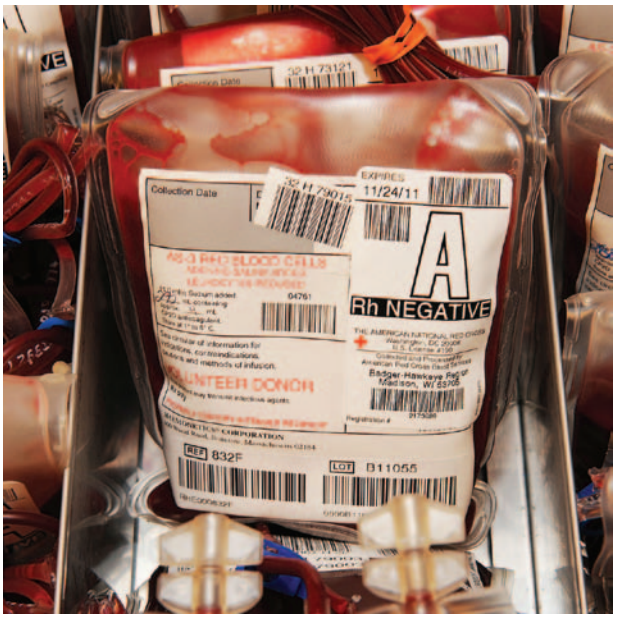
Understanding the Erythrocyte Storage Lesion

THE methodology to store erythrocytes for transfusion has markedly enhanced the supportive care capacity in hematology, surgery, trauma, and critical care medicine. However, the limitations of stored blood have come under recent scrutiny and great controversy. In this issue of ANESTHESIOLOGY, Lei *et al.*[1] at Massachusetts General Hospital show that transfusion with stored erythrocytes increases tissue injury of hemorrhagic shock more than transfusion with fresh erythrocytes, using an experimental rodent model of blood transfusion. Furthermore, they find that preexisting endothelial dysfunction caused by a high-fat diet increases the risk of this adverse response to transfused stored blood, modeling a common scenario under which human patients might receive transfusions for traumatic emergencies.



"... this [work and others] supports a credible hypothesis that the blood storage lesion effect on morbidity and mortality is seen primarily in patients with preexisting risk factors for cardiovascular disease during critical illness."

Over the years, several observational studies have hinted that critically ill patients are at greater risk of dying if they receive transfusions of multiple units of erythrocytes, suggesting an adverse property of stored erythrocytes. This finding is hotly debated, because it is essentially impossible to erase the confounding issue that the patients who require the most transfusions inherently are also the sickest and most likely to die. Do the sickest patients die because of blood transfusions or despite blood transfusions? This is a sensitive but crucial question.

Pathologic changes in blood during storage, often called the blood storage lesion, is a rapidly growing area of biomedical research. Before 2005, this was an esoteric subject, addressed in five peer-reviewed articles each year on average. Since that year, nearly 20 articles per year on the average fuel the debate with new research on the storage lesion. Stored erythrocytes undergo morphologic changes, metabolic alterations, and some degree of hemolysis during storage. During storage, erythrocytes undergo depletion of potassium, 2,3-diphosphoglycerate, adenosine triphosphate, lipids, and membrane, with increased erythrocyte rigidity and impaired oxygen delivery.[2] The stored units accumulate microvesicles derived from erythrocytes, free hemoglobin and biologically active lipids, demonstrating increased proinflammatory and procoagulant activity. Modern blood-bank preservative solutions provide glucose and other stabilizing substances to minimize these undesirable changes that might adversely affect the transfusion recipient.[3]

Despite these stabilizers, detectable hemolysis occurs in the storage bag and in the patient. The Food and Drug Administration mandates minimal standards that less than 1% of the erythrocytes lyse in the storage bag, and no more than 25% of the cells cleared from the recipient within 24 h after infusion, which likely correspond to cells lysed *in vivo*.[3] These round number thresholds are arbitrarily established long ago and not derived by any empirical scientific testing.

This debate more recently has been focused on a single hot button: duration of erythrocyte storage. Patients who received newer blood serve as experimental controls for those who receive older blood. Wang *et al.*[4] performed a meta-analysis of 21 independent observational studies evaluating associations of transfusion with erythrocytes stored for longer duration. Older blood was associated with a significantly increased risk of death (odds ratio, 1.16; 95% CI, 1.07–1.24). These authors provocatively estimate that if all cardiac surgery and trauma patients were transfused with

Photo: J. P. Rathmell.

Accepted for publication July 18, 2012. Dr. Kato receives research funding from the Division of Intramural Research of the National Heart, Lung and Blood Institute at the National Institutes of Health (Bethesda, Maryland; grant number 1 ZIA HL006014-03 and others).

 Anesthesiology 2012; 117:1159-61

◆ This Editorial View accompanies the following article: Lei C, Yu B, Shahid M, Beloiartsev A, Bloch KD, Zapol WM: Inhaled nitric oxide attenuates the adverse effects of transfusing stored syngeneic erythrocytes in mice with endothelial dysfunction after hemorrhagic shock. ANESTHESIOLOGY 2012; 117:1190–202.

fresher rather than older blood, one additional life would be saved for each 97 transfused with standard blood.

