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RESEARCH ARTICLE

Erythrocytes in COVID-19: Effects on Morphology, Function, and Potential Role in Disease Pathogenesis

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ABSTRACT

Since the SARS CoV-2 virus was first identified in December 2019, huge scientific endeavor has occurred in order to characterize the pathogenesis of this virus and how best to treat it. Early observations noted marked cytokine release, coagulopathy and a prothrombotic phenotype associated with severe disease. The potential contribution of red blood cells to these findings remains an area of ongoing investigation.

While there is no evidence of direct infection of red blood cells by the SARS CoV-2 RNA virus, anaemia and increased variability in shape and size of red cells have been shown to be associated with adverse outcomes in COVID-19 infection. This is likely related to the impact of inflammatory cytokine-induced oxidative stress on erythrocytes, where decreased levels of reducing agents have been shown to correlate with disease severity. The consequences of increased oxidative stress on red cells include membrane damage leading to the morphological abnormalities seen in patients, and increased rates of programmed red cell death with resultant anaemia. Production of nitric oxide by red cells is altered, possibly as a means to alleviate tissue hypoxia in these patients, and red cells may also demonstrate enhanced lactate influx, possibly reducing circulating levels at a time of increased glycolysis.

In this review we discuss the currently available evidence describing the impact of SARS CoV-2 infection on erythrocytes and the possible roles they play in patients with COVID-19 infection.

Introduction

The COVID-19 pandemic, caused by infection with the Severe Acute Respiratory Syndrome Coronavirus-2 (SARS CoV-2) virus has caused high levels of mortality and morbidity. Since the start of the pandemic in December 2019 there have been over 600 million documented cases leading to approximately 6.5 millions deaths world-wide [1]. Severe COVID-19 disease is associated with an endotheliopathy and excess production of inflammatory cytokines, the so-called cytokine storm [2,3]. This dysregulated immune response can lead to development of coagulopathy and a prothrombotic state [4,5]. The part that erythrocytes play in the pathogenesis and response to SARS CoV-2 infection remains incompletely characterised. In this review we summarise the current understanding of the role of erythrocytes in COVID-19 infection, shown in Figure 1.

