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Review Article

Role of Free Radicals in the Alterations of Immune Responses in Hemolytic Disorders

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Introduction

An estimated 250 millions of Hb molecules are packed in a red blood cell. During intravascular hemolysis, the destruction of RBCs results in release of billions of Hb molecules in circulation. The cell-free Hb in flowing blood can contribute towards many cytotoxic effects including tissue damage and organ dysfunction. Cell-free Hb has been shown to be associated with the pathogenesis of endothelial dysfunction, intravascular thrombosis, altered immune responses along with acute inflammations, and lung and renal injury in different disease conditions such as Paroxysmal Nocturnal Hemoglobinuria (PNH) and Sickle Cell Disease (SCD) [1,2]. A number of iatrogenic diseased states are resulted due to excessive release of cell-free Hb during hemodialysis, cardiac bypass, and transfusion therapies.

Extensive studies have shown that the accumulation of cell-free Hb causes great deal of cytotoxicity [3,4]. The oxidation products of Hb are the most potent mediator that manifests the Hb toxicity *via* free radicals. Heme, one of the degradation products of Hb, induces oxidative damages *via* ROS generation. Heme triggers production of inflammatory mediators through activation of selective signaling pathways in immune cells. Under normal conditions this potential source of oxidative hazard is minimized by Hp and Hpx, which bind free Hb and heme respectively and facilitate their removal from blood. However, during the hemolytic disease conditions, the oxidized Hb or Hb derivatives can increase many of the above clinical consequences including inflammations. Overwhelmed with the excessive amount of cell-free Hb in circulations, the scavenging system fails to work adequately further exacerbating the oxidative stress. The prevailing pro-oxidant conditions may lead to the development of pro-inflammatory symptoms, which promotes tissue injuries and organ damages. The present review focuses on the pro-oxidant properties of cell-free Hb and its general consequences on immune components in hemolytic disorders.

Cell-free Hb: A Pro-oxidant

Normally the removal of senescent RBCs from the circulation leads to certain degree of intravascular hemolysis, which is readily cleared up by the conventional Hb- scavenging mechanisms. However, in patients with hemolytic disorders such as PNH and SCD, the scavenging systems are overwhelmed with excess of cell-free Hb. The breakdown products of Hb such as heme exhibit intrinsic toxicity. Heme interferes with the oxidant and anti-oxidant balance in the tissue [5]. The cell-free Hb produces harmful reactive oxygen species (ROS) such as superoxide anion and hydrogen peroxide (H_2O_2) [6]. Furthermore, in presence of ROS the free-Hb is oxidized into methhemoglobin (MetHb), chracterized by the change in the oxidation state of Iron (Fe) from ferrous (Fe^{+2}) to ferric (Fe^{+3}). The pro-oxidant effects of cell-free Hb are mostly related to conformational change observed in the Hb molecule (related to oxidation state of iron) which also affects its ability to interact with the cellular receptors [7].

Hb exists in a dynamic equilibrium state of tetramer and αβ-unit heterodimer where the dimer state predominates at low plasma Hb concentration. Dimers being small in size (~32kDa) have an easy access to tissues and cells and can even be translocated across endothelial barrier thereby increasing cellular susceptibility of oxidant-mediated cell injury [8,9]. After tissue extravasation, the cell-free Hb is oxidised in presence of ROS leading to formation of MetHb and superoxide [10]. Being unstable, MetHb readily releases heme. Once released, free heme causes oxidative damage and inflammation *via* Fenton chemistry, thus acting as a prototypic damage-associated molecular pattern (DAMP) [11,12]. These pro-oxidant effects are specific to MetHb than OxyHb through oxidation *via* H_2O_2 or oxygen radicals [7].

Dismutaion of superoxide anions formed during autoxidation of Hb dimers results in H_2O_2 formation [13], which is further involved in the secondary oxidative reactions with Hb [14]. Cell-free Hb is highly reactive with H_2O_2 gaining pseudo-peroxidase properties [15]. Fe^{2+} of Hb reacts with

H_2O_2 *via* Fenton reaction generating highly reactive hydroxyl radical (·OH), which can further amplify the toxicity and the damage induce by free Hb [16]. H_2O_2 produced oxidizes ferrous and ferric Hb to Fe (Fe^{4+})-ferryl Hb and Oxyferryl Hb, respectively. Ferryl-Hb further reacts with H_2O_2 producing heme degradation products and free iron, which is responsible for various pathophysiological conditions [17]. An increase in these heme degradation products indicates an increased potential for oxidative stress, though the specific reactions involving these products remains elusive [18]. Besides auto-oxidation, Hb may also remove Nitric Oxide (NO) and react with inorganic and organic peroxides and lipids, leading to the generation of oxygen-, heme-, and globin based radicals as well as other mediators [19].