This analysis has unleashed a volley of caveats regarding the intrinsic limitations of these retrospective analyses of observational data.[5] A patient receiving multiple transfusions is likelier to receive at least 1 unit of blood that has been stored longer than average, confounding the age of blood with the amount of blood and the severity of illness. There is no standard, accepted method that accurately scores the properties of the stored blood, even storage duration, when multiple units of blood are administered, partly because many factors are known to change in stored blood as part of the storage lesion. Assumptions during the analysis can introduce statistical artifact and biases that may either obscure or overestimate the association of storage duration to subsequent outcomes.[5] Randomized controlled trials certainly could produce data with fewer such issues.

Three randomized controlled trials have been initiated in cardiac surgery,[6] critically ill patients,[7] and in neonates,[8] but no final results are yet published. In an effort to reach answers more quickly and mechanistically, several investigators have conducted physiologic studies of stored blood in animal models and in humans. Many of the researchers have experimentally investigated vasodilatory dysfunction in recipients of stored blood products attributable to extracellular free hemoglobin. *In vitro*, extracellular free hemoglobin avidly scavenges nitric oxide, a signal transduction ligand that orchestrates a broad program of vascular homeostasis. Additional human and animal research studies have evaluated the contribution of nitric oxide scavenging by free hemoglobin to physiologic consequences of the blood storage lesion.[9] Alternatively, some investigators have emphasized oxidant stress as the principal mechanism mediating free hemoglobin toxicity to endothelial function and vascular smooth muscle tone.[10] Still others even implicate oxidant stress from free heme or iron released from hemoglobin.[11] Whatever the dominant mechanism, many diverse lines of investigation implicate hemolysis of stored blood as pathogenic.

Regardless of exact mechanism, a growing number of publications report evidence that stored blood impairs normal vasodilation and blood flow and implicate extracellular hemoglobin as the offending molecule.[12] Free hemoglobin and hemoglobin-containing erythrocyte microvesicles accumulate during blood storage and consume nitric oxide *in vitro*. Transfusion of these forms of extracellular hemoglobin induces vasoconstriction in rats.[13] Haptoglobin, the endogenous scavenger of free hemoglobin, in a guinea pig model blunts adverse effects of stored blood, including intravascular hemolysis, acute hypertension, vascular injury, and kidney dysfunction, supporting a role in vascular pathophysiology of extracellular hemoglobin among the other potentially pathogenic molecules released during hemolysis.[14] Transfusion of blood stored for 40 days into lambs induces pulmonary vasoconstriction and acute pulmonary hypertension preventable with inhaled nitric oxide.[15] The effectiveness of either haptoglobin or nitric oxide breathing is consistent with free hemoglobin as the pathogenic agent in stored blood, whether mediated by nitric oxide scavenging or oxidative stress.

It may be possible that the effect of the blood storage lesion on outcome is clinically significant only when combined with additive or synergistic risk factors. The Zapol group has found that known risk factors for vasomotor dysfunction, including diabetes,[16] high-fat diet, and hemorrhagic shock,[1] amplify the adverse effect of blood storage duration on vascular function and outcome in animal models. In clinical practice, this supports a credible hypothesis that the blood storage lesion effect on morbidity and mortality is seen primarily in patients with preexisting risk factors for cardiovascular disease during critical illness.

This hypothesis would help to explain why the effect of stored blood on vascular physiology is not apparent in healthy volunteers.[17] Future investigations may yield more informative results if focused on transfusion with stored blood under life-threatening conditions compounded by other vascular risk factors. Until then, clinical use of erythrocytes stored up to 42 days remains the standard of care.

Gregory J. Kato, M.D., Hematology Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland. gkato@mail.nih.gov

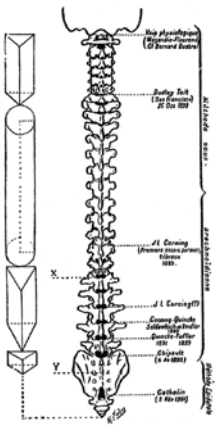
References

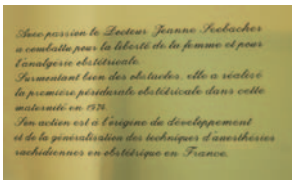
1. Lei C, Yu B, Shahid M, Beloiartsev A, Bloch KD, Zapol WM: Inhaled nitric oxide attenuates the adverse effects of transfusing stored syngeneic erythrocytes in mice with endothelial dysfunction after hemorrhagic shock. Anesthesiology 2012; 117:1190-202
2. Kim-Shapiro DB, Lee J, Gladwin MT: Storage lesion: Role of red blood cell breakdown. Transfusion 2011; 51:844–51
3. Hess JR: An update on solutions for red cell storage. Vox Sang 2006; 91:13–9
4. Wang D, Sun J, Solomon SB, Klein HG, Natanson C: Transfusion of older stored blood and risk of death: A meta-analysis. Transfusion 2012; 52:1184–95
5. Triulzi DJ, Yazer MH: Clinical studies of the effect of blood storage on patient outcomes. Transfus Apher Sci 2010; 43:95–106
6. Steiner ME, Assmann SF, Levy JH, Marshall J, Pulkrabek S, Sloan SR, Triulzi D, Stowell CP: Addressing the question of the effect of RBC storage on clinical outcomes: The Red Cell Storage Duration Study (RECESS) (Section 7). Transfus Apher Sci 2010; 43:107–16
7. Lacroix J, Hébert P, Fergusson D, Tinmouth A, Blajchman MA, Callum J, Cook D, Marshall JC, McIntyre L, Turgeon AF; ABLE study group: The Age of Blood Evaluation (ABLE) randomized controlled trial: Study design. Transfus Med Rev 2011; 25:197–205
8. Fergusson D, Hutton B, Hogan DL, LeBel L, Blajchman MA, Ford JC, Hebert P, Kakadekar A, Kovacs L, Lee S, Sankaran K, Shapiro S, Smyth JA, Ramesh K, Bouali NR, Tinmouth A, Walker R: The age of red blood cells in premature infants (ARIPI) randomized controlled trial: Study design. Transfus Med Rev 2009; 23:55–61
9. Gladwin MT, Kim-Shapiro DB: Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis. Curr Opin Hematol 2009; 16:515–23