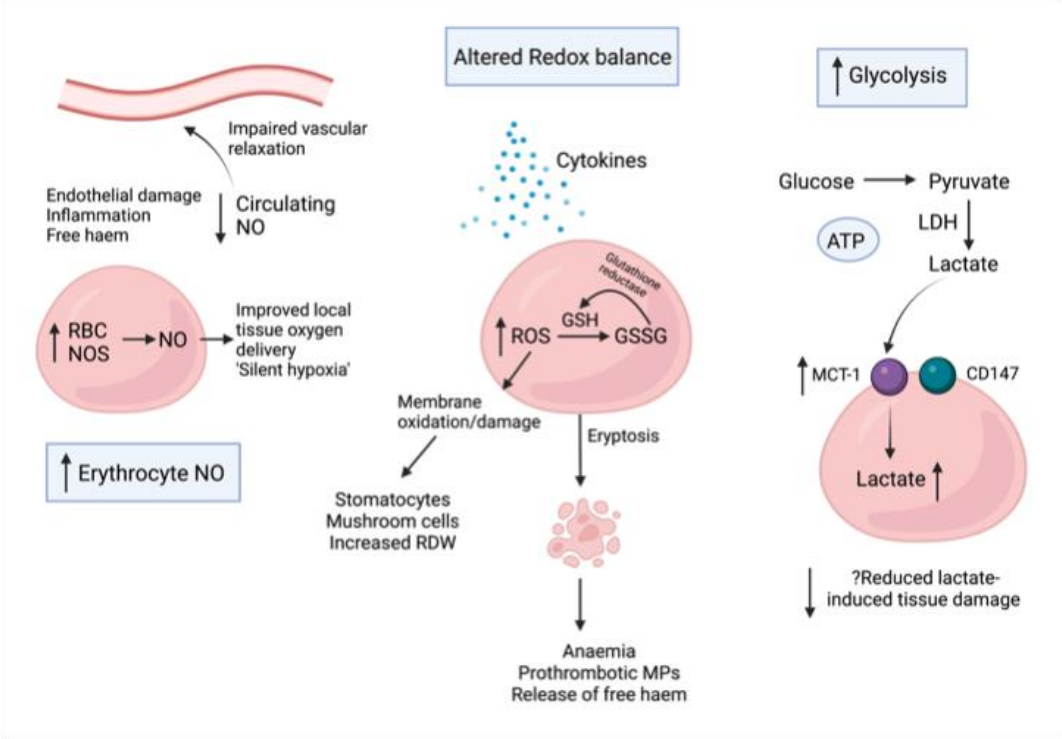

Figure 1. Erythrocytes in COVID-19: increased production of NO by RBC NOS, increased oxidative stress causing membrane damage and promoting eryptosis, increased glycolysis and possible increase in lactate influx into erythrocytes.

Morphological changes in erythrocytes

The impact of COVID-19 on erythrocyte structure and function has not been fully determined. Anaemia and an increased red cell distribution width (RDW) have been shown to be associated with increased mortality in hospitalized COVID-19 patients [6,7]. One group analysed peripheral blood morphology in 115 patients, categorizing them as having no red cell changes, changes in <10% of red cells and changes in ≥10%. Mortality correlated significantly with increasing erythrocyte abnormalities from 12.5% to 41.9% across the three groups. Patients with more erythrocyte abnormalities were also more likely to have lymphopenia and thrombocytopenia, suggestive of worsened disease severity [8]. Despite early claims, there is no unequivocal evidence that SARS CoV-2 can specifically infect red cells. In active COVID-19 infection, SARS CoV-2 RNA is only detectable in a minority of blood products at low viral levels, with no direct evidence of transfusion related COVID-19 infection [9].

Does SARS CoV-2 directly affect haemoglobin?

Various putative binding sites for SARS CoV-2 have been proposed in erythryocytes. The angiotensin-

converting enzyme 2 (ACE2) receptor is expressed by haematopoietic progenitor cells but has not been shown to be involved in direct erythrocyte infection [10]. CD147 and CD26 were suggested as potential targets, however the available evidence does not support this [11,12]. A resonance recognition model suggested that the SARS CoV-2 spike protein could bind to erythrocyte band3 protein [13]. While this theory remains unproven, interestingly, red cell morphological anomalies seen in COVID-19 patients include stomatocytes and mushroom cells, typical in patients with band3 defects [14,15].

It also does not appear that patients with COVID-19 have altered oxygen binding capabilities. SARS CoV-2 has a number of open reading frame (ORF) polyproteins, responsible for replicating viral RNA [16]. One group used homology modelling and molecular docking algorithms to identify potential interactions between ORF polyproteins. They reported that several ORF proteins might be able to dissociate iron from porphyrin in haemoglobin [17]. The methods used in this paper were later criticized [18]. Nor were they corroborated by another study, which identified increased levels of the porphyrin by-products uroporphyrin I and coproporphyrin I and the metabolite coproporphyrin III in the serum of COVID-19 patients. Accumulation of these byproducts was thought to exacerbate the heme shortage seen, whereas the normal levels of protoporphyrin IX identified suggested that SARS CoV-2 is not directly competing with the heme group for the iron atom as stated by the previous publication [19]. Moreover, subsequent studies have not found convincing evidence of altered haemoglobin oxygen affinity in these patients, further refuting these claims [20,21].

Impact of red blood cell groups

Red cell blood group may alter the risk of contracting COVID-19 and degree of disease severity [22]. The ABO blood group consists of glycosylated cell surface glycoproteins and glycolipids which form the A and B antigens. Individuals who lack expression of A and B are termed group O and rapidly form anti-A and anti-B antibodies during early life [23]. Globally, the most common ABO group is O, followed by A, B and finally AB. A number of studies have published higher rates of COVID-19 in Group A individuals, particularly compared with Group O [24-28]. One such paper compared COVID-19 patients with healthy controls, reporting a significantly higher proportion of Group A patients than controls (38% vs 32%) and a lower proportion of Group O patients (26% vs 34%). The Group A results were likely confounded by increased prevalence of comorbidities in this cohort, but other authors have reported similar findings [24,25]. ABO group may also influence disease severity. A prospective study of 95 critically ill COVID-19 patients reported an increased risk of requiring mechanical ventilation in non-Group O cases [29]. Various hypotheses have been suggested to explain this observed association including whether A and B antigens may serve as low-affinity SARS CoV-2 receptors, or if anti-A antibodies might alter viral ability to infect target cells, as seen in a preclinical Severe Acute Respiratory Syndrome (SARS) study [30]. Reduced antibody titres in patients of all ABO groups were reported in one study, compared with healthy controls [31]. Conversely, a recent meta-analysis of over 233,000 cases did not find an association with ABO group and severe disease, defined by need for mechanical ventilation, or mortality [32]. In addition, no definitive evidence of if or how ABO group interacts with the SARS CoV-2 has so far been produced.