Interaction of Cell-Free Hb with NO

In recent years, a great deal of interest has been directed towards the reaction of NO with Hb [20]. Interaction of Hb with NO is among the most studied aspect of Hb induced toxicity. The consumption of NO and subsequent oxidation of Hb occur through mechanisms either by NO dioxygenation of oxy-Hb, which generates nitrate (NO_3^-) and ferric Hb (Hb-Fe^{3+}) or by nitrosylation of iron in deoxy-Hb, which occurs when NO directly binds to iron in non-liganded ferrous form [8]. The Hb-Fe^{3+} formed in the blood vessels can readily accumulate in tissues as well as in circulation inducing oxidative stress [21]. Free Hb in plasma scavanges NO thereby reducing the availability of NO for various other essential physiological functions especially regulation of vascular tone and vasodilatation [22,23], relaxation of smooth muscle, neutrophil adhesion to endothelial cells and platelet activation etc. [24]. Moreover, reaction of superoxide to NO results in generation of peroxynitrite which can oxidize oxy-Hb forming ferryl-Hb [25]. Hemolysis also results in loss of erythrocyte arginase, an enzyme that metabolizes l-arginine. Reduction in the amount of l-arginine available for conversion to NO, further contributes to endothelial dysfunction [26,27]. Besides, the effects of NO on the oxidation and nitrate generation by free-Hb are also considered to be the inducer for cytotoxicity.

Heme Mediated Toxicity

The free Hb interacts with NO to generate Hb-Fe^{3+} in tissues, which further promotes release of heme and its transfer to other proteins/lipids leading to secondary toxicity. Heme transfers its reactive porphyrin to membranes or soluble plasma and cell proteins and lipids resulting in formation of heme-albumin or heme lipid complexes. Free heme acts as a ligand for molecular signalling and interactions [8,28]. Under specific physiological conditions free-iron protoporphyrin of heme can act as an intermediate to transform recipient molecule into a reactive end product. The most identifiable toxic end product of heme in plasma is the oxidized low density lipoprotein (oxLDL) [29]. LDL oxidation and associated inflammatory and cytotoxic effects mediated by cell-free Hb contributes significantly to the vascular injury [29,30]. Heme can alter cell activation state and physiology by selectively binding to several receptors, transcription factors and enzymes. Interaction of heme with Bach-1 (a transcriptional repressor of heme-oxygenase 1 or HO-1) is well characterized and seems crucial for clearance of increased level of intravascular Hb [31]. Heme is also reported as a ligand for nuclear receptor REV-ERB-αβ thereby playing important roles in regulation of circadian rhythm, adipogenesis and metabolism [32]. Reversible inhibition of proteosome activity by heme has also been reported [33,34]. These interactions of heme might outline several new roles for heme in cell-free Hb induced toxicity in biological systems.

Clearing of Free-Hb and its Derivatives by Immune System

Body harbors a repertoire of scavenger proteins, receptors, and enzymes that accomplishes mechanisms related to clearance and detoxification of cell-free Hb and heme. The clearance pathway primarily involves the transport of cell-free Hb to the liver or macrophages, breakdown of the porphyrin by the HO-1 system into bilirubin and carbon monoxide and ultimately the recovery of the iron for *de novo* erythropoiesis. The Hb scavenger system comprises of several soluble plasma proteins viz. haptoglobin (Hp) and hemopexin (Hpx) along with the cellular receptors that bind protein-Hb, or protein-heme complexes and provide protection against systemic Hb and heme toxicity. The most studied pathway involved the plasma Hb scavenger Hp and the monocyte/macrophage Hb-Hp scavenger receptor CD163. Endocytosis of the Hb-Hp complex results in release of heme and up-regulation of hemoxygenase-1 (HO-1) thereby providing evidence for HO-1 dependent macrophage mediated clearance of heme. The scavenging of free-Hb by these phagocytes can be modulated by anti-inflammatory cytokines such as IL-4 and IL-10. Recently Du et al. identified an Hb interactome comprising of Hb, Hp and lipid-free apolipoprotein A-I (apoAI). Apolipoprotein A-I, acts as a secondary antioxidant that interacts with Hb and quench the redox activity of Hb. It facilitates the uptake of Hb by interacting with a scavenger receptor class B type 1 (SR-B1) exhibited on macrophages and hepatocytes suggesting a novel receptor-mediated mechanism for clearance of cytotoxic Hb from plasma.

Hpx, the primary scavenger for free heme complements the plasma Hb-binding capacity provided by Hp. Mainly expressed in the liver Hpx is classified as class I acute phase protein induced by pro-inflammatory cytokines (i.e., IL-1 and IL-6). Interaction of free heme with Hpx is reported to be the strongest among the plasma heme-protein interaction. Heme-Hpx binding and its subsequent uptake by the macrophage-associated LRP-1 receptor removes the heme from the circulation. The relevance of Hpx in clearance of cell-free Hb was highlighted in Hp/Hpx double knockout mice that are susceptible to severe liver inflammation, splenomegaly, and Hb induced oxidative damages. The Hb:Hp and heme:Hpx complexes are finally cleared by their respective endocytosis receptors CD163 and CD91/LRP before they are degraded by lysosomal proteases.