10. Boretti FS, Buehler PW, D'Agnillo F, Kluge K, Glaus T, Butt OI, Jia Y, Goede J, Pereira CP, Maggiorini M, Schoedon G, Alayash AI, Schaer DJ: Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs. J Clin Invest 2009; 119:2271–80
11. Hod EA, Spitalnik SL: Stored red blood cell transfusions: Iron, inflammation, immunity, and infection. Transfus Clin Biol 2012; 19:84–9
12. Roback JD: Vascular effects of the red blood cell storage lesion. Hematology Am Soc Hematol Educ Program 2011; 2011:475–9
13. Donadee C, Raat NJ, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT: Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation 2011; 124:465–76
14. Baek JH, D'Agnillo F, Vallelian F, Pereira CP, Williams MC, Jia Y, Schaer DJ, Buehler PW: 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; 122:1444–58
15. Baron DM, Yu B, Lei C, Bagchi A, Beloiartsev A, Stowell CP, Steinbicker AU, Malhotra R, Bloch KD, Zapol WM: Pulmonary hypertension in lambs transfused with stored blood is prevented by breathing nitric oxide. Anesthesiology 2012; 116:637–47
16. Yu B, Lei C, Baron DM, Steinbicker AU, Bloch KD, Zapol WM: Diabetes augments and inhaled nitric oxide prevents the adverse hemodynamic effects of transfusing syngeneic stored blood in mice. Transfusion 2012; 52:1410–22
17. Berra L, Coppadoro A, Yu B, Lei C, Spagnolli E, Steinbicker AU, Bloch KD, Lin T, Sammy FY, Warren HS, Fernandez BO, Feelisch M, Dzik WH, Stowell CP, Zapol WM: Transfusion of stored autologous blood does not alter reactive hyperemia index in healthy volunteers. Anesthesiology 2012; 117:56–63

ANESTHESIOLOGY REFLECTIONS FROM THE PIERRE VIARS MUSEUM

Epidural Anesthesia at the Pitié-Salpêtrière Hospital: From Fernand Cathelin (1901) to Jeanne Seebacher (1974)





The discovery of the epidural technique for locoregional anesthesia has been attributed to two independent French physicians, Jean Athanase Sicard (1872–1929) and Fernand Cathelin (1873–1945). However, for many French historians, it seems that the credit of the discovery should be attributed to Cathelin and that Sicard was something of an opportunist, being aware of the research conducted by Cathelin at the Pitié-Salpêtrière hospital. An image from the book of Cathelin published in 1903 is shown (*left*) and represents the main historical steps in the development of epidural injections from 1885 to 1901. In 1974, in the Pitié-Salpêtrière, the anesthesiologist Jeanne Seebacher (*top right*) introduced epidural anesthesia in obstetrics for the first time in France. In the obstetric department, a memorial tablet celebrates that event (*bottom right*): "With passion, Jeanne Seebacher struggled for the freedom of women and for obstetric analgesia. Overcoming many obstacles, she performed the first obstetric epidural anesthesia in 1974 in France in this hospital. Her action was the first step toward a widespread use of epidural and intrathecal anesthesia for obstetrical analgesia in France."

Jean-Bernard Cazalaà, M.D., President of Club d'Histoire de l'Anesthésie et de la Réanimation (French Association for the History of Anesthesiology and Critical Care), France (www.char-fr.net), and Musée Viars, CHU Pitié-Salpêtrière, Paris, France.

Inhaled Nitric Oxide Attenuates the Adverse Effects of Transfusing Stored Syngeneic Erythrocytes in Mice with Endothelial Dysfunction after Hemorrhagic Shock

Chong Lei, M.D., Ph.D.,* Binglan Yu, Ph.D.,† Mohd Shahid, Ph.D.,† Arkadi Beloiartsev, M.D.,* Kenneth D. Bloch, M.D.,‡ Warren M. Zapol, M.D.§

ABSTRACT

Background: The authors investigated whether transfusion with stored erythrocytes would increase tissue injury, inflammation, oxidative stress, and mortality (adverse effects of transfusing stored erythrocytes) in a murine model of hemorrhagic shock. They tested whether the adverse effects associated with transfusing stored erythrocytes were exacerbated by endothelial dysfunction and ameliorated by inhaling nitric oxide.

Methods: The authors studied mice fed a high-fat diet (HFD-fed; to induce endothelial dysfunction) or a standard diet for 4–6 weeks. Mice were subjected to 90 min of hemorrhagic shock, followed by resuscitation with leukoreduced syngeneic erythrocytes stored less than 24 h (fresh erythrocytes) or stored for 2 weeks (stored erythrocytes).

