REVIEW ARTICLE

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Cell-Free Hemoglobin in the Pathophysiology of Trauma: A Scoping Review

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OBJECTIVES: Cell-free hemoglobin (CFH) is a potent mediator of endothelial dysfunction, organ injury, coagulopathy, and immunomodulation in hemolysis. These mechanisms have been demonstrated in patients with sepsis, hemoglobinopathies, and those receiving transfusions. However, less is known about the role of CFH in the pathophysiology of trauma, despite the release of equivalent levels of free hemoglobin.

DATA SOURCES: Ovid MEDLINE, Embase, Web of Science Core Collection, and BIOSIS Previews were searched up to January 21, 2023, using key terms related to free hemoglobin and trauma.

DATA EXTRACTION: Two independent reviewers selected studies focused on hemolysis in trauma patients, hemoglobin breakdown products, hemoglobin-mediated injury in trauma, transfusion, sepsis, or therapeutics.

DATA SYNTHESIS: Data from the selected studies and their references were synthesized into a narrative review.

CONCLUSIONS: Free hemoglobin likely plays a role in endothelial dysfunction, organ injury, coagulopathy, and immune dysfunction in polytrauma. This is a compelling area of investigation as multiple existing therapeutics effectively block these pathways.

KEYWORDS: haptoglobin; hemolysis; hemopexin; multiple trauma; oxidative str ess

BACKGROUND

Cell-free hemoglobin (CFH) is a potent mediator of endothelial and organ injury in sepsis and other hemolytic disorders, but its effects on the pathophysiology of trauma are less well understood. Hemoglobin is released from hemolyzed RBCs in a wide range of pathologic states and during RBC storage (1). In physiologic circumstances, this "cell-free" hemoglobin is cleared by robust scavenging mechanisms. However, when these systems are overwhelmed, CFH in the blood and its breakdown products scavenge nitric oxide, mediate oxidative damage, and have widespread transcriptional and immunomodulatory effects. Increased levels of CFH are associated with mortality in sepsis (2, 3), where CFH is a critical mediator in endothelial dysfunction (4), acute kidney injury (5), and acute lung injury (4).

The role of CFH in the pathophysiology of trauma is potentially complex. Major trauma produces CFH levels equivalent to those shown to induce organ injury in sepsis (6, 7). Markers of hemolysis in trauma patients correlate with injury severity and are associated with worse outcomes (8). In addition, the effects of endogenous hemolysis may be compounded by the transfusion of stored RBCs (9). CFH is released from stored RBCs and, when transfused, causes endothelial dysfunction and oxidative injury through the

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KEY POINTS

Question: What is known about the role of hemolysis in the pathophysiology of trauma, and what can we take from our understanding of the impact of hemolysis in sepsis and hemoglobinopathies to inform the care of critically injured trauma patients?

Findings: Hemolysis associated with major trauma and transfusion produces sufficient free hemoglobin to overwhelm the body's scavenging mechanisms. Free hemoglobin is a critical mediator of endothelial dysfunction, kidney, and lung injury in sepsis and hemolytic disorders and likely plays a role in polytrauma.

Meaning: Understanding the timing and impact of hemolysis in trauma could open up a new treatment strategy for severely injured patients as existing therapeutics effectively target these pathways.

same mechanisms that have been demonstrated in sepsis (7). However, the role of endogenous CFH as a mediator of organ injury in trauma patients, and the potential contributions and interactions of endogenous CFH and transfused CFH are not well understood. CFH is a compelling target of investigation in trauma not only because of the significant recent advances in our understanding of its role in sepsis and hemoglobinopathies but also because data from multiple animal and observational studies demonstrate that existing therapeutics effectively alter these pathways (2, 10). A scoping review format was chosen to best define our current understanding of the pathophysiologic effects of CFH and its products and to identify the major gaps in our knowledge of how these effects might affect patients following traumatic injury.

Therefore, the aims of this scoping review are three-fold. First, to describe the pathophysiology of CFH-mediated injury in hemolysis, highlighting mechanisms that have been demonstrated in trauma or sepsis. Second, to review the experimental evidence for the role of CFH in transfusion-related injury in trauma. Lastly, to review the available animal and clinical studies on the use of existing therapeutics to prevent or treat CFH-mediated injury in trauma.

MATERIALS AND METHODS

Search Strategy

The search strategy was developed by a medical librarian (A.C.S.) and based on the Preferred Reporting Items for Systematic Reviews and Meta-Analysis (PRISMA) extension for Scoping Reviews checklist (11). On January 21, 2023, a systematic search of the following databases was conducted: Ovid MEDLINE, Embase (Elsevier), Web of Science Core Collection, and BIOSIS Previews (Clarivate Web of Science). Eligibility criteria included English-language journal articles, without date restriction. After the Ovid MEDLINE search strategy was refined, translation to the other databases was partially automated using the Polyglot Search Translator (12). Reproducible search strategies are provided in **Supplemental file** (http://links.lww.com/CCX/B311).

Study Selection

The abstracts of all studies were reviewed by two investigators (J.T.R. and A.J.R.). Studies focused on hemolysis in trauma patients, hemoglobin breakdown products, hemoglobin-mediated injury in trauma, transfusion, or sepsis, or therapeutics aimed at these pathways were included. Studies focused on pregnancy, subarachnoid hemorrhage, extracorporeal membrane oxygenation or cardiopulmonary bypass, atherosclerosis, or cancer were excluded. Full texts were then independently screened by two reviewers (J.T.R. and A.J.R.). Studies that focused exclusively on structural hemoglobin variants, synthetic oxygen carriers, and bacterial-hemoglobin interactions were excluded. In vitro studies were excluded except for cell-culture experiments that directly addressed mechanisms of hemoglobin or heme-mediated injury. Relevant references from the selected manuscripts were also reviewed for inclusion.