Oxidative stress in COVID-19 erythrocytes

The cytokine storm seen in COVID-19 is highly prothrombotic. Cytokines lead to upregulation of tissue factor on endothelial cells and monocytes [33,34], activate platelets [35], alter fibrinolysis [36] and promote formation of neutrophil extracellular traps (NETs). NETosis is increased particularly in severe COVID-19 infection [37], where extracellular histones within NETs cause platelet activation and thrombosis [38]. Increased oxidative stress is a well-recognized occurrence in severe viral infections where neutrophils move towards sites of infection and release reactive oxygen species (ROS) to aid in pathogen killing [39,40]. Oxidative stress itself refers to increased production or reduced elimination of ROS, whereby the balance of the redox state is disrupted with potentially deleterious consequences. An increased neutrophil:lymphocyte ratio in COVID-19 is associated with the presence of increased levels of neutrophil-derived ROS and with an increased mortality rate [41,42]. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2), an enzyme involved in production of ROS is also overexpressed in hospitalized patients with COVID-19 [43]. ROS stimulate further NETosis in SARS CoV-2 infected patients [44], promoting a positive feedback loop and worsening the proinflammatory, prothrombotic milieu.

Binding of the SARS CoV-2 virus to ACE2 may itself trigger an increase in ROS production. Binding to

ACE2 prevents conversion of angiotensin II (Ang II), leading to increased levels. Ang II binds the angiotensin type 1 receptor (AT1R), stimulating NADPH to produce more ROS [45]. ACE2 deficiency has been shown to lead to increased ROS levels, although not specifically in the context of COVID-19 infection [46].

Erythrocytes are particularly vulnerable to oxidative stress based on their primary function of oxygen transport. Intracellular defense against oxidative stress relies upon the presence of reducing agents. Glutathione (gamma-glutamyl-cysteinyl-glycine) plays an important role in this process. It exists as oxidized GSSG and reduced GSH and is made in the cytoplasm of all mammalian cells [47,48]. GSH reduces ROS producing GSSG, which is subsequently reduced by glutathione reductase [49]. Reduced GSH levels have been identified in COVID-19 patients, with degree of abnormality of GSH/GSSG ratio correlating with disease severity [50,51]. Thomas *et al*. reported increased GSSG levels in erythrocytes from COVID-19 patients alongside evidence of membrane damage including oxidation and fragmentation of ankyrin, spectrin beta and the N-terminal cytosolic domain of Band3 with altered lipid metabolism. Alongside increased GSSG levels they showed reduced levels of antioxidant enzymes including catalase, peroxiredoxins 1, 2 and 6, glutathione peroxidases 1 and 4, and superoxide dismutase [52]. Other key regulatory genes may also be affected following SARS CoV-2 infection. The NF-E2 related factor 2 (NRF2) transcription factor regulates genes required for ROS scavenging [53]. NRF2 gene expression has been shown to be reduced in biopsies from COVID-19 patients, although not specifically within erythrocytes. Interestingly, treatment with a NRF2 agonist also inhibited viral replication in cell lines [54].