Results: In standard-diet–fed mice at 2 h after resuscitation, transfusion with stored erythrocytes increased tissue injury more than transfusion with fresh erythrocytes. The adverse effects of transfusing stored erythrocytes were more marked in HFD-fed mice and associated with increased lactate levels and short-term mortality. Compared with fresh erythrocytes, resuscitation with stored erythrocytes was associated with a reduction in P_{50}, increased plasma hemoglobin levels, and increased indices of inflammation and oxidative stress, effects that were exacerbated in HFD-fed mice. Inhaled nitric oxide reduced tissue injury, lactate levels, and indices of inflammation and oxidative stress and improved short-term survival in HFD-fed mice resuscitated with stored erythrocytes.

Conclusions: Resuscitation with stored erythrocytes adversely impacts outcome in mice with hemorrhagic shock, an effect that is exacerbated in mice with endothelial dysfunction. Inhaled nitric oxide reduces tissue injury and improves short-term survival in HFD-fed mice resuscitated with stored erythrocytes.

What We Already Know about This Topic

- Transfusion of stored (more than 14 days) blood is associated with an increased rate of infection, multiorgan failure, and mortality
- The precise mechanisms involved in these adverse effects remain uncertain

What This Article Tells Us That Is New

- In a murine model of hemorrhagic shock, endothelial dysfunction (induced by feeding a high-fat diet) exacerbates, whereas breathing nitric oxide ameliorates, the adverse effects of resuscitation with stored erythrocytes

BLOOD transfusion is a lifesaving treatment for hemorrhagic shock (HS). During *ex vivo* storage, erythrocytes undergo numerous biochemical, structural, and functional alterations, which are collectively termed the "storage lesion."[1,2] In 2007, the average duration of storage of transfused units was 19.5 days in the United States and 20 ± 11 (mean ± SD) days for trauma patients.[3] A number of clinical studies have documented that transfusion of blood stored for more than 14 days is associated with an increased rate

◆ This article is accompanied by an Editorial View. Please see: Kato GJ: Understanding the erythrocyte storage lesion. ANESTHESIOLOGY 2012; 117:1159–61.

* Postdoctoral Fellow, † Instructor in Anesthesia, ‡ William Thomas Green Morton Professor of Anesthesia, § Reginald Jenney Professor of Anesthesia, Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care and Pain Medicine, Harvard Medical School at Massachusetts General Hospital, Boston, Massachusetts.

Received from the Anesthesia Center for Critical Care Research, Department of Anesthesia, Critical Care and Pain Medicine, Harvard Medical School at Massachusetts General Hospital, Boston, Massachusetts. Submitted for publication February 6, 2012. Accepted for publication July 19, 2012. Dr. Zapol receives royalties from patents on inhaled nitric oxide licensed by Massachusetts General Hospital to Linde Corp, Munich, Germany, and Ikaria Inc., Clinton, New Jersey. Dr. Bloch has received grants from Ikaria Inc. to study inhaled nitric oxide. Drs. Zapol and Yu have applied for patents on inhaled nitric oxide and blood transfusion. The remaining authors report no conflicts of interest. Supported by grants from the National Natural Science Foundation of China, Xi'an, China (to Dr. Lei; #81000232), an Eleanor and Miles Shore 50th Anniversary Fellowship of Harvard Medical School, Boston, Massachusetts (to Dr. Yu; #217249), and a research grant from the Fondation LeDucq, Paris, France (to Dr. Bloch). Drs. Lei and Yu contributed equally to this work.

Address correspondence to Dr. Zapol: Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, 55 Fruit Street, Thier 503, Boston, Massachusetts 02114. wzapol@partners.org. This article may be accessed for personal use at no charge through the Journal Web site, www.anesthesiology.org.

 Anesthesiology 2012; 117:1190-202

of infection,[4] multiorgan failure,[5] extended length of stay in hospital,[6] and mortality.[7–10] In contrast, other clinical studies of selected patient populations, including those undergoing cardiac surgical procedures, trauma victims, and critically ill patients, reported no association between the duration of erythrocyte storage and adverse clinical outcomes.[5,11–15]

The precise mechanisms responsible for the adverse clinical effects seen after transfusion of erythrocytes stored for prolonged periods are uncertain. One possibility is that these adverse effects are attributable to release of free hemoglobin from stored erythrocytes, both in solution and as lipid-enclosed microparticles.[16] Hemoglobin in plasma can scavenge nitric oxide more avidly than erythrocyte-encapsulated hemoglobin[17] and can cause vasoconstriction,[18] inflammation, and platelet activation.[19] Increased plasma hemoglobin levels induce a "nitric oxide deficiency" state, contributing to the complications of various genetic and acquired hemolytic disorders such as sickle cell disease, malaria, and hemolysis-associated smooth muscle dystonia.[20]

Our previous studies of infusing hemoglobin-based oxygen carriers or tetrameric hemoglobin indicated that scavenging of endothelium-derived nitric oxide by plasma hemoglobin produced vasoconstriction in both mice and sheep.[18] We reported that preexisting endothelial dysfunction (a deficiency of vascular nitric oxide availability commonly associated with diabetes and atherosclerosis) dramatically enhanced the susceptibility to hemoglobin-based oxygen carriers or hemoglobin-induced systemic vasoconstriction in mice.[21] Moreover, we reported that breathing nitric oxide before infusing a hemoglobin-based oxygen carrier or tetrameric hemoglobin in mice prevented the vasoconstriction.[18]