RESULTS

A total of 4865 articles underwent initial screening and 290 articles were fully reviewed for eligibility. Of these, 51 studies and their relevant references were included in this review. See the PRISMA flow diagram in **Figure 1**.

Endogenous Mechanisms of Cell-Free Hemoglobin Clearance

CFH and its breakdown products are highly biologically active. Multiple systems have evolved to maintain

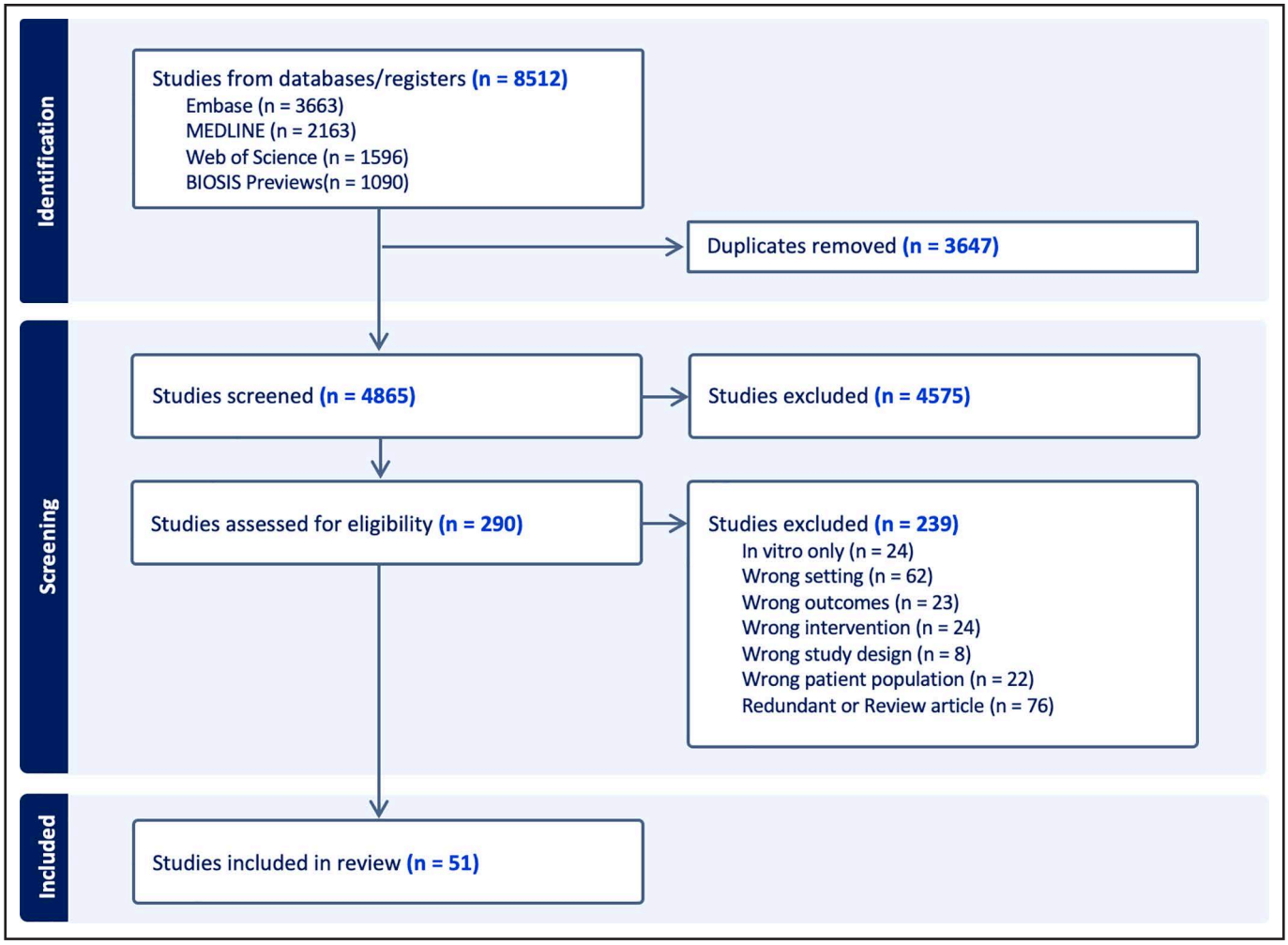

Figure 1. Preferred Reporting Items for Systematic Reviews and Meta-Analysis flow diagram.

the redox state of hemoglobin in RBCs and to neutralize and clear hemoglobin and its breakdown products that escape from damaged RBCs into circulation. These mechanisms are summarized in **Figure 2**.

Hemoglobin released from RBCs is scavenged by circulating haptoglobin, and taken up by monocytes and macrophages via CD163 (13, 14). Smaller amounts of hemoglobin are also taken up through megalin and cubilin receptors in the renal proximal tubule, and via apoA1 (15, 16). There are three haptoglobin genotypes in humans, that impact an individual's susceptibility to hemoglobin-mediated injury (21). When these systems are overwhelmed, CFH tetramers break down to dimers and ultimately release unbound or free heme, a single iron atom in a porphyrin ring. Cell-free heme is bound by circulating hemopexin, which is taken up by hepatocytes via the receptor CD91 (22). Heme also binds with lower affinity to albumin and lipoproteins in circulation, which contribute to its clearance (17).