Pathophysiology of the Hb-Induced Oxidative Stress

Being an oxygen carrier, Hb may act as a signalling molecule, activating oxygen sensitive transcription factors and target genes. Nevertheless, excessive cell-free Hb pose a severe pathological risk for vital systems, including renal, cardiovascular, gastrointestinal, neural, immunologic, coagulation related and many others [20,35]. Vasculature, particularly the endothelial cells due to their direct contact with plasma borne Hb, is among the most sensitive targets of the oxidation products generated by cell-free Hb. Cell-free Hb induced ROS increases pulmonary micro vascular endothelial permeability through an oxidant-dependent, mitochondrial-mediated pathway [36]. Interactions of Hb with hydrogen peroxide are known to alter thiol levels that might modulate endothelial cell

survival [37]. Accumulation of Hb dimers in kidney tubules results in acute renal dysfunction which can be attributed to Hb associated oxidative reactions [38]. Studies explain that the Hb derivative, heme stimulate the secretion of chemokine Monocyte Chemo-Attractant Protein-1 (MCP-1) and Transforming Growth Factor b1 isoform1 (TGF beta-1) via NF-κB dependent pathway [39,40]. Also MetHb has been reported to stimulate lung epithelial cell to secrete chemokines via NF-κB and MAPK dependent pathway [41] suggesting a crucial pro-inflammatory role of cell-free Hb in development of acute lung injury.

At cellular level, heme and the ferryl radical can damage DNA and oxidize lipids and proteins, and thus perturb the cellular integrity. The intrinsic peroxidase activity of Hb also contribute to the oxidation of LDL and Polyunsaturated Fatty Acids (PUFAs) present in cell membrane, and Induces Intercellular Adhesion Molecule-1 (ICAM-1) and Vascular Cell Adhesion Molecule-1 (VCAM-1) [42]. It also impairs the activity of cytosolic enzymes such as glucose-6-phosphate dehydrogenase and glutathione reductase. Heme is also known to induce activation of cell-damaging enzymes such as caspases and cathepsins [43,44]. Apart from the above mentioned functions, the oxidized Hb potentially modulates immune cell functions [24]. The free heme increases number of regulatory T cells (Treg) and promotes neutrophil activation, which further leads to inflammation and tissue extravasation [24]. The free Hb increases the survivability of neutrophils and prevents apoptosis via HO-1 and ferretin dependent mechanism. Recent report from our laboratory describes that the circulating monocytes are transformed into pro-inflammatory subsets, when engulf Hb activated platelets [45].

Cell-Free Hb Modulates Immune Responses

Cell-free Hb acts as host-derived Danger-Associated Molecular Patterns (DAMPs) that interacts with the components of innate immune system either directly or via binding to Pathogen-Associated Molecular Patterns (PAMPs). PAMPs (LPS and LTA) trigger the pseudo-peroxidase activity of Hb resulting in superoxide anion production which enhances the inflammatory potential of cell-free Hb [46]. The components of innate immune system (dendritic cells/DC, macrophages, monocytes and neutrophils) have been reported to exhibit altered phenotypes and functional responses in hemolytic conditions. Reports also highlight the effect of cell-free Hb in altering function of T and B lymphocytes [47]. Among Hb degradation products, heme has been reported to exhibit several pro-inflammatory activities related to leukocyte activation, and migration, expression of adhesion molecules and induction of cytokines and acute phase response proteins [48]. Interestingly, mammalian cells have been reported to express surface protein that can bind heme [49]. Neutrophils among the immune cells are first to respond to infection or damage. The migration of neutrophils to the site of inflammation is mediated by PAMPs or DAMPs derived from disrupted host cells. Heme has been shown to induce ROS and stimulate neutrophil migration utilizing the signature signalling pathways characteristic of chemoattractant molecules suggesting its role in amplification of inflammatory responses [50]. Oxygen/nitrogen based reactive species are also known to regulate Toll-like receptor (TLR)-mediated signalling [51,52]. Heme has been shown to utilize pattern recognition receptors like TLRs (Toll like Receptors) to mediate activation of the cells of innate immune system. Heme also induces oxidative burst, neutrophil recruitment using TLR4 signalling mechanisms [52]. Similarly, the MetHb also behaves as DAMPs, but unlike heme it signals through TLR2 mediated NF-kB dependent pathway to stimulate neutrophil ROS production and induce pro-inflammatory cytokines and neutrophil apoptosis [53]. Heme induces assembly of p62/SQTM1 ubiquitinated proteins known as Aggresome-Like Induced Structures (ALIS). ALIS formation is particularly mediated by ROS generated in response to Hb degradation products through activation of the transcription factor NRF2 thereby modulating heme induced inflammation [54]. Recent study from Vinchi and colleagues showed that heme and its iron moiety, brings about phenotype change in macrophages leading them toward M1-like proinflammatory phenotype, a response that is specifically mediated by TLR4 signalling and ROS [55]. Heme *via* modulation of HO-1 also regulates DC maturation and function by modulating the p38 MAPK-CREB/ATF1 signalling [56]. Also there are reports that suggest that ROS induced by heme is responsible for necrotic cell death of macrophages via TNF-α dependent mechanism [57]. Several other studies have shown that Hb treatment with multiple TLR ligands or PAMPs synergistically induces cytokines, such as IL-1b, IL-6, IL-8, and TNF-α in macrophages [58,59]. The heme induced levels of ROS apart from inducing cell death might prime the responsiveness of cells of the innate immune system [60]. Taken together, all these reports collectively suggest a high likelihood of degradation products of Hb binding to certain DAMPs/PAMPs thereby inducing ROS that can further enhance the pro-inflammatory signalling in the immune cells.