The findings reported by Thomas *et al*. suggest a significant impact of infection on erythrocyte membrane integrity, which could potentially alter their deformability, potentiating haemolysis, reduced oxygen delivery to tissues and thrombosis [52]. *In* vitro data in the pre-COVID-19 era showed that inducing oxidative stress, demonstrated by a decrease in the GSH/GSSG ratio, decreased red cell deformability and increased blood viscosity. This effect was more pronounced in NRF2 knockout mice [55]. One group reported altered red cell deformability in 50 COVID-19 patients compared with 42 healthy controls, with evidence of increased ROS detected within female patients only [56]. A further recent publication reported conflicting results in COVID-19 patients with acute respiratory distress syndrome (ARDS) requiring intensive care support, showing normal erythrocyte deformability on admission and over the course of one week [57]. Another group hypothesized that endothelial dysfunction in COVID-19 might be partly mediated by erythrocyte handling of ROS. Rat aortic rings were incubated with erythrocytes from COVID-19 patients and healthy controls. Endothelium-dependent and independent relaxation (EDR, EIR) were both impaired in COVID-19 samples, however the addition of apocynin (a NOX inhibitor) had no impact on either EDR or EIR [58].

At our hospital, we measured ROS in red blood cells from COVID-19 patients and healthy controls by incubating cells with 2'-7'-dichlorofluorescin (DCF) and measuring mean fluorescence intensity (MFI) by flow cytometry. There was a significantly higher increase in MFI in the COVID-19 patients following incubation with hydrogen peroxide (H_2O_2), which correlated with CRP level [59]. Pre-incubation with the anti-oxidant N-acetyl cysteine (NAC, a precursor of GSH) partially reversed the ROS generation indued by H_2O_2 [59] as has been previously shown in normal red cells and those from various disorders [60]. Elevated CRP has been shown to be predictive of severe disease and adverse outcomes by several studies in COVID-19 [61-63]. The positive correlation between increased ROS and CRP in our study provides further support to the hypothesis that the cytokine storm is a key factor in development of a state of oxidative stress following SARS CoV-2 infection. Eryptosis, or programmed erythrocyte death, may be triggered by oxidative stress via activation of cation channels and calcium influx into cells [64,65]. Caspases are activated by oxidative stress, which cleave Band3, leading to translocation of phosphatidylserine from the inner layer of the bileaflet red cell membrane to the erythryocyte surface [66,67]. Oxidative stress also stimulates chloride channels leading to cell shrinking, a hallmark of eryptosis [68]. Increased red cell death in states of high oxidative stress, such as severe COVID-19 infection, may partly explain the anaemia observed in critically ill cases. Furthermore, exposure of phosphatidylserine is prothrombotic, and can lead to interactions between dying red cells, the endothelium and platelets [69,70], although we did not show this in our work.

Several groups have trialed use of NAC to treat COVID-19 patients with variable success. A retrospective cohort study of approximately 900 patients found no impact of NAC on in-hospital

mortality or ICU admission, but a shorter admission duration in NAC-treated individuals [71]. Another retrospective analysis of around 2000 patients treated with NAC reported a significant mortality benefit, independent of concomitant corticosteroid use and comorbidities, but no effect on ICU admission or requirement for mechanical ventilation [72].

Nitric oxide in COVID-19 erythrocytes

Nitric oxide (NO) is a free radical gas which induces relaxation of vascular smooth muscle via the activation of soluble guanylate cyclase, plays a key role in the pathogenesis of inflammation and controls mitochrondrial oxygen consumption by inhibiting cytochrome c oxidase [2,73]. The majority of vascular NO produced is made by endothelial nitric oxide synthase (eNOS) [74]. Erythrocyte intracellular NO may be produced by the RBC NOS enzyme, may enter red cells by binding to the haemoglobin beta chain as S-nitrosohaemoglobin (SNO-Hb) or may be derived by reduction of nitrite by deoxyhaemoglobin [75-77]. Tissue hypoxia stimulates release of NO from SHO-Hb and cellular stress triggers activation of RBC NOS to produce NO, thereby leading to vasodilation in areas of tissue hypoxia to improve oxygen delivery [78,79]. NO levels are reduced in COVID-19 infection, potentially contributing to the prothrombotic milieu observed in these patients. Decreased NO is likely due to a combination of vascular dysfunction, inflammation and endothelial cell damage [80-82]. Free heme, potentially released by red cell haemolysis may also bind NO, generating methaemoglobin and nitrates. Indeed, methaemoglobin level was shown to be slightly but statistically significantly higher in COVID-19 patients by one group [83].