Heme proteins taken up by macrophages, hepatocytes, and in the renal proximal tubules are broken down by a trio of enzymes (Nicotnamide adenine dinucleotide phosphate) cytochrome P450 reductase, heme oxygenase [HO], and biliverdin reductase) to biliverdin, carbon monoxide, and iron (23). Most of the residual iron is bound by ferritin, which prevents it from engaging in further Fenton and Haber-Weiss reactions (24). HO is the rate-limiting step in this series and the key point of regulation (25). The heme degradation products, biliverdin, and carbon monoxide, also have important anti-inflammatory and antioxidant effects (23).

The systems that exist to manage extracellular hemoglobin are redundant and tightly controlled. However, the degree of hemolysis that exists in major trauma, sepsis, and RBC transfusion is sufficient to overwhelm the capacity of these systems. The widespread pathologic effects of CFH and its breakdown products are outlined here and summarized in **Figure 3**.

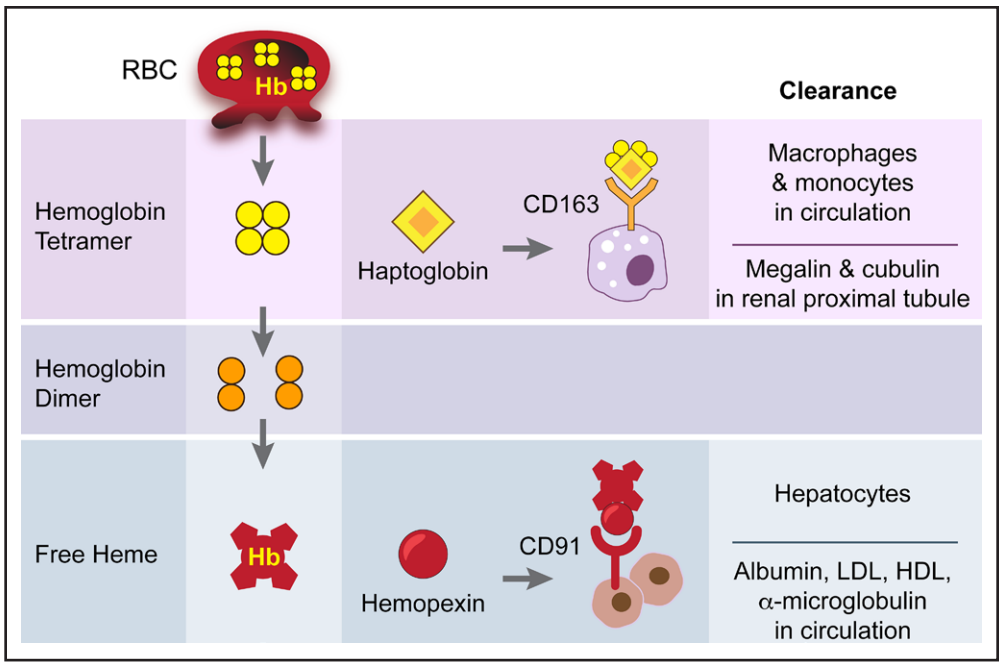

Figure 2. Endogenous mechanisms of cell-free hemoglobin and heme clearance. RBC lysis releases free hemoglobin. Cell-free hemoglobin is bound primarily by circulating haptoglobin, and the resulting hemoglobin–haptoglobin complexes are taken via CD163 (13, 14). Smaller quantities of cell-free hemoglobin are also bound by megalin and cubilin receptors in the renal proximal tubule (15), and by apoA1 (16). Cell-free hemoglobin that is not cleared breaks down to hemoglobin dimers, and ultimately to free heme, free heme is bound by circulating hemopexin and taken up via the receptor CD91 (17–19). Free heme also binds with low affinity to albumin, high-density lipoprotein (HDL), low-density lipoprotein (LDL), and α_1-microglobulin, all of which likely contribute to heme clearance (17, 20).

Pathophysiology of Cell-Free Hemoglobin-Mediated Injury

Depletion of Nitric Oxide. Oxygen-carrying hemoglobin reacts with nitric oxide to form methemoglobin and nitrate. This process is so rapid that reactions of nitric oxide with hemoglobin are thought to be the major pathway limiting nitric oxide bioavailability at the human vascular endothelium (26). Typically, the RBC membrane provides enough of a diffusion barrier to slow NO consumption. However, CFH rapidly consumes local nitric oxide. This effect is exacerbated by the release of arginase from damaged RBCs, which competes with nitric oxide synthase for their shared substrate, L-arginine (40).

Nitric oxide is a critical regulator of the vascular endothelium and its consumption has profound local effects (26). Constitutive production of nitric oxide by endothelial nitric oxide synthase downregulates the expression of endothelial adhesion molecules including P-selectin (41), and inhibits the synthesis of endothelin-1, a potent vasoconstrictor (42). Nitric oxide also inhibits platelet activation and thrombosis (26). Loss of this basal nitric oxide level contributes to vasoconstriction, endothelial dysfunction, platelet activation, adhesion, and thrombosis. Nitric oxide scavenging and its associated endothelial dysfunction and thrombosis are implicated in the adverse effects seen in trials of hemoglobin-based blood substitutes (1, 43).