Reproducible animal models of erythrocyte storage can provide important insights into the effects of transfusing stored blood in an HS model and aid in the development of methods to prevent stored blood toxicity. Our study had three objectives. First, we measured tissue injury, hemodynamic changes, and survival rate in mice subjected to HS for 90 min and resuscitated with erythrocytes stored for less than 24 h (fresh erythrocytes) or with erythrocytes stored for 2 weeks (stored erythrocytes). Second, we studied the impact of endothelial dysfunction (induced by feeding mice a high-fat diet [HFD-fed] for 4–6 weeks) after resuscitation from HS with fresh or stored erythrocytes. Third, we studied whether nitric oxide inhalation during and for 2 h after resuscitation could prevent or reduce the adverse effects (tissue injury, organ dysfunction, inflammation, oxidative stress, and mortality) of resuscitation with stored erythrocytes in HFD-fed mice. We report that resuscitation with stored erythrocytes induced greater tissue injury, inflammatory response, oxidative stress, mortality, and plasma hemoglobin levels than did resuscitation with fresh erythrocytes. All these adverse effects were exacerbated in HFD-fed mice resuscitated with stored erythrocytes after HS. Inhaled nitric oxide reduced tissue injury, plasma hemoglobin levels, and oxidative stress and improved the survival rate of HFD-fed mice transfused with stored erythrocytes.

Materials and Methods

Animal Studies

Animal studies were approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital, Boston, Massachusetts. Eight- to 10-week–old male C57BL/6J mice were obtained from Jackson Laboratory (Bar Harbor, ME). Mice were fed either a standard diet (10% of calories from fat) or a HFD (60% of calories from fat; Research Diets, Inc., New Brunswick, NJ) for 4–6 weeks. Murine blood was collected and stored as previously described.[22]

We studied seven groups of C57BL/6 mice. Sham control groups fed the standard diet or HFD-fed mice (n = 8/group) received anesthesia, mechanical ventilation, and vascular cannulation but were not subjected to hemorrhage or resuscitation. We studied the effects of resuscitation with fresh erythrocytes (n = 16–17) and stored erythrocytes (n = 16–18) after HS in both standard diet– and HFD-fed mice. An additional group of HFD-fed mice (n = 16) was resuscitated with stored erythrocytes and breathed 80 parts per million (ppm) nitric oxide commencing 10 min before blood and fluid resuscitation and ending 2 h after fluid resuscitation was completed. Inhaled nitric oxide was delivered *via* the mechanical ventilator, as previously described.[18]

At 2 h after resuscitation, mice were extubated, and vascular catheters were removed. Cefazoline (400 mg/kg; Sandoz Inc., Princeton, NJ) was administered intraperitoneally at 2 h after resuscitation and readministered every 24 h. In a separate study, additional mice (5–7/group) were euthanized at 2 h after resuscitation to obtain blood and tissue samples.

Mouse HS Model

Mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (4 mg/kg) and mechanically ventilated at a respiratory rate of 120/min and tidal volume of 10 μl/g with an inspired oxygen fraction of 0.21 (Mini Vent 845; Harvard Apparatus, Holliston, MA). Both femoral arteries were cannulated with heparinized polyethylene-10 tubing (BD Diagnostics, Franklin Lakes, NJ). One arterial line was used for continuously measuring and recording arterial blood pressure (Ponemah Physiology Platform, Valley View, OH). The other arterial line was used for blood withdrawal and fluid infusion. Rectal temperature was maintained between 36.5 and 37°C using a heating pad. Anesthetized mice were bled over 10 min to a mean arterial pressure of 40 mmHg. A stable mean arterial pressure of 40 mmHg was maintained for 80 min by either withdrawing additional blood or infusing lactated Ringer's solution. This is a model of severe blood loss, approximately 40% of the blood volume is acutely shed (table 1). After 90 min of HS, mice were resuscitated over 20 min by infusing a volume

of fresh or stored erythrocytes equal to the shed blood volume with an equal volume of lactated Ringer's solution. We measured the survival rate up to 7 days after HS and resuscitation.

Blood Chemistry

Blood samples were obtained only from mice killed at 2 h after resuscitation by cardiac puncture. Standard base excess, arterial pH, Pa_{O_2}, Pa_{CO_2}, and Hco_3^- were measured using a blood gas analyzer (ABL 800 FLEX; Radiometer Medical, Brønshøj, Denmark). Lactate levels were measured using a Blood Lactate Measuring Meter (Lactate Plus; Nova Biomedical, Waltham, MA). Hematocrit was measured by centrifuging 70 μl of heparinized blood.

At 2 h after resuscitation, oxygen dissociation curves were determined with a Hemox-Analyzer (TCS Scientific Corp., New Hope, PA). P_{50}, defined as the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen, was calculated from the oxygen dissociation curve. Erythrocyte 2,3-diphosphoglycerate (2,3-DPG) concentration was measured with a kit (Roche Diagnostics, Mannheim, Germany).

Measurements of Plasma Hemoglobin, Haptoglobin, and Hemopexin Levels

Two hours after resuscitation with fresh or stored erythrocytes, plasma hemoglobin levels were determined with a QuantiChrom Hemoglobin Assay Kit (BioAssay Systems, Hayward, CA). Haptoglobin and hemopexin levels were measured with murine haptoglobin and hemopexin enzyme-linked immunosorbent assay kits (Life Diagnostics Inc., West Chester, PA).