Hb Triggers Inflammatory Responses via Oxidative Stress

The pro-oxidative properties the free Hb also triggers inflammatory signaling [21] which further impact the functional state and responsiveness of immune cells and acts as a causal agent for cell damage. Heme induced ROS has been shown to induce expression of pro-inflammatory adhesion molecules on endothelium and blood cells and increase vascular permeability leading to greater leukocyte influx [61]. It also interacts with the cells of innate immune with unknown mechanism and contributes to the pathogenesis of several diseases [21,62]. Neutrophils in response to heme have been shown to generate ROS through NADPH oxidase-dependent Protein Kinase C (PKC) activity leading to cytoskeleton reorganization [63]. Heme has potential role in activation and acceleration of inflammation in both sterile and infectious condition [62] and triggers inflammasome activation playing active role in pathogenesis of hemolytic disorders [62]. Inflammasome, an innate immune component, act as receptor and sensor for PAMPs and DAMPs and regulate the caspase-1 activation which facilitate chronic inflammation [64]. It consists of several Pattern Recognition Receptors (PRRs) including NOD-like Receptors, (NLRs) and the Absent in Melanoma 2 (AIM)-like Receptors (ALRs) which oligomerize to form caspase-1-activating scaffold and cleave the IL-1 family cytokines to generate active form of IL1b and IL-18. The continuous presence of these cytokine leads to chronic inflammation and cell death (pyrotopsis) [62,65,66]. The general and common molecules which oligomerise to form inflammasome in cytosol are Apoptosis-associated Speck-like protein (ASC) and Caspase Activation and Recruitment Domain (CARD). ASC multimers interact with CARD and upstream sensor molecule which act as scaffold in activation of procaspase-1 and initiate the chronic inflammatory cascade [67]. Depending upon

the nature of interacting receptors in inflammasome complex it is broadly categorized into two major forms, canonical and non-canonical inflammasome. NLRP1, NLRP3, NLRC4, AIM2 and pyrin which comprises of Nucleotide Oligomerization Domain (NOD), Leucine-Rcich Repeat (LRR)-containing proteins comes under canonical forms. Non-canonical inflammasome however, are not well characterized but complement the function of canonical inflammasome in both mice and human by targeting caspase-11 and caspase-4 and/or caspase-5 respectively [66,68].

Heme, an auto-oxidation product of Hb induces the activation of NLRP3 inflammasome. Heme stimulates spleen tyrosine kinase (syk) via an unknown receptor involved in direct alterations of lipid rafts leading to mitochondrial ROS generation and NLRP3 activation [12]. The hemolysis increases the cell-free Hb which has inflammatory effects and act as one of the cause of cell death. The activation of inflammatory cascade by heme specifically in macrophages primed with LPS enhances the IL1b processing via nucleotide-binding domain and Leucine Rich Repeat (LRR) containing family, pyrin domain containing 3 (NLRP3). The activation of NLRP3 via heme in macrophages depends on spleen tyrosine kinase, NADPH oxidase-2, mitochondrial reactive oxygen species, and K^+ efflux. This finding suggests the potential role of heme in the induction of hemolysis induced-lethality [11].