Oxidative Injury. In RBCs, the central iron atom in hemoglobin is maintained in its redox 2^+ (ferrous) form by strict compartmentalization and a robust antioxidant system (37). Once free in circulation, hemoglobin in the 2^+ state is oxidized in reactions with nitric oxide (as described above), or through Fenton and Haber-Weiss reactions that produce free radicals from hydrogen peroxide. The resulting 3^+ (ferric) hemoglobin is unstable and can break down, either releasing free heme or further oxidizing to the 4^+ (ferryl) state.

Free heme is hydrophobic and intercalates readily into endothelial cell membranes. Here, it catalyzes peroxidation of the lipid membrane by H_2O_2 or by oxidants from activated neutrophils and macrophages (44). Ferryl (4^+) hemoglobin drives further Fenton, Haber-Weiss, and peroxidase reactions, including reacting directly with membrane phospholipids to form lipid peroxyl species (45). These reactive oxygen species (ROS) further deplete local nitric oxide. Heme also inhibits the proteasome, which is normally responsible for identifying and removing damaged proteins, contributing to the accumulation of lipid peroxide-modified proteins in the cell (46).

Endothelial Dysfunction. CFH and its products cause endothelial injury through oxidative stress, endothelial activation, and barrier dysfunction (28). Oxidative stress includes the lipid peroxidation described above, and activation of the nucleotide-binding domain, leucine-rich-containing family,

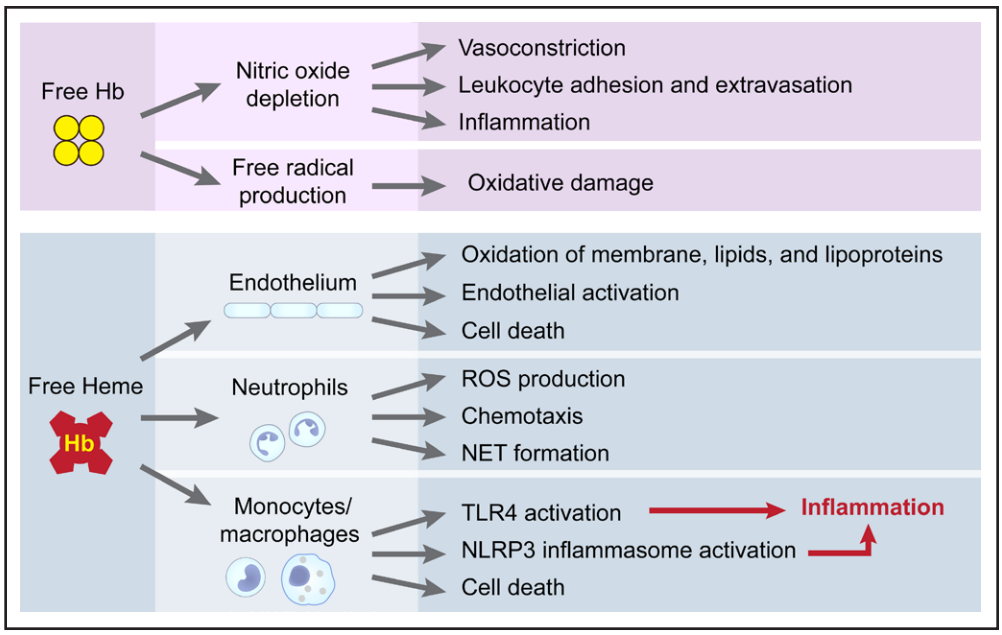

Figure 3. Mechanisms of cell-free hemoglobin (Hb)- and heme-mediated injury. Cell-free Hb rapidly depletes local nitric oxide (26), leading to vasoconstriction, up-regulation of endothelial adhesion molecules, platelet activation, and thrombosis. Hb also catalyzes free radical production and the resulting reactive oxygen species (ROS) damage membrane lipids and proteins. Free heme also activates the endothelium, increasing local inflammation triggering complement activation, and targeting the endothelium (27). Heme also disrupts the endothelial barrier function through a disruption of tight and adherens junctions and via ROS-mediated necroptosis (28–30). Free heme also acts as a damage-associated molecular pattern by binding to Toll-like receptor 4 (TLR4) on neutrophils and monocytes/macrophages (31–33). These effects lead to broad activation of proinflammatory pathways including via the nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (NLRP3) inflammasome, formation of neutrophil extracellular traps (NET) (34–36) apoptosis of immune cells (37), and impaired phagocytosis (31, 38, 39).

pyrin domain-containing-3 inflammasome, promoting IL-1β production (37). Heme-mediated endothelial activation is driven primarily through toll-like receptor 4 (TLR4). Heme-binding stimulates rapid degranulation of Weibel-Palade bodies on the endothelial cell surface, releasing a host of vasoactive proteins including P-selectin, endothelin-1, endothelin-converting enzyme, tissue-type plasminogen activator, and von Willebrand Factor (17, 47). TLR4 activation also upregulates the production of the inflammatory cytokines IL-6 and IL-8 and the adhesion molecule E-selectin (48). Heme/TLR4-mediated P-selectin release triggers complement activation and targeting of the endothelium (27). Heme leads to further barrier dysfunction through disruption of tight and adherens junctions between endothelial cells and via ROS-mediated necroptosis of endothelial cells (28–30).