Measurement of Liver, Kidney, and Muscle Injury

Plasma aspartate aminotransferase (AST) and creatine phosphokinase (CPK) concentrations, and blood urea nitrogen (BUN) levels, were measured by spectrophotometric analysis with commercial assay kits (BioAssaySystems, Hayward, CA) at 2 h after resuscitation with either fresh or stored erythrocytes.

Evaluation of Inflammation and Oxidative Stress

As a measure of inflammation, plasma interleukin (IL)-6 level and pulmonary myeloperoxidase activity were determined. IL-6 levels were measured using a Duoset® murine IL-6 enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN). Myeloperoxidase activity was evaluated in lung homogenates, as described previously.[23]

As an index of *in vivo* oxidative stress,[24] levels of 4-hydroxynonenal (4-HNE)-histidine protein adducts were measured in tissue homogenates using a commercial enzyme-linked immunosorbent assay kit (Cell Biolaboratories, San Diego, CA). To evaluate oxidative damage to DNA, levels of 8-hydroxy-2-deoxy guanosine (8-OHdG) were measured in DNA extracted from lung, liver, and kidney using a commercial enzyme immunoassay kit (Cayman

Table 1. Comparison of Volumes of Shed Blood and Transfused Fluids during Hemorrhagic Shock and Resuscitation, and Blood Chemistry and Hematocrit at 2 h after Resuscitation with Fresh or Stored Erythrocytes in Standard-Diet– and High-fat Diet–fed Mice

	Standard Diet		High-fat Diet		
	Fresh	Stored	Fresh	Stored	Stored + iNO
Body weight, g	26.1 ± 0.2	25.8 ± 0.2	34.4 ± 0.7	34.2 ± 0.8	32.2 ± 0.5
Estimated blood volume, ml	1.8 ± 0.0	1.8 ± 0.0	2.1 ± 0.1	2.4 ± 0.1	2.3 ± 0.0
Shed blood volume, ml	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.8 ± 0.0	0.8 ± 0.0
Lactated Ringer's infused during shock, ml	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0.0	0.7 ± 0.1	0.6 ± 0.1
Lactated Ringer's and blood infused during resuscitation, ml	1.4 ± 0.0	1.4 ± 0.0	1.5 ± 0.0	1.5 ± 0.0	1.5 ± 0.0
Arterial pH	7.25 ± 0.02	7.26 ± 0.01	7.26 ± 0.01	7.23 ± 0.01	7.26 ± 0.01
Pa_{O_2}, mmHg	100 ± 10	120 ± 11	120 ± 5	102 ± 8	96 ± 3
Pa_{CO_2}, mmHg	31 ± 2	27 ± 3	30 ± 3	31 ± 6	28 ± 2
Hco_3^-, mM	14 ± 0	15 ± 1	15 ± 1	14 ± 1	16 ± 1
SBE, mM	−13 ± 0	−12 ± 1	−13 ± 1	−13 ± 1	−12 ± 2
Lactate, mM	1.6 ± 0.2	1.9 ± 0.3	1.5 ± 0.1	4.5 ± 0.9*	1.9 ± 0.1†
Hematocrit, %	51 ± 2	52 ± 2	47 ± 3	44 ± 2	46 ± 2

Values are mean ± SD. Fresh, resuscitated with fresh erythrocytes stored for less than 24 h (n = 6); High-fat diet, WT mice fed a high-fat diet for 4–6 weeks; Standard diet, WT mice fed a standard diet; Stored, resuscitated with erythrocytes stored for 2 weeks (n = 6); Stored + iNO, resuscitated with stored erythrocytes, while breathing 80 ppm nitric oxide from 10 min before resuscitation until 2 h after resuscitation (n = 6).

*$P < 0.05$ differs *vs*. fresh erythrocytes in high-fat diet–fed mice; †$P < 0.05$ differs *vs*. stored erythrocytes in high-fat diet–fed mice.

iNO = inhaled nitric oxide (80 ppm); SBE = standard base excess; WT = wild type.

Table 2. Primers Used for qRT-PCR

Gene	mRNA Reference	Primer Sequence	
		Forward	Reverse
IL-6	NM_031168	TAGTCCTTCCTACCCCAATTTCC	TTGGTCCTTAGCCACTCCTTC
TF	NM_010171	AACCCACCAACTATACCTACACT	GTCTGTGAGGTCGCACTCG
HO-1	NM_010442	AAGCCGAGAATGCTGAGTTCA	GCCGTGTAGATATGGTACAAGGA
18S	NM_001081135	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT

18S = 18S ribosomal RNA; HO-1 = heme oxygenase-1; IL-6 = interleukin-6; mRNA =messenger ribonucleic acid; qRT-PCR = quantitative reverse transcription-polymerase chain reaction; TF = tissue factor.

Chemical, Ann Arbor, MI).[25] Total lung protein levels were measured using bicinchoninic acid protein reagent (Thermo Scientific, Rockford, IL).