However, analysis of 14 patients with COVID-19 and four healthy controls revealed increased levels of erythrocyte-derived NO in the COVID-19 patients, irrespective of the presence or absence of hypoxia. The authors hypothesized that this might potentially account for the phenomenon of 'silent hypoxia' seen in COVID-19 patients, where patients are observed to be relatively asymptomatic despite measurable hypoxaemia [84].

Red blood cell glycolysis in COVID-19

Glycolysis is a critical cellular metabolic pathway. Under aerobic conditions, glucose is converted into pyruvate and NADH. Pyruvate is turned into acetyl CoA which is used in oxidative phosphorylation to make ATP. Under hypoxic conditions, pyruvate is reduced into lactate by lactate dehydrogenase (LDH), compromising the production of ATP. The glycolytic pathway produces two ATP molecules for each glucose metabolized, compared with oxidative phosphorylation, which produces 32 [85,86]. In hypoxia, when cells are reliant upon glycolysis, this leads to increased glucose use termed hyperglycolysis, and increased production of lactate [86,87].

There is some evidence in COVID-19 infection of increased glycolysis within monocytes from bronchoalveolar lavage samples [88]. This seems to potentiate cytokine production and may be exacerbated under conditions of hypoxia in patients with severe disease, although this theory remains unproven. As a consequence of enhanced glycolysis, high lactate levels may be expected in patients with SARS CoV-2 infection, however clinical data has not mirrored this, with lower-than-expected lactate levels seen, even in critically ill individuals [89].

In order to regulate intracellular acid-base, cells have a variety of pH regulators including monocarboxylate transporters (MCT). MCTs 1-4 are involved in glycolysis, with activities including transport of L-lactate and pyruvate [90]. MCT1 is the only MCT expressed by human red cells [91]. Its expression is regulated by CD147 which can be used as a surrogate marker. The presence of CD147 markedly increases movement of lactate into cells by increasing its concentration at the extracellular entry site to MCT1 [92,93]. We analysed CD147 expression on the red cells of COVID-19 patients and healthy controls and found that mean CD147 expression was significantly higher in the COVID-19 group than controls. There was also a significant positive correlation between serum lactate and red cell CD147 expression, whilst the subset of patients who died from complications of SARS CoV-2 infection had significantly higher serum lactate and red cell CD47 levels that patients who survived [59]. We, thus, hypothesize that, in severe SARS CoV-2 infection, increased expression of CD147/MCT1 on red blood cells facilitates transport of lactate from plasma into erythrocytes to protect against lactic acid-induced organ damage [94]. This might account for the lower than expected levels seen in these patients [89]. However, further research, such as measurement of erythrocyte membrane expression of MCT1 protein by western blot and lactate distribution between plasma and red blood cells, is required to further this hypothesis.

Conclusions

Over the past few years, the SARS CoV-2 virus has had a huge impact on the World, accounting for over 6.5 million deaths. Understanding of this virus has expanded dramatically, with the production of safe and effective vaccines occurring at record speed. The role of erythrocytes within COVID-19 infection has yet to be fully elucidated. While there is no evidence to support direct infection of red cells, morphological red cell anomalies alongside anaemia and an elevated RDW are associated with adverse outcomes. Changes in erythrocyte metabolic pathways, notably those regulating the redox status, production of NO and handling of lactic acid, occur in response to viral infection, and in some cases may impact pathogenesis.

As mentioned, a number of groups have investigated the role of oxidative stress as a potential therapeutic tool in COVID-19. NAC may be beneficial, although the evidence is not consistent [71,72]. Much focus has been placed on ameliorating the cytokine storm caused by SARS CoV-2 infection. Tocilizumab was approved for use in this setting based on results of the RECOVERY and EMPACTA trials. Both studies showed a reduced likelihood of progressing to mechanical ventilation, whereas only RECOVERY found a survival benefit [95,96]. Whether administration of tocilizumab causes appreciable changes in haemoglobin concentration or erythrocyte morphology has not been reported. It is therefore not known if dampening down the cytokine storm can reverse the deleterious effects of oxidative stress on red cells in patients with COVID-19, and whether this contributes to the improved outcomes observed.

Although much progress has been made in the fight against SARS CoV-2, further research is required to clarify the exact role of red cells in COVID-19 infection and to determine the associated potential therapeutic implications.

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Conflicts of interest: none

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