Interestingly, although initial exposure of the vascular endothelium to CFH promotes the production of inflammatory signals and sensitizes the endothelium to oxidative injury, prolonged exposure of cultured endothelial cells to methemoglobin renders the endothelium relatively resistant to oxidative damage (44). This adaptation appears to be mediated in part by up-regulation of HO-1 and ferritin.

Coagulopathy. Hemolysis is associated with coagulopathy and thrombosis through its effects on platelets, the endothelium, and macrophages. Human platelets exposed to heme or hemoglobin show a dose-dependent increase in surface P-selectin and activated glycoprotein IIb/IIIa (49). This effect depends, at least in part, on heme-binding through TLR4, which leads to inhibition of platelet mitochondrial complex V, increased production of mitochondrial ROS, and release of a subset of platelet granules (49). Heme-activated platelets release thrombospondin-1, which potentiates platelet activation and aggregation, enhances leukocyte migration, and inhibits endothelial nitric oxide signaling (49, 50). Heme exposure also boosts tissue factor expression in the endothelium and in macrophages (51, 52).

Immunomodulatory Effects. Free heme acts as a damage-associated molecular pattern (DAMP) by binding to TLR4 on monocytes, macrophages, and endothelial cells (31–33). TLR4 activation leads to transcriptional activation of proinflammatory pathways through MyD88 and nuclear factor-kappa B (NF-κB) (34). Free heme also promotes the release and potentiates the proinflammatory effects of high mobility box group 1 (53).

Free heme has widespread effects on neutrophil and macrophage populations. Heme induces neutrophil migration and activation, and can trigger the oxidative burst and NETosis (35, 36), but suppresses

tumor necrosis factor-alpha (TNF-α) and interferon gamma, and impairs phagocytosis (31, 38, 39). Heme also promotes the mobilization of immature granulocytes from the bone marrow, and upregulates HO-1 in immature neutrophils, limiting their capacity to kill bacteria through oxidative burst (38). Heme-mediated activation of TLR4 on macrophages stimulates the secretion of TNF-α and promotes a shift toward the pro-inflammatory M1 phenotype (37). However, similar to its effects on the endothelium, heme induces up-regulation of HO-1 and ferritin in macrophages, which both have anti-inflammatory effects (54, 55). Heme also acts through monocytes to promote an anti-inflammatory polarization of the T cell population (38).

Heme has important effects on other aspects of the innate immune system. Heme exposure modifies immunoglobulin function, forming heme-immunoglobulin complexes with altered affinities for various self and bacterial antigens (37). Interestingly, although heme-mediated effects on the endothelium activate complement and promote endothelial injury (27), heme inhibits C1q the first step in the classical pathway, which is involved in pathogen recognition and the clearance of apoptotic cells (56). There is also evidence that heme triggers trained immunity, which may have a protective effect against bacterial infection in the short term but is deleterious in the medium term (32, 57).

Taken together, these findings suggest that acute exposure to CFH triggers a significant host immune response. Interestingly, prolonged heme exposure appears to promote a relatively anti-inflammatory immune phenotype that may protect against future heme-mediated injury but impair the body's ability to recognize and defend against pathogens. These mechanisms are highly relevant in trauma patients as they may contribute to the finding that patients who survive their initial injuries have increased susceptibility to infection at sites remote from their initial injury (58).

Evidence of Hemoglobin-Mediated Pathophysiology From Trauma Patients and Models

Although the pathophysiologic significance of CFH in trauma patients was recognized as early as the 1970s (59), relatively few mechanistic studies have been performed in trauma models compared with the progress made in sepsis models. Two critical studies highlight the relevance of hemoglobin-mediated pathophysiology in trauma.

Lee et al (6) demonstrated an approximately 10-fold increase in free heme in trauma patients 24 hours after injury. The authors investigated the immunomodulatory effects of free heme in a mouse model of liver crush injury followed by bacterial inoculation into the lungs. Mice who underwent laparotomy with liver crush injury were less able to clear either *Staphylococcus aureus* or *Escherichia coli* from the lungs compared with animals who had laparotomy or bacterial challenge alone. Hemopexin knockout mice ($Hpx^{-/-}$) subjected to liver crush injury and *S. aureus* pulmonary inoculation showed a similar rise in free heme but heme clearance was delayed, and animals showed significantly impaired bacterial clearance in the lungs compared with wild-type animals. The authors showed that liver crush injury led to the recruitment of large numbers of neutrophils to the liver. Liver crush injury did not affect the number of neutrophils recruited to the lungs in the subsequent bacterial challenge, but the population of neutrophils recruited was less mature and relatively dysfunctional compared with those recruited after the bacterial challenge alone.

Rittirsch et al (60) examined the expression of components of the hemoglobin degradation pathway in the circulating leukocytes of severely injured trauma patients over a period of 21 days after injury. The authors found that early haptoglobin expression correlated with the predicted risk for massive transfusion at presentation, and with the number of RBC units received. Expression of the components of the hemoglobin degradation pathway then followed the clinical course, with haptoglobin, CD163, HO-1, and biliverdin reductase expression clustered with ICU length of stay. Patients who developed sepsis showed further up-regulation of haptoglobin and HO-1 compared with patients who did not develop sepsis. This study highlights the importance of the hemoglobin degradation pathway in severely injured trauma patients but was not able to distinguish the impact of endogenous hemolysis from that of transfusion and other interventions.