Quantification of Tissue Messenger RNA Levels

Total messenger ribonucleic acid (mRNA) was extracted from murine lungs and livers using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA). Complementary DNA was synthesized by the reverse transcriptase reaction (MMLV-RT; Invitrogen Life Technologies). Real-time amplification of transcripts was performed by the Synergy Brands method using an Eppendorf Mastercycler Realplex (Eppendorf, Hamburg, Germany). The relative expression of target transcripts was normalized to the levels of 18S ribosomal RNA and analyzed using the relative C_T method. The sequences of primers used are listed in table 2.

Measurement of Vascular Reactivity

Standard-diet– and HFD-fed mice were euthanized with pentobarbital (200 mg/kg, intraperitoneal). A second-order mesenteric artery was dissected free, cleared of surrounding fatty tissue, and mounted onto a glass cannula in a pressure myograph (DMT-USA Inc., Ann Arbor, MI). Vessels were perfused with Krebs solution for 45 min at 60 mmHg. Vessels were constricted with phenylephrine (10^{-5} M). The vasodilator response to increasing concentrations of acetylcholine (10^{-9}–10^{-5} M) was measured and calculated, as previously reported.[26]

Statistical Analysis

All values are expressed as mean ± SD. We verified that all variables were normally distributed by Shapiro-Wilk test. Data were analyzed using a one-way analysis of variance with a *post hoc* Newman-Keuls test (GraphPad Prism; GraphPad Software Inc., La Jolla, CA). Survival rates were compared with a log-rank test. A two-way analysis of variance with repeated measures was performed to determine the effect of diet on mesenteric luminal dilation over a wide range of concentrations of acetylcholine (fig. 1). Relationships between plasma hemoglobin levels *versus* plasma AST, CPK, and BUN levels were analyzed by linear regression correlation. Plasma hemoglobin levels *versus* tissue 4-HNE and 8-OHdG levels and plasma hemoglobin levels *versus* pulmonary myeloperoxidase activity were also analyzed by linear regression correlation. Probability values were two-tailed, and a P value less than 0.05 was considered significant.

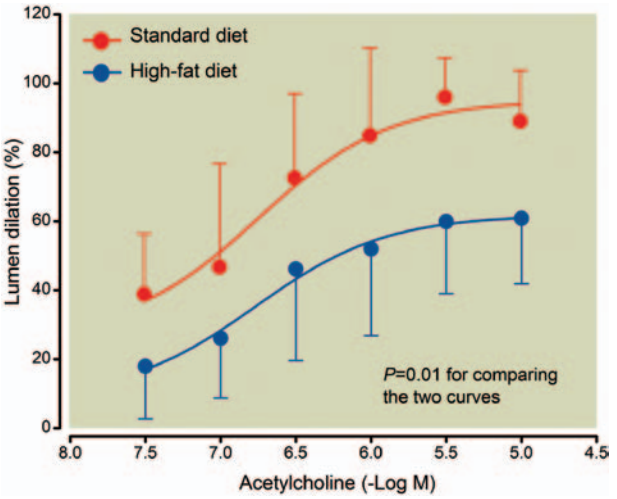


Fig. 1. High-fat diet–fed mice exhibit endothelial dysfunction. Endothelial function was measured by the vasorelaxation response induced by acetylcholine (10^{-9}–10^{-5} M) in phenylephrine (10^{-5} M) precontracted second-order mesenteric arteries. Standard diet, wild-type (WT) mice fed a standard diet (n = 7); High-fat diet, WT mice fed a high-fat diet for 4–6 weeks (n = 8). Data are expressed as mean ± SD. P = 0.01 by two-way analysis of variance with repeated measures.

Results

Endothelial Function Is Impaired in Second-order Mesenteric Arteries from Standard Diet– and HFD-fed Mice

Previous studies from our laboratory reported that diabetic mice exhibited endothelial dysfunction and were sensitized to the vasoconstrictor effects of infusing tetrameric hemoglobin and stored erythrocytes.[21,22] However, in pilot studies of db/db mice subjected to HS and resuscitation, none of them survived the 90 min of HS. Previous study showed that wild-type mice consuming an HFD for 9 days developed endothelial dysfunction.[27] Thus, we chose to study HFD-fed mice to learn whether their host factors would contribute to the adverse effects after transfusing stored erythrocytes. To confirm that

Table 3. Biochemical Characterization of Murine Plasma and Tissue at 2 h after Hemorrhagic Shock and Resuscitation with Fresh or Stored Erythrocytes

	Standard Diet		
	Sham	Fresh	Stored
Plasma AST, units/l	32 ± 3	132 ± 34†	185 ± 23†‡
Plasma BUN, mg/dl	21 ± 5	35 ± 7†	45 ± 4†‡
Plasma CPK, units/l	122 ± 38	315 ± 55†	379 ± 119†
Plasma interleukin-6, ng/ml	0.2 ± 0.1	0.3 ± 0.1†	1.8 ± 1.0†‡
Pulmonary myeloperoxidase activity, units/g tissue	10 ± 4	19 ± 5†	25 ± 5†‡
Pulmonary levels of 4-HNE adducts, μg/mg protein	150 ± 24	189 ± 78	252 ± 86
Hepatic levels of 4-HNE adducts, μg/mg protein	155 ± 20	175 ± 32	167 ± 26
Kidney levels of 4-HNE adducts, μg/mg protein	162 ± 34	148 ± 20	197 ± 64
Pulmonary levels of 8-OHdG, μg/g DNA	41 ± 18	48 ± 7	54 ± 15
Hepatic levels of 8-OHdG, μg/g DNA	68 ± 4	99 ± 54	123 ± 16
Kidney levels of 8-OHdG, μg/g DNA	67 ± 31	92 ± 54	221 ± 103
Plasma hemoglobin, μM	3.8 ± 0.8	8.7 ± 1.2†	30.3 ± 11.7†‡
Plasma haptoglobin, mg/dl	7 ± 2	9 ± 4	3 ± 2†‡
Plasma hemopexin, mg/dl	74 ± 13	57 ± 12†	40 ± 12†‡

