence of ATP-dependent blood flow regulation which is a possible mechanism underlying the vascular complications of these diseases[84,85]. A study by Zhu *et al*. revealed that RBC storage lesion and development of transfusion-induced lung injury and pulmonary hypertension are associated with compromised ATP release from RBC[86]. The authors claimed a cause-effect relationship for inhibited ATP release from stored RBC and transfusion-related toxicity in the lung specifically by showing that RBC-derived ATP is: 1) important in preventing stored RBC-dependent increases in pulmonary arterial pressure in isolated perfused lungs; and 2) important in preventing RBC adhesion to endothelial cells *in vitro* and in the lung after transfusion. The *in vivo* mouse transfusion

experiments suggested that, under physiological conditions, RBC adhesion is suppressed by RBC-derived ATP with the pathological consequences in the lungs only manifesting when ATP release is inhibited. Clearly, this study demonstrates that compromised purinergic signalling due to decreased intracellular ATP concentration and ATP release in stored RBCs is a new mechanism underlying the transfusion-induced lung injury and inflammation that are associated with storage lesion. Moreover, ATP released from RBCs contributes to the elevation of circulating adenosine which has multiple functions in both RBCs and other organs as well[87].

## Adenosine signalling in RBC storage and transfusion

### Adenosine metabolism and signalling

Adenosine is ubiquitously produced in almost all of the cells in our bodies under physiological conditions and further produced under hypoxia or conditions of energy depletion. It is generated intracellularly by the breakdown of both AMP and S-adenosylhomocysteine. Adenosine is generated extracellularly from the degradation of adenine nucleotides by the consecutive action of ectonucleotidase CD39 and ecto-5'-nucleotidase CD73. Circulating adenosine is maintained at low levels by adenosine deaminase and bi-directional equilibrative nucleoside transporters (ENTs) in the plasma membrane[87]. Extracellular adenosine is also the ligand for four distinct G-protein coupled receptors: A1, A2A, A2B and A3. However, only the ADORA2B receptor has a HIF1-α binding site at its promoter region and is up-regulated during hypoxia[88,89]. Research has shown that CD39 and CD73 are up-regulated by HIF1-α and the ENTs are down-regulated[90,91]. In response to hypoxia, ATP is released from cells and converted to adenosine, increasing the extracellular adenosine levels from nanomolar range up to micromolar range[90,92]. Thus, the co-ordinated cellular response to hypoxia is to promote the production of increased extracellular adenosine and enhanced signalling through the ADORA2B. This is accomplished by increased release of ATP, the upregulation of ectonucleotidases CD39 and CD73 to convert the extracellular ATP to adenosine, reduced production of the ENTs to prevent cellular uptake of adenosine, and enhanced production of the ADORA2B.

### Adenosine signalling increases RBC $O_2$ release in sickle cell disease and leads to disease progression

Blood transfusion in intensive care is associated with the need to restore tissue oxygenation and blood viscosity in response to haemorrhagic shock. Long-stored human RBCs are characterised by a higher $O_2$ affinity[93]. Such effects are promoted by the storage-dependent consumption of 2,3-BPG. This compound is known to act as allosteric effectors as they stabilise the "T" (deoxygenated) state of haemoglobin (Hb) and thus decrease Hb-$O_2$ affinity. Similar to most of the purinergic receptors, adenosine receptors are widely expressed in various types of cells, including RBCs[87]. However, the important role of adenosine signalling in RBCs were not on the horizon of adenosine and RBCs research before recently published studies that showed adenosine signalling plays important roles in regulating RBCs 2,3-BPG production in both sickle cell disease (SCD)[94] and high-altitude hypoxia[53]. By treating SCD mice with polyethylene glycol-modified adenosine deaminase (PEG-ADA), an FDA-approved drug for treatment of human ADA genetic deficiency, Zhang *et al*. saw decreased sickling compared with controls. Subsequently, they found that levels of 2,3-BPG were elevated in red blood cells of SCD patients and mice. Interestingly, after treatment with PEG-ADA, Zhang *et al.* observed decreased RBC 2,3-BPG levels in SCD mice. Moreover, the *in vitro* experiments also showed that the potent, non-metabolisable adenosine analogue, 5'-N-ethylcarboxamidoadenosine (NECA), can increase 2,3-BPG production in normal cultured human and mouse RBCs[94]. Hypothesising that the adenosine-mediated 2,3-BPG increase is through adenosine receptors, Zhang *et al*. tested NECA-mediated 2,3-BPG induction using RBCs from wild-type control mice and RBCs from each of the four adenosine receptor deficient mice. Only in RBCs lacking the ADORA2B was NECA unable to induce 2,3-BPG levels. The importance of the ADORA2B receptor was also confirmed pharmacologically using ADORA2B antagonists. The ADORA2B receptor coupled with stimulatory G-protein (Gs), and activates adenylyl cyclase resulting in increased cAMP levels and protein kinase A (PKA) activation[94]. Pharmacological experiments determined that PKA was responsible for the induction of 2,3-BPG production by adenosine through A2B signalling. This was the first evidence that adenosine signalling can regulate the $O_2$ delivery ability of RBCs through increasing the production of 2,3-BPG, which provides a new way of maintaining 2,3-BPG levels in stored blood by boosting adenosine-ADORA2B signalling.