Impact of Cell-Free Hemoglobin From Stored RBCs

Blood transfusion can compound the effects of endogenous hemolysis after injury (61). RBCs undergo hemolysis and fragmentation into microparticles during storage leading to the transfusion of CFH with each unit of RBCs. The RBCs that remain undergo a range

of morphologic and biochemical changes that increase susceptibility to later hemolysis (7, 62). However, the relationship between the age of stored blood, the amount of CFH transfused, the degree of subsequent hemolysis, and its downstream impacts are poorly understood.

In one study of trauma patients who received total transfusion volumes of at least 5 L, CFH rose and haptoglobin fell in the recipient with each liter of blood transfused (9). Patients who received older units of blood tended to have lower haptoglobin levels after transfusion, but the age of the blood transfused did not correlate with CFH levels. Further, Schaid et al (8) demonstrated not only that hemolysis markers were elevated before transfusion, but that severely injured patients who received greater than 10 RBC units in the first 6 hours of treatment had evidence of increased hemolysis from 6 to 12 hours.

Wagener et al (63) conducted an elegant series of experiments in which mice with hemorrhagic shock were resuscitated with either fresh or stored mouse RBCs and, 48 hours later, were challenged with intratracheal *Pseudomonas aeruginosa*. Both groups had similar initial responses to resuscitation, but levels of both CFH and heme were higher in mice that received stored RBCs. After intratracheal *P. aeruginosa*, mice that received stored RBCs had more pulmonary edema, higher bacterial levels in the lungs, and higher mortality. The effect of stored RBCs was reduced if mice were given hemopexin before resuscitation, suggesting that the lung injury was heme-dependent. Of note, hemopexin treatment also improved survival after *P. aeruginosa* in mice resuscitated with fresh RBCs, suggesting that heme-mediated toxicity after transfusion of fresh RBCs remains important.

The importance of storage time in RBC transfusion has been the topic of intensive research and a full discussion is beyond the scope of this review. However, as the examples above illustrate, the existing literature is difficult to interpret in part because of the ethical limitations in studying transfusion of aged blood, the relative scarcity of patients in the studies who received massive transfusions, and the multiple overlapping effects that are being studied (64). Thus, we propose four distinct phases of hemolysis that must be considered in future experiments; hemolysis of autologous RBCs related to the initial trauma, hemolysis during RBC storage, hemolysis of stored RBCs after transfusion, and hemolysis associated with resuscitation and procedural interventions.

Therapeutics Aimed at Ameliorating the Effects of Cell-free Hemoglobin

Although few therapies have been tested to reduce the pathologic effects of CFH in trauma patients, there are many potential therapies that have been studied in sepsis, transfusion-related injury, and hemolytic diseases. There are also Food and Drug Administration (FDA)-approved drugs that act on relevant parts of the pathway.

The most effective therapeutic strategy would likely be to prevent or reduce the degree of hemolysis. As there is typically a delay between the initial injury and reaching the hospital, reducing hemolysis associated with the initial injury may not be possible. However, the initial resuscitation strategy and early interventions including the composition, age, and volume of transfusions may be relevant. Although all are important areas of ongoing investigation, a full discussion is beyond the scope of this review. Of note, there have also been reports of using plasma exchange to treat hemolysis in cardiopulmonary bypass (65), and multiple groups are working on novel peptides or chemical strategies to remove CFH and its degradation products (66, 67). However, here we will focus on drugs that are in current clinical use or have been studied extensively in animals.

Exogenous human haptoglobin and hemopexin are both potential therapies. In addition to binding and ultimately clearing CFH and heme, there is some evidence that infusion of exogenous haptoglobin and hemopexin have direct anti-inflammatory effects, mediated in part through induction of HO-1 and suppression of NF-κB activation (68). Traditional antioxidants (e.g., niacin, glutamine, ascorbate) (69, 70) and anti-inflammatory drugs (e.g., acetaminophen) (71) are also potential therapeutics. There have also been efforts to restore nitric oxide homeostasis (e.g., inhaled NO, S-nitrosoalbumin, or arginine supplementation) (10, 72), to augment CD163 expression with glucocorticoids (73), and to block regulators further along the injury pathways (e.g., TLR4 inhibition or up-regulation of HO-1 expression). One challenge in reviewing animal studies of potential therapeutics is that many rely on pretreatment or coadministration with CFH or heme, which is typically not

possible in trauma. Another challenge is ensuring that the outcomes are clinically relevant to trauma patients. **Table 1** summarizes therapeutic studies in animal hemolysis models.

Haptoglobin. As the initial line of defense against CFH, haptoglobin supplementation is an appealing therapeutic option (74, 80). The effectiveness of haptoglobin in trauma will likely depend on the kinetics of hemolysis and hemoglobin-mediated injury in trauma patients. If the majority of hemolysis occurs within minutes of injury, and a large portion of hemoglobin breaks down rapidly to release free heme, then even haptoglobin treatment upon arrival to a trauma center may be too late to prevent injury. However, if hemolysis in trauma patients is more episodic, subacute, and sufficiently predictable—occurring at the initial trauma, but also at the time of transfusions and procedures—then haptoglobin could be an effective therapy. Human plasma-derived haptoglobin is approved for clinical use in Japan for the treatment of hemolysis in massive transfusion following trauma, severe burns, and cardiopulmonary bypass but outcomes data are limited (81).

