The potential adverse effects of haemolysis

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Abstract

Haemolysis occurs in many haematologic and non-haematologic diseases. Transfusion of packed red blood cells (pRBCs) can result in intravascular haemolysis, in which the RBCs are destroyed within the circulation, and extravascular haemolysis, in which RBCs are phagocytosed in the monocyte-macrophage system. This happens especially after RBCs have been stored under refrigerated conditions for long periods. The clinical implications and the relative contribution of intra- *vs* extra-vascular haemolysis are still a subject of debate. They have been associated with adverse effects in animal models, but it remains to be determined whether these may be involved in mediating adverse effects in humans.

Keywords: intravascular haemolysis, extravascular haemolysis, red blood cells, transfusion, blood storage.

Haemolysis occurs in many haematologic and non-haematologic diseases and can be defined as the removal of senescent or damaged red blood cells (RBCs) from the circulation[1]. Haemolysis also occurs after transfusion of stored blood. In particular, there is increasing evidence to suggest that increasing the storage period between blood donation and transfusion results in a decrease in RBC recovery and consequently an increase in post-transfusional haemolysis[2,3]. However, the storage time-related adverse effects and the potential mechanisms associated with transfusion-related toxicity remain controversial, and the relative contribution of intra- *vs* extra-vascular haemolysis is still under discussion.

In fact, haemolysis can be distinguished as intravascular haemolysis, in which the RBCs are destroyed within the circulation and release free haemoglobin (Hb) and RBC contents into the bloodstream, and extravascular haemolysis, in which RBCs are phagocytosed in the monocyte-macrophage system of organs such as the liver and the spleen[4,5]. To the extent that clinically-relevant adverse effects of transfusions exist, it is likely that they are due to a combination of intra- and extra-vascular haemolysis, as shown by some animals models designed to describe the consequences of massive transfusions[6]. Of course, the complex biochemical and structural changes occurring during blood storage, and generally referred to as the Storage Lesion, can also contribute to these effects.

Intravascular haemolysis

The primary acute pathophysiological responses to extracellular Hb in plasma are increased blood pressure[7] and pro-oxidative toxicity occurring in vascular and renal tissues[6,8]. Pulmonary arterial pressure (PAP) was also observed to increase after exposure to free Hb[9,10]. During intravascular haemolysis, some toxic compounds typically compartmentalised within RBCs, such as haemoglobin and haeme, are released into the circulation. The adverse clinical effects associated with intravascular haemolysis are thought to be caused by: 1) extravascular translocation of haemoglobin and other RBC content; 2) imbalance between nitric oxide (NO) and reactive oxygen species (ROS); 3) platelet and haemostatic activation; and (4) haeme, haemoglobin and ATP-mediated activation of the innate immune system. The scavenger systems to limit the toxicity of the RBC contents include soluble plasma proteins, among which haptoglobin and haemopexin are considered to be the most important.

The first line of defence is haptoglobin, which irreversibly binds to the released haemoglobin. The resulting complex is rapidly cleared from the circulation via receptor-mediated endocytosis (CD-163 scavenger receptor[11,12]) and degraded in the liver, leading to a reduction in plasma haptoglobin. Cell-free plasma haemoglobin may overwhelm this scavenger system causing an intensified consumption of the endogenous NO and the formation of methemoglobin, which releases free haeme. Haemopexin, an acute phase protein primarily expressed in the liver, binds haeme and in addition this complex is removed by receptor-mediated endocytosis.

Various adverse effects, such as vascular dysfunction, injury, and inflammation, can be caused by the presence of free haemoglobin and free haeme in the circulation[13]. The first mechanism causing these effects is the imbalance between NO, a critical regulator of vasodilation and vascular homeostasis, and ROS. NO produced by endothelium and oxyhaemoglobin can quickly and irreversibly react, but this process is usually limited by compartmentalisation of haemoglobin inside the erythrocyte. During intravascular haemolysis, haemoglobin circulates in vessels free or in small microvesicles that can react faster with NO via the NO deoxygenation reaction and iron nitrosylation

reactions, as shown in some animal models[7,14]. In particular, it has been shown that more than 0.01 g/dL of free haemoglobin in plasma can potently inhibit NO-dependent vasodilation *in vivo*[15,16]. The decrease in NO availability during intravascular haemolysis can also be due to other mechanisms. Free haeme can cause NO consumption and vasoconstriction by increasing adhesion molecule expression and endothelial activation, serving as a pro-inflammatory ligand of innate immune receptors (e.g., TLR4). This process also promotes inflammatory cell recruitment, platelet aggregation, and oxidation of low-density lipoprotein[17-20]. In addition, during RBC haemolysis significant concentrations of the enzyme arginase 1 are released into the circulation. Arginase 1 can metabolise L-arginine to ornithine, reducing the available L-arginine which is required for NO synthesis by the endothelial NOS (NO synthase) enzyme.

Therefore, during intravascular haemolysis, low levels of decompartmentalised or cell-free plasma Hb can impair NO signalling, reducing its bioavailability and producing vasomotor instability, endothelial dysfunction and systemic vasocontriction that clinically results in an increase in systemic vascular resistance and, as a consequence, a rising systolic, diastolic and mean arterial blood pressure with a decrease in or either unchanged cardiac output[8,9,15], and a decreased perfusion to some organ systems, such as kidneys[21]. NO supplementation before free Hb exposure seems to attenuate these phenomena and the consequent clinical effects[9,22].