### Beneficial role of adenosine-ADORA2B signalling in increasing $O_2$ release to counteract high-altitude hypoxia

High altitude is a challenging condition caused by insufficient $O_2$ supply, which can also happen to trauma patients who need transfusion. Inability to adapt to hypoxia may lead to pulmonary edaema, stroke, cardiovascular dysfunction, and even death. Using high-throughput, unbiased metabolomic profiling, Liu *et al.* reported that the metabolic pathway responsible

for the production of RBC 2,3-BPG was significantly induced in 21 healthy humans within two hours of arrival at an altitude of 5,260 m and further increased after 16 days at 5,260 m[53]. This finding led the authors to discover that plasma adenosine concentrations and free CD73 activity rapidly increased at high altitude and were associated with elevated RBC 2,3-BPG levels and $O_2$ releasing capacity. Mouse genetic studies demonstrated that elevated CD73 contributed to hypoxia-induced adenosine accumulation and that elevated adenosine-mediated RBC A2B adenosine receptor activation was beneficial by inducing 2,3-BPG production and triggering $O_2$ release to prevent multiple tissue hypoxia, inflammation, and pulmonary vascular leakage[53]. Mechanistically, Liu *et al*. demonstrated that RBC AMP-activated protein kinase was activated in humans at high altitude and that AMP-activated protein kinase (AMPK) is a key protein functioning downstream of the ADORA2B, phosphorylating and activating BPG mutase, and thus inducing 2,3-BPG production and $O_2$ release from RBCs. Moreover, pre-clinical studies demonstrated that activation of AMPK enhanced BPG mutase activation, 2,3-BPG production, and $O_2$ release capacity in CD73-deficient mice, RBC-specific A2B adenosine receptor knockouts, and wild-type mice. In turn, AMPK activation reduced tissue hypoxia and inflammation[53]. Interestingly, several studies based on mice with genetic deficiency of AMPK revealed a very peculiar anaemic phenotype due to shortened RBC lifespan[95,96]. Although these studies claimed that AMPK deletion leads to accumulation of oxidative stress due to the incapacity of producing antioxidant, it is worth investigating if these mice also have low 2,3-BPG levels which may undermine the function of RBCs in delivering $O_2$.