In a beagle model of *S. aureus* pneumonia and septic shock, animals that received bolus doses and then infusion of haptoglobin required less vasopressor support and had reduced markers of lung injury compared with septic controls (75). In septic animals who received an exchange transfusion with 7-day-old blood, animals that also received haptoglobin treatment required less vasopressor support, reduced markers of lung injury, and lower mortality at 96 hours. These experiments are particularly encouraging in the trauma context because the therapeutic effect of haptoglobin was evident even when the initial haptoglobin bolus was given 4 hours after the beginning of the infection. However, in a series of follow-up experiments that generated very high CFH levels with repeated exchange transfusions of older blood, haptoglobin treatment improved shock scores but did not improve lung injury or mortality (82). This was attributed in part to the saturation of the animals' ability to clear hemoglobin–haptoglobin complexes and highlights a potential limitation of haptoglobin therapy alone in cases of massive hemolysis. The FDA-designated human plasma-derived haptoglobin as an orphan drug for the treatment of sickle cell disease in 2013 and subarachnoid hemorrhage in 2020.

Hemopexin. Hemopexin is also a promising therapeutic (77–79). Although it has no effect on CFH, it binds and clears free heme, the primary driver of oxidative tissue injury. Hemopexin might be used alone or in combination with haptoglobin, to clear a critical breakdown product of CFH that has escaped control by haptoglobin therapy.

Several promising animal trials have highlighted the therapeutic potential of hemopexin. In a mouse CLP model, intraperitoneal hemopexin treatment reduced serum markers of liver, kidney, and cardiac injury, and decreased mortality (53). In a mouse model of hemorrhagic shock and resuscitation with aged RBCs, coinfusion of a single dose of hemopexin reduced mortality at 48h (76). Hemopexin was designated an orphan drug by the FDA for the treatment of sickle cell disease in 2020.

Acetaminophen. Therapeutic concentrations of acetaminophen can inhibit the peroxidase activity of CFH by reducing the ferryl (4+) to the ferric (3+) state. Although acetaminophen acts relatively late in the pathway of hemoglobin-mediated injury, it is a widely used drug with a well-established safety profile. Initial studies have highlighted a potential role in renal protection. Experiments in a rat model of rhabdomyolysis demonstrated that pretreatment with acetaminophen inhibited lipid peroxidation, and attenuated the rise in creatinine associated with rhabdomyolysis (71). In a randomized controlled trial of patients with severe sepsis and detectable CFH, acetaminophen reduced oxidative injury and improved renal function compared with placebo (83).

DISCUSSION

Our scoping review highlights the steady progress that has been made over the last 20 years in improving our understanding of the mechanisms of free hemoglobin- and heme-mediated injury and in the identification and preclinical testing of a range of potential therapeutics. However, our structured search highlighted multiple areas in which studies on the potential role of hemolysis in trauma are limited or absent.

We know from clinical studies that major trauma is associated with endogenous hemolysis and that markers of hemolysis before transfusion correlate with physiologic markers of injury severity, and poor outcomes (6, 8). Clinical studies also demonstrated that large-volume transfusion (> 5 L) is associated with a

TABLE 1.
Therapeutic Studies in Animal Hemolysis Models

Therapeutic	Mechanism	Stressor	Organism	Dose (Route)	Timing	Outcome	References
Acetaminophen	Inhibits lipid peroxidation	Glycerol-induced rhabdomyolysis	Rat	100 mg/kg (IP)	Pretreatment and posttreatment	• Reduced markers of lipid peroxidation (F_2-isoprostanes) • Decreased creatinine rise in rhabdomyolysis	Boutaud et al (71)
Haptoglobin	Binding and clearance of cell-free hemoglobin	Transfusion of aged RBCs	Guinea pig	750 mg (IV)	Coinfusion	• Blocks hypertension associated with transfusion of old blood • Attenuates iron deposition and coagulative necrosis in the aortic arch • Attenuates creatinine elevation and hemoglobin in urine	Baek et al (74)
		Staphylococcus aureus pneumonia ± exchange transfusion	Dog	100 mg/kg (IV) ×2, 600 mg/kg (IV) over 48 hr	4 hr and 7 hr bolus, then 48 hr infusion	• Decreased vasopressor requirements • Reduced markers of lung injury • Decreased mortality (in septic animals with exchange transfusion)	Remy et al (75)
		Hemorrhagic shock with transfusion of aged RBCs	Mouse	7.5 mg per mouse (IV)	Coinfusion	• Decreased mortality at 48 hr • Increased urine output • Decreased kidney injury (kidney injury molecule 1, NGAL) and iron deposition	Graw et al (76)
Hemopexin	Binding and clearance of free heme	Phenylhydrazine-induced hemolysis	Mouse	100–500 mg/kg (IV)	Pretreatment	• Decrease in cell-free hemoglobin and increase in hemoglobin: haptoglobin× complexes at 6 hr • Reduced kidney injury (creatinine and BUN) • Reduced renal complement deposition (C3b/iC3b staining) at 6 hr	Poillerat et al (77)
		Cecal ligation and puncture	Mouse	50 mg/kg (IP)	2, 12, 24, and 36 hr	• Decreased markers of liver, kidney, and cardiac injury • Decreased mortality	Larsen et al (53)

(*Continued*)