Hb-Mediated Apoptosis of Immune Cells

The pro-apoptotic roles of free Hb have been suggested promoting various inflammatory impacts on immune cells. Apoptosis is a process, which is required for maintenance of cellular homeostasis. Besides having beneficial role, dysregulation in this cellular process can lead to tissue damage, chronic inflammation and can also promote cancer [69]. The free-Hb initiates inflammatory responses and the persistence inflammations may leads to apoptosis and cell death [70]. It is well documented that cell-free Hb leads to platelet activation and its apoptosis. Hb binds to platelets *via* GP1bα, which leads to its activation and enhanced expression of pro-apoptotic proteins Bak, Bax, cytochrome C and activated caspase-9 and caspase-3 [71]. The direct effect of cell-free Hb on immune cell apoptosis is yet to be explored. However, the impact of cell-free Hb on innate immune cells majorly on monocytes which is one of the important phagocytic cells of immune system has been explored by our group. Our study reported that the monocytes in hemolytic diseases such as PNH and SCD, show the elevation in expression of phosphatidylserine (PS). This finding was correlated with cell-free Hb and platelets activated with cell-free Hb in the *in vitro* study where we have treated the monocytes with Hb and Hb activated platelets and observed the same [45]. The finding of the other independent study suggest that cell-free Hb and its component i.e. heme has anti-apoptotic role in neutrophils, which is the innate immune cells and classically known as the first cell which migrates at the site of inflammation. Heme modulates expression of pro- and anti-apoptotic Bcl-2 family members. Heme induced Bcl-XL synthesis and promoted Bad degradation. Other study has shown that heme preserves mitochondria stability and shifts the Bcl- XL/Bad ratio in a ROS-dependent manner [72]. Heme has been shown to delay apoptosis in neutrophils. The effect is heme oxygenase and ROS dependent which protect the neutrophil apoptosis via ERK, PI3K and NF-kB dependent pathways. The delay in apoptosis or the increased life-span of neutrophil may have impact on the trigger of inflammatory response in the hemolytic conditions [72]. Heme induced ROS has been reported to destabilize macrophage membrane leading to necrosis. Moreover, heme activated TNFR1 and TLR4/MyD88-dependent production of tumor necrosis factor (TNF) has been suggested to induce macrophage necrosis [60].

Conclusion

The cell-free Hb as well as the derivaties of its oxidative products such as heme is a hallmark of hemolysis that may laed to various clinical consequences in diseases such as SCD, PNH, polycythemia, and drug-induced hemolytic anemia. Extensive survey of literature suggests that pro-oxidant, cytotoxic and inflammatory attributes of the Hb derivatives play crucial role in the pathogenesis of various cellular functions including immune responses. However, a clear insight into the mechanisms of the patho-physiology of oxidative damages induced by the derivatives of Hb is challanging. Although several studies have described the mechanism of inflammasome activation and immune modulation by the Hb/heme induced oxidative stress but the crucial gaps for developing targeted therapeutics to counteract these clinical consequences in hemolytic conditions remains unfilled.

Several studies have explored the use of scavenger proteins like haptoglobin (Hp) and hemopexin (Hpx) as therapeutic agents to abrogate the cytotoxic effect of cell-free Hb and heme. Apart from using scavenger protein as a part of treatment regime, therapeutic strategies should be developed to utilize anti-oxidants or other drugs exhibiting anti-oxidant and anti-inflammatory properties to attenuate the damaging effects of cell-free Hb induced ROS. Considering the anti-inflammatory potential of steroids, use of glucocorticoids is being explored for treatment of hemolysis induced inflammations in PNH and SCD. Furthermore, Vallelian et al. reported that glucocorticoid therapy in patients polarized monocytes into a M2/alternatively activated phenotype with higher expression of Hb-scavenger receptor (CD163) further enhancing its Hb clearance capacity [73]. The synthetic anti-oxidants like carbazole and its derivatives as therapeutics can be potential but are of limited use because of its toxicity [74]. In addition, anti-oxidants such as vitamin E and polyphenols when given together with iron chelators, may provide a substantial improvement in the pathophysiology of hemolytic anaemias and particularly in thalassemia and may also prove important in ameliorating oxidative stress parameters [75]. Flavonoids (like kaempferol, quercetin, morin and rutin) can be used as natural antioxidants for the treatment and prevention of patho-physiological conditions which occurs as a result of oxidative stress during red blood cell hemolysis [76,77] has been used in the treatment of anemia. All these reports culminate to conclusion that more extensive investigations are needed to understand the pathways that can be therapeutically targeted for treating pathological conditions associated with intravascular hemolysis.