**Manipulate adenosine-ADORA2B signalling for improved quality of stored RBCs and decreased storage lesion**

One of the biggest challenges facing blood transfusion is the increased $O_2$ affinity in stored RBCs that prevents the release of $O_2$ to the ischaemic and hypoxic tissues. Classic studies have clearly shown a role for the RBC energetic state (in particular ATP and 2,3-BPG levels) in mediating RBC survival upon transfusion[97]. Routine storage results in the rapid loss of 2,3-BPG and ATP, with obvious consequences in haemoglobin $O_2$-binding/off-loading kinetics[98]. These changes are also accompanied by a progressive loss of metabolic modulation (the capacity to activate the PPP to generate reducing equivalents, such as NADPH), and impaired synthesis of reduced glutathione. All these metabolic lesions result in the progressive accumulation of oxidative stress, which in turn target proteins and lipids[46]. The observations in normal and SCD RBCs in mechanistic studies with genetic mice indicate that adenosine signalling through ADORA2B plays an unanticipated role in RBC metabolic modulation. In particular, metabolomics analyses of RBCs from 21 healthy young individuals exposed to high altitude hypoxia revealed a strong positive correlation between the levels of plasma adenosine and key glycolytic metabolites[52,53]. Therefore, a metabolic linkage exists between adenosine and glycolysis. Consistently, adenosine conversion to inosine by adenosine deaminase disrupts the signalling cascade owing to the inability of inosine to activate ADORA2B. Taken together, these studies provide strong support for the notion of manipulating adenosine-ADORA2B signalling to improving quality of stored RBCs and decrease storage lesion. For example, ADORA2B agonist may be supplemented to the storage solution to maintain the high activity of glycolysis and ATP and 2,3-BPG production. BAY 60-6583, a potent ADORA2B agonist that demonstrated cardioprotective role by attenuating infarct size in a mouse model of myocardial ischaemia[99], can be tested. However, caution must be exercised, since the adenosine-ADORA2B signalling also regulates many other important processes.

**Enhance extracellular adenosine to counteract transfusion-related acute lung injury**

One of the common and most severe complications associated with blood transfusion is the transfusion-related acute lung injury (TRALI)[100], which characterised by as pulmonary edaema and severe hypoxia. TRALI is a rare but potentially fatal complication of blood product transfusion. It has been defined by both a National Heart, Lung, and Blood Institute (NHLBI) working group as well as a Canadian Consensus Conference, as new acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) occurring during or within six hours after blood product administration. Historical estimates suggest that TRALI occurs at a rate of approximately 0.04-0.1% of transfused patients or in approximately 1 in 5,000 transfused blood components[100]. It is the leading cause of transfusion-related mortality in the United States. However, due to a lack of understanding of the molecular mechanism involved in the development and progression of TRALI, no effective therapeutic options are available. Several groups reported that adenosine serves beneficial functions on features of ALI such as enhanced alveolar-capillary barrier function and dampened inflammation, and substantially protects against ALI resulting from hypoxia or ischaemia[101]. Follow-up genetic and pharmacological studies reported that the adenosine-mediated beneficial role in ALI is *via* ADORA2B in a CD73-dependent manner, suggesting that adenosine converted from released ATP plays a

role[101]. These studies further indicate that RBCs ATP release and adenosine signalling play an important role in transfusion-induced tissue and organ damage.

## Conclusions

With the improvement in *ex vivo* blood storage, the field of transfusion medicine has achieved a huge success with approximately 108 million units donated per year. However, despite the fact that recent clinical studies confirmed the safety and effectiveness of transfusion therapies, a 30% decrease in blood usage in the last five years has been attributed to the concern over the "storage lesion". Although some retrospective clinical studies raised concerns about the use of RBC units stored longer than two weeks as issuable blood-derived therapeutics, it is unclear whether and to what extent these lesions might end up compromising the safety and effectiveness of the transfusion therapy[7]. Despite reassuring clinical prospective evidence, an accumulating body of evidence from biochemical, morphological, and omics investigations suggests that RBCs stored longer than 14 days are characterised by the accumulation of a series of lesions that make them qualitatively different from fresh RBCs; lesion(s) that can be counteracted with the introduction of alternative storage strategies and solutions.

Sustaining the function of RBCs, and mitigating the storage lesion by maintaining normal metabolism, is the primary goal in current RBC storage protocols. Here we reviewed studies on the central role of purinergic signalling through receptor A2B in RBC metabolic modulation in normal and sickle RBCs[53,94]. These observations offer the opportunity to prevent or counteract the RBC storage lesion by controlling purinergic signalling in stored RBCs. These observations are suggestive of an unappreciated metabolic linkage between adenosine signalling and glycolysis, one that can be present in stored human RBCs, and may thus be potentially exploited to better preserve ATP and 2,3-BPG for a longer period during storage, theoretically enhancing the safety and effectiveness of the transfusion therapy.

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