TABLE 1. (*Continued*)
Therapeutic Studies in Animal Hemolysis Models

Therapeutic	Mechanism	Stressor	Organism	Dose (Route)	Timing	Outcome	References
		Endogenous hemolysis	β-thal and SCD mice	700 µg (IP)	Twice a week for 1 mo	• Reduced iron accumulation in aortic endothelium • Reduced expression of endothelial adhesion molecules • Normalization of blood pressure and cardiac output in SCD mice	Vinchi et al (78)
		Endogenous hemolysis	SCD mice	4 mg (IP)	Weekly for 3 wk	• Decreased M1 polarization of liver macrophages	Vinchi et al (79)
		Phenylhydrazine-induced hemolysis	Mouse	40 µmol/kg (IP)	Pretreatment	• Reduced complement deposition in vascular endothelium of the liver	Merle et al (27)
		Hemorrhagic shock with transfusion of aged RBCs	Mouse	7.5 mg per mouse (IV)	Coinfusion	• Decreased mortality at 48 hr	Graw et al (76)
Protein cocktail[a]	Clearance of Fe, hemoglobin, heme	Exchange transfusion with hemolyzed blood	Hamster	5% of animal's hemoglobin mass	Coinfusion	• Decreased markers of liver injury (aspartate aminotransferase, alanine transaminase) • Decreased markers of kidney injury (creatinine, BUN, urinary NGAL)	Pires et al (67)
Hydroxyurea	NO donor	Water-induced hemolysis	C57BL/6 Mouse	250 mg/kg (IV)	Pretreatment	• Reduced inflammation (myeloperoxidase activity), leukocyte rolling, adhesion, and extravasation	Almeida et al (72)
Diethylamine NONOate	NO donor	Water-induced hemolysis	C57BL/6 Mouse	250 mg/kg (IV)	Pretreatment	• Reduced inflammation (myeloperoxidase activity), leukocyte adhesion, and extravasation	Almeida et al (72)
Inhaled NO	NO donor	Water-induced hemolysis	Dog	80 ppm (inhaled)	Continuous	• Attenuated hemolysis-induced increases in mean arterial pressure and systemic vascular resistance index, and decrease in cardiac index	Minneci et al (10)

BUN = blood urea nitrogen, Cr = creatinine, NGAL = neutrophil gelatinase-associated lipocalin, NO = nitric oxide, SCD = sickle cell disease.
[a]Protein cocktail generated by tangential flow filtration of human Cohn fraction IV, which includes albumin (40%), transferrin (35%), haptoglobin (10%), hemopexin (5%), ceruloplasmin (5%), and vitamin D-binding protein (5%). Note that ceruloplasmin catalyzes oxidation of Fe^{2+} to Fe^{3+} and vitamin D-binding protein scavenges actin which is released during hemolysis (67).
Due to space constraints, studies in which cell-free hemoglobin or its breakdown products were directly administered and their actions were modulated by administration of a therapeutic were not included.

dose-dependent rise in circulating free hemoglobin (9) and that patients who receive large-volume transfusions demonstrate evidence of increased hemolysis in the subsequent 6 hours (8). However, the mechanisms and relative importance of trauma-induced endogenous hemolysis are unknown. Further, we do not understand the interaction of newly transfused RBCs with the disordered endothelial, immune, and coagulation environment of the severely injured patient. For example, does the damaged endothelium lead to increased lysis of the transfused erythrocyte compared with transfusion into a less injured patient?

With regard to the pathophysiologic mechanisms of CFH and free heme, the available data are mixed. There is strong clinical and preclinical evidence supporting the role of CFH in nitric oxide depletion, vasoconstriction, and thrombosis though no strong data in trauma patients (41–43, 45). There are clinical data supporting the association of free hemoglobin levels with oxidative injury (F_2-isoprostane levels) and mortality in sepsis (2), and a small randomized trial in patients with sepsis that demonstrated reduced F_2-isoprostane levels after 2 days of acetaminophen treatment and reduced creatinine after 3 days (83). However, there are no strong clinical data demonstrating a link between CFH or heme and oxidative injury in trauma patients. Experiments in the ex vivo perfused human lung demonstrate that CFH increases endothelial permeability (84, 85), but this has not been tested in trauma patients. The contribution of heme-activated platelets to coagulopathy has been studied in isolated human platelets in vitro but not yet tested in trauma patients (49). The immunomodulatory effects of CFH and free heme are varied, with the majority of data coming from small animal models of hemolysis. However, the study by Rittirsch et al (60) suggests a link between the expression of elements of the hemolysis pathways in a cohort of trauma patients and later septic complications. We have developed the following list of high-priority open questions using this scoping review.

Open Questions

- What are the mechanisms of endogenous hemolysis in traumatic injury?
- What are the relative contributions of endogenous hemolysis, transfusion-related hemolysis, and hemolysis related to other interventions after traumatic injury?
- Are there certain patient populations that are more or less susceptible to hemolysis-related damage after traumatic injury (e.g., carriers of specific haptoglobin phenotypes)?
- Do CFH and heme have a unique pathophysiology in trauma (as suggested in sepsis) or are they simply two of many DAMPs acting through traditional pathways?
- To what extent do CFH and free heme contribute to immune dysfunction after traumatic injury?

CONCLUSIONS

CFH and its breakdown products are critical mediators of endothelial dysfunction, organ injury, and immunomodulation in hemolysis. Although the majority of research to date has been focused on the role of these pathways in sepsis-associated hemolysis, hemoglobinopathies, and transfusion, these pathways are also implicated in trauma and massive transfusion. A concerted research effort is needed to clarify and contextualize the relative contributions of these pathways to the pathophysiology of trauma. This is particularly important as existing therapeutics have been shown to reduce hemoglobin-mediated injury and could have a major impact on improving trauma outcomes.

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