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References

1. Janz DR, Ware LB. The role of red blood cells and cell-free hemoglobin in the pathogenesis of ARDS. J Intensive Care. 2015; 3: 20.
2. Qian Q. Hemolysis and acute kidney failure. Am J Kidney Dis. 2010; 56: 780-784.
3. Baek JH. Hemoglobin-driven pathophysiology is an in vivo consequence of the red blood cell storage lesion that can be attenuated in guinea pigs by haptoglobin therapy. J Clin Invest. 2012; 122: 1444-1458.
4. Gladwin MT, Kanias T, Kim-Shapiro DB. Hemolysis and cell-free hemoglobin drive an intrinsic mechanism for human disease. J Clin Invest. 2012; 122: 1205-1208.
5. Alayash AI. Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? Nat Biotechnol. 1999; 17: 545-549.
6. Motterlini R. Oxidative-stress response in vascular endothelial cells exposed to acellular hemoglobin solutions. Am J Physiol. 1995; 269: 648-655.
7. Bastarache JA, Roberts LJ 2nd, Ware LB. Thinking outside the cell: how cell-free hemoglobin can potentiate acute lung injury. Am J Physiol Lung Cell Mol Physiol. 2014; 306: 231-232.
8. Schaer DJ. Hemolysis and free hemoglobin revisited: exploring hemoglobin and hemin scavengers as a novel class of therapeutic proteins. Blood. 2013; 121: 1276-1284.
9. Nakai K. Permeability characteristics of hemoglobin derivatives across cultured endothelial cell monolayers. J Lab Clin Med. 1998; 132: 313-319.
10. Chirico EN, Pialoux V. Role of oxidative stress in the pathogenesis of sickle cell disease. IUBMB Life. 2012; 64: 72-80.
11. Dutra FF. Hemolysis-induced lethality involves inflammasome activation by heme. Proc Natl Acad Sci U S A. 2014; 111: 110-118.
12. Sadrzadeh SM. Hemoglobin. A biologic fenton reagent. J Biol Chem. 1984; 259: 14354-14356.
13. Kohen R, Nyska A. Oxidation of biological systems: oxidative stress phenomena, antioxidants, redox reactions, and methods for their quantification. Toxicol Pathol. 2002; 30: 620-650.
14. Rifkind JM, Mohanty JG, Nagababu E. The pathophysiology of extracellular hemoglobin associated with enhanced oxidative reactions. Front Physiol. 2014; 5: 500.
15. Kawano T, Hosoya H. Oxidative burst by acellular haemoglobin and neurotransmitters. Med Hypotheses. 2002; 59: 11-15.
16. Hebbel RP. Auto-oxidation and a membrane-associated 'Fenton reagent': a possible explanation for development of membrane lesions in sickle erythrocytes. Clin Haematol. 1985; 14: 129-140.
17. Nagababu E, Rifkind JM. Reaction of hydrogen peroxide with ferrylhemoglobin: superoxide production and heme degradation. Biochemistry. 2000; 39: 12503-12511.
18. Nagababu E. Heme degradation and oxidative stress in murine models for hemoglobinopathies: thalassemia, sickle cell disease and hemoglobin C disease. Blood Cells Mol Dis. 2008; 41: 60-66.
19. Simoni J, Simoni G, Moeller JF. Intrinsic toxicity of hemoglobin: how to counteract it. Artif Organs. 2009; 33: 100-109.
20. Rother RP, Bell L, Hillmen P, Gladwin MT. The clinical sequelae of intravascular hemolysis and extracellular plasma hemoglobin: a novel mechanism of human disease. JAMA. 2005; 293: 1653-1662.
21. Schaer DJ, Buehler PW. Cell-free hemoglobin and its scavenger proteins: new disease models leading the way to targeted therapies. Cold Spring Harb Perspect Med. 2013; 3.
22. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO 3rd, Schechter AN, et al. Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. Nat Med. 2002; 8: 1383-1389.
23. Yeo TW, Lampah DA, Tjitra E, Gitawati R, Kenangalem E, Piera K, et al. Relationship of cell-free hemoglobin to impaired endothelial nitric oxide bioavailability and perfusion in severe falciparum malaria. J Infect Dis. 2009; 200: 1522-1529.
24. Dutra FF, Bozza MT. Heme on innate immunity and inflammation. Front Pharmacol. 2014; 5: 115.
25. Romero N, Radi R, Linares E, Augusto O, Detweiler CD, Mason RP, et al. Reaction of human hemoglobin with peroxynitrite. Isomerization to nitrate and secondary formation of protein radicals. J Biol Chem. 2003; 278: 44049-44057.
26. Yeo TW, Lampah DA, Gitawati R, Tjitra E, Kenangalem E, McNeil YR. Recovery of endothelial function in severe falciparum malaria: relationship with improvement in plasma L-arginine and blood lactate concentrations. J Infect Dis. 2008; 198: 602-608.
27. Morris CR, Kuypers FA, Kato GJ, Lavrisha L, Larkin S, Singer T, et al. Hemolysis-associated pulmonary hypertension in thalassemia. Ann N Y Acad Sci. 2005; 1054: 481-485.
28. Miller YI, Shaklai N. Kinetics of hemin distribution in plasma reveals its role in lipoprotein oxidation. Biochim Biophys Acta. 1999; 1454: 153-164.
29. Jeney V, Balla J, Yachie A, Varga Z, Vercellotti GM, Eaton JW, et al. Pro-oxidant and cytotoxic effects of circulating heme. Blood. 2002; 100: 879-887.
30. Nagy E, Eaton JW, Jeney V, Soares MP, Varga Z, Galajda Z, et al. Red cells, hemoglobin, heme, iron, and atherogenesis. Arterioscler Thromb Vasc Biol. 2010; 30: 1347-1353.
31. Ogawa K, Sun J, Taketani S, Nakajima O, Nishitani C, Sassa S, et al. Heme mediates derepression of Maf recognition element through direct binding to transcription repressor Bach1. EMBO J. 2001; 20: 2835-2843.
32. Raghuram S, Stayrook KR, Huang P, Rogers PM, Nosie AK, McClure DB, et al. Identification of heme as the ligand for the orphan nuclear receptors REV-ERBalpha and REV-ERBbeta. Nat Struct Mol Biol. 2007; 14: 1207-1213.
33. Tanaka K, Ichihara A. Autodegradation of rat liver proteasomes (large multicatalytic proteinase complexes). Biochem Biophys Res Commun. 1989; 158: 548-554.
34. Santoro AM, Lo Giudice MC, D'Urso A, Lauceri R, Purrello R, Milardi D. Cationic porphyrins are reversible proteasome inhibitors. J Am Chem Soc. 2012; 134: 10451-10457.
35. Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM. Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. JAMA. 2008; 299: 2304-2312.
36. Kuck JL, Dikalov SI, Fessel JP, Shaver CM, Bastarache JA, Ware L. Cell-Free Hemoglobin Causes Oxidative Stress, Mitochondrial Dysfunction, and Increased Endothelial Permeability in Human Pulmonary Microvascular Endothelial Cells. Am J Respir Crit Care Med. 2016; 193: A4777.
37. D'Agnillo F, Alayash AI. Interactions of hemoglobin with hydrogen peroxide alters thiol levels and course of endothelial cell death. Am J Physiol Heart Circ Physiol. 2000; 279: H1880-H1889.
38. Ackers GK, Halvorson HR. The linkage between oxygenation and subunit dissociation in human hemoglobin. Proc Natl Acad Sci U S A, 1974; 71: 4312-4316.
39. Kanakiriya SK, Croatt AJ, Haggard JJ, Ingelfinger JR, Tang SS, Alam J, et al. Heme: a novel inducer of MCP-1 through HO-dependent and HO-independent mechanisms. Am J Physiol Renal Physiol. 2003; 284: F546-F554.
40. Tracz MJ, Alam J, Nath KA. Physiology and pathophysiology of heme: implications for kidney disease. J Am Soc Nephrol. 2007; 18: 414-420.
41. Mumby S, Ramakrishnan L, Evans TW, Griffiths MJD, Quinlan GJ. Methemoglobin-induced signaling and chemokine responses in human alveolar epithelial cells. Am J Physiol Lung Cell Mol Physiol. 2014; 306: L88-L100.