An increase in ROS production is also observed during haemolysis. In fact, free Hb auto-oxidises to methemoglobin and participates in a catalytic pseudoperoxidase cycle producing ROS. Haeme, which contains iron, is also responsible for the production of ROS through the Fenton reaction and by other distinct signalling pathways[23,24].

During intravascular haemolysis, platelet and haemostatic activation can occur. *In vitro* experiments demonstrate that NO inhibits both platelet aggregation and endothelial adhesion molecule expression. Thus, during intravascular haemolysis, the acute reduction in NO bioavailability can lead to the activation of platelets and the haemostatic system[25,26]. Furthermore, NO may affect coagulation by inhibiting Factor XIII, enhancing clot stability and reducing clot dissolution[27]. Finally, RBCs contain high levels of ADP, the release of which can activate platelets via the P2Y receptors[28].

As mentioned before, haeme and haemoglobin can mediate the activation of the innate immune system causing macrophage and neutrophil migration to the lung and the release of DNA neutrophil extracellular traps (NETs)[29-31] within the lung parenchyma. This process induces activation of inflammation and thrombosis, through endothelial activation, RBC and activated platelet recruitment, and fibrin deposition. Haeme may also trigger pro-inflammatory and pro-thrombotic pathways through the stimulation of macrophage and endothelial cell toll-like receptor 4 (TLR)-4, involving Weibel-Palade body degranulation and nuclear factor-kappa B (NF-κB) activation[18,19]. Finally, intravascular haemolysis leads to ATP release, which can activate inflammatory pathways leading to sterile inflammation[32]. Therefore, intravascular release of RBC content after transfusion of older stored blood could contribute to cardiovascular and renal dysfunction, as well as inflammation, thrombosis, and enhanced susceptibility to infection, in severely ill patients.

Extravascular haemolysis

Damaged or aged RBCs accumulate over time within stored blood bags. Some degree of acute haemolysis occurs after transfusion through phagocytosis by the macrophage-monocyte system of the liver or spleen. This process is called extravascular haemolysis, and it classically occurs to eliminate senescent circulating RBCs displaying surface markers that identify them as cells requiring removal. During extravascular haemolysis, the RBC content is not found in plasma because the cell is lysed inside the macrophage. The degradation products deriving from this process are salvaged and recycled. In particular, the iron derived from haemoglobin is either stored intracellularly in ferritin deposits or returned to the plasma to be bound by transferrin and transported to the erythroid marrow for erythropoiesis and to other tissues for re-use. In circulation, iron (Fe^{3+}) is carried by transferrin, which binds it with high affinity and renders it unable to react with ROS and other substances. Furthermore, at a steady state, the rate of RBC destruction is equal to the rate of red cell production, generating an equilibrium between waste production and re-use. However, this process is intensified after transfusion, when an average of up to 25% of the transfused RBCs can be cleared from the circulation according to regulatory agency criteria for blood storage[3]. The majority of the storage-damaged RBCs are cleared from the circulation very rapidly (within the first hour after transfusion[33]), causing an excessive rate of delivery of haeme-iron to reticuloendothelial macrophages. Consequently, the rate of release of iron into the circulation can surpass the rate of uptake by transferrin, producing circulating non-transferrin-bound iron (NTBI)[3,34]. NTBI is a heterogeneous group of iron complexes, mainly Fe^{3+}-citrate or albumin complexes, which is considered potentially toxic. A fraction of NTBI, known as labile plasma iron (LPI), is very loosely bound to proteins and is highly redox active, and is probably the main cause

of iron-mediated oxidative damage[35,36]. NTBI and LPI can also enter many cell types, such as liver, pancreas, endocrine glands cells and cardiomyocytes by non-transferrin dependent pathways, resulting in increased labile intracellular iron (LIC)[37], a highly reactive form of iron. LIC can generate ROS from reactive oxygen intermediates, over-riding the cell antioxidant defences and compromising cell integrity and causing organ damage and failure. Under normal conditions, NTBI and LPI should not be found in plasma. However, NTBI can be detected in the plasma as soon as transferrin becomes more saturated[38] , and rises significantly when transferrin saturation exceeds 70-80%[38-40].

The full implications of the increased extravascular haemolysis after transfusion of stored blood, regardless of the chronic or acute nature of the transfusions, remain to be determined. However, animal studies using mice[41] and dogs[42] suggest that there is a pro-inflammatory response following transfusion of older, stored RBCs. This can exacerbate an underlying systemic inflammatory response syndrome (SIRS)[41], increase alloimmunogenicity to RBC antigens[43], and enhances proliferation of certain pathogens[3,44,45]. Thus, as expected, multiple observational studies have suggested an association between transfusion of RBC stored for longer durations and worse clinical outcomes[46] (e.g., increases in sepsis, pneumonia, multi-organ failure, myocardial infarction, acute renal failure, thrombosis, and mortality). However, these studies have significant flaws, mainly owing to the difficulty in disentangling the contribution of the age of the RBCs from the increased underlying disease severity in patients receiving more, and therefore older, units of RBCs. Thus, despite the completed and ongoing controlled trials designed to address these questions, the issue of whether transfusion of RBCs stored for a prolonged period is harmful is still controversial.

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