42. Cheng TM, Mao SJ, Lai ST, Chang CC, Yang MC, Chen NC, et al. Haemoglobin-induced oxidative stress is associated with both endogenous peroxidase activity and H2O2 generation from polyunsaturated fatty acids. Free Radic Res. 2011; 45: 303-316.

43. Tracz MJ, Juncos JP, Grande JP, Croatt AJ, Ackerman AW, Rajagopalan G, et al. Renal hemodynamic, inflammatory, and apoptotic responses to lipopolysaccharide in HO-1-/- mice. Am J Pathol. 2007; 170: 1820-1830.

44. Su D, Roth RI, Yoshida M, Levin J. Hemoglobin increases mortality from bacterial endotoxin. Infect Immun. 1997; 65: 1258-1266.

45. Singhal R, Chawla S, Rathore DK, Bhasym A, Annarapu GK, Sharma V, et al. Development of pro-inflammatory phenotype in monocytes after engulfing Hb-activated platelets in hemolytic disorders. Clin Immunol. 2017; 175: 133-142.

46. Jiang N, Tan NS, Ho B, Ding JL. Respiratory protein-generated reactive oxygen species as an antimicrobial strategy. Nat Immunol. 2007; 8: 1114-1122.

47. Bao W, Zhong H, Manwani D, Vasovic L, Uehlinger J, Lee MT. Regulatory B-cell compartment in transfused alloimmunized and non-alloimmunized patients with sickle cell disease. Am J Hematol. 2013; 88: 736-740.

48. Wagener FA, Volk HD, Willis D, Abraham NG, Soares MP, Adema GJ, et al. Different faces of the heme-heme oxygenase system in inflammation. Pharmacol Rev. 2003; 55: 551-571.

49. Shayeghi M, Latunde-Dada GO, Oakhill JS, Laftah AH, Takeuchi K, Halliday N, et al. Identification of an intestinal heme transporter. Cell. 2005; 122: 789-801.

50. Porto BN, Alves LS, Fernández PL, Dutra TP, Figueiredo RT, Graça-Souza AV, et al. Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors. J Biol Chem. 2007; 282: 24430-24436.

51. Nicholas SA, Sumbayev VV. The role of redox-dependent mechanisms in the downregulation of ligand-induced Toll-like receptors 7, 8 and 4-mediated HIF-1 alpha prolyl hydroxylation. Immunol Cell Biol. 2010; 88: 180-186.

52. Figueiredo RT, Fernandez PL, Mourao-Sa DS, Porto BN, Dutra FF, Alves LS, et al. Characterization of heme as activator of Toll-like receptor 4. J Biol Chem. 2007; 282: 20221-20229.

53. Bastarache JA, Wynn JL, Ware LB. Fanning the Fire: Can Methemoglobin Enhance Neutrophil Activation? EBioMedicine. 2015; 2: 184-185.

54. Vasconcellos LR, Dutra FF, Siqueira MS, Paula-Neto HA, Dahan J, Kiarely E, et al. Protein aggregation as a cellular response to oxidative stress induced by heme and iron. Proc Natl Acad Sci U S A. 2016; 113: E7474-E7482.

55. Vinchi F, Costa da Silva M, Ingoglia G, Petrillo S, Brinkman N, Zuercher A, et al. Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. Blood. 2016; 127: 473-486.

56. Al-Huseini LM, Aw Yeang HX, Hamdam JM, Sethu S, Alhumeed N, Wong W, et al. Heme oxygenase-1 regulates dendritic cell function through modulation of p38 MAPK-CREB/ATF1 signaling. J Biol Chem. 2014; 289: 16442-16451.

57. Fortes GB, Alves LS, de Oliveira R, Dutra FF, Rodrigues D, Fernandez PL, et al. Heme induces programmed necrosis on macrophages through autocrine TNF and ROS production. Blood. 2012; 119: 2368-2375.

58. Bodet C, Chandad F, Grenier D. Hemoglobin and LPS act in synergy to amplify the inflammatory response. J Dent Res. 2007; 86: 878-882.

59. Lin T, Kwak YH, Sammy F, He P, Sujatha T, Sun G, et al. Synergistic inflammation is induced by blood degradation products with microbial Toll-like receptor agonists and is blocked by hemopexin. J Infect Dis. 2010; 202: 624-632.

60. Powers KA, Szászi K, Khadaroo RG, Tawadros PS, Marshall JC, Kapus A, et al. Oxidative stress generated by hemorrhagic shock recruits Toll-like receptor 4 to the plasma membrane in macrophages. J Exp Med. 2006; 203: 1951-1961.

61. Wagner KR, Packard BA, Hall CL, Smulian AG, Linke MJ, De Courten-Myers GM, et al. Protein oxidation and heme oxygenase-1 induction in porcine white matter following intracerebral infusions of whole blood or plasma. Dev Neurosci. 2002; 24: 154-160.

62. Daniels MJ, Brough D. Unconventional Pathways of Secretion Contribute to Inflammation. Int J Mol Sci. 2017; 18.

63. Graça-Souza AV, Arruda MA, de Freitas MS, Barja-Fidalgo C, Oliveira PL. Neutrophil activation by heme: implications for inflammatory processes. Blood. 2002; 99: 4160-4165.

64. Guo H, Callaway JB, Ting JP. Inflammasomes: mechanism of action, role in disease, and therapeutics. Nat Med. 2015; 21: 677-687.

65. Strowig T, Henao-Mejia J, Elinav E, Flavell R. Inflammasomes in health and disease. Nature. 2012; 481: 278-286.

66. Lamkanfi M, Dixit VM. Inflammasomes and their roles in health and disease. Annu Rev Cell Dev Biol. 2012; 28: 137-161.

67. Latz E, Xiao TS, Stutz A. Activation and regulation of the inflammasomes. Nat Rev Immunol. 2013; 13: 397-411.

68. Broz P, Dixit VM. Inflammasomes: mechanism of assembly, regulation and signalling. Nat Rev Immunol. 2016; 16: 407-420.

69. Ichim G, Tait SW. A fate worse than death: apoptosis as an oncogenic process. Nat Rev Cancer. 2016; 16: 539-548.

70. Gram M, Sveinsdottir S, Cinthio M, Sveinsdottir K, Hansson SR, Mörgelin M, et al. Extracellular hemoglobin - mediator of inflammation and cell death in the choroid plexus following preterm intraventricular hemorrhage. J Neuroinflammation. 2014; 11: 200.

71. Singhal R, Annarapu GK, Pandey A, Chawla S, Ojha A, Gupta A, et al. Hemoglobin interaction with GP1balpha induces platelet activation and apoptosis: a novel mechanism associated with intravascular hemolysis. Haematologica. 2015; 100: 1526-1533.

72. Arruda MA, Rossi AG, de Freitas MS, Barja-Fidalgo C, Graça-Souza AV. Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-kappaB. J Immunol. 2004; 173: 2023-2030.

73. Tang YZ, Liu ZQ. Free-radical-scavenging effect of carbazole derivatives on AAPH-induced hemolysis of human erythrocytes. Bioorg Med Chem. 2007; 15: 1903-1913.

74. Fibach E, Rachmilewitz EA. The role of antioxidants and iron chelators in the treatment of oxidative stress in thalassemia. Ann N Y Acad Sci. 2010; 1202: 10-16.

75. Vallelian F, Schaer CA, Kaempfer T, Gehrig P, Duerst E, Schoedon G, et al. Glucocorticoid treatment skews human monocyte differentiation into a hemoglobin-clearance phenotype with enhanced heme-iron recycling and antioxidant capacity. Blood. 2010; 116: 5347-5356.

76. Asgary S, Naderi G, Askari N. Protective effect of flavonoids against red blood cell hemolysis by free radicals. Exp Clin Cardiol. 2005; 10: 88-90.

77. Banerjee A, Kunwar A, Mishra B, Priyadarsini KI. Concentration dependent antioxidant/pro-oxidant activity of curcumin studies from AAPH induced hemolysis of RBCs. Chem Biol Interact. 2008; 174: 134-139.