Values are mean ± SD. Fresh, resuscitated with fresh erythrocytes stored less than 24 h (n = 6/group); High-fat diet, WT mice fed a high-fat diet for 4–6 weeks; iNO, inhaled 80 ppm nitric oxide; Sham, no hemorrhagic shock and resuscitation (n = 6/group); Standard diet, WT mice fed a standard diet; Stored, resuscitated with erythrocytes stored for 2 weeks (n = 6/group); Stored+iNO, resuscitated with stored erythrocytes and breathed 80 ppm nitric oxide (n = 6/group).

*P < 0.05 differs *vs*. standard diet–fed mice resuscitated with stored erythrocytes, †P < 0.05 differs *vs*. corresponding sham, ‡P < 0.05 differs *vs*. corresponding fresh erythrocytes, ¶P < 0.05 differs *vs*. corresponding stored erythrocytes, §P < 0.05 differs *vs*. standard diet–fed mice resuscitated with fresh erythrocytes, #P < 0.05 differs *vs*. sham-operated standard diet–fed mice.

4-HNE = 4-hydroxynonenal (4-HNE)-histidine protein adducts; 8-OHdG = 8-hydroxy-2-deoxy guanosine; AST = aspartate aminotransferase; BUN = blood urea nitrogen; CPK = creatine phosphokinase; WT = wild type.

mice fed an HFD for 4–6 weeks would develop endothelial dysfunction, we compared the ability of acetylcholine to dilate second-order mesenteric arteries obtained from standard-diet– and HFD-fed mice. We found that HFD-fed mice have a significantly impaired vasodilator response to acetylcholine (P = 0.01 by two-way analysis of variance with repeated measures, fig. 1). These results suggest that HFD-fed mice provide an animal model of endothelial dysfunction.

Liver, Kidney, and Muscle Injury after Resuscitation with Fresh or Stored Erythrocytes

To learn whether resuscitation with stored erythrocytes after HS would cause injury to liver, kidney, or muscle in mice, we measured plasma levels of AST, BUN, and CPK at 2 h after resuscitation. Baseline levels of plasma AST, BUN, and CPK did not significantly differ between standard-diet– and HFD-fed mice. In standard-diet–fed mice, resuscitation with either fresh or stored erythrocytes increased plasma AST, BUN, and CPK levels compared with standard-diet–fed mice that were not challenged with HS (table 3). AST and BUN levels were greater in standard-diet–fed mice resuscitated with stored erythrocytes than in standard-diet–fed mice resuscitated with fresh erythrocytes. In HFD-fed mice, resuscitation with fresh or stored erythrocytes induced higher levels of AST, BUN, and CPK than in HFD-fed mice that were not subjected to HS. AST, BUN, and CPK levels were much greater in HFD-fed mice resuscitated with stored erythrocytes than in HFD-fed mice resuscitated with fresh erythrocytes. Furthermore, AST, BUN, and CPK levels were greater in HFD-fed mice resuscitated with stored erythrocytes than in standard-diet–fed mice resuscitated with stored erythrocytes (P < 0.05 for each comparison, table 3). In HFD-fed mice resuscitated with stored erythrocytes that breathed nitric oxide, the AST, BUN, and CPK levels were less than in HFD-fed mice resuscitated with stored erythrocytes that breathed air without nitric oxide. Our data suggest that resuscitation with stored erythrocytes induced greater liver, kidney, and muscle injury in both standard diet– and HFD-fed mice than did resuscitation with fresh erythrocytes. Tissue injury associated with resuscitating HS with stored or fresh erythrocytes was exacerbated in HFD-fed mice, and the injury caused by transfusing stored erythrocytes in HFD-fed mice was attenuated by breathing nitric oxide.

Effects of Resuscitation with Fresh or Stored Erythrocytes and Nitric Oxide Breathing on Survival after HS

Despite the large volume of blood that mice acutely shed to produce severe hypotension and the variation of body weight between groups, there were no significant differences within groups for body weight, estimated blood volume, shed blood

High-fat Diet			
Sham	Fresh	Stored	Stored + iNO
54 ± 18	415 ± 148†	1150 ± 415†‡*	745 ± 76†‡¶
23 ± 5	43 ± 8†	55 ± 9†‡*	40 ± 6†¶
137 ± 79	454 ± 121†	814 ± 166†‡*	576 ± 169†¶
0.5 ± 0.2	20.8 ± 9.9†§	47.3 ± 26.2†‡*	39.8 ± 27.1†
19 ± 7	24 ± 2	38 ± 11†‡*	23 ± 6¶
236 ± 72	332 ± 101	505 ± 108†‡*	294 ± 54¶
215 ± 26	229 ± 55	354 ± 114†‡*	201 ± 21¶
193 ± 67	184 ± 34	294 ± 48†‡*	210 ± 36¶
29 ± 11	43 ± 10	92 ± 51†‡*	45 ± 7¶
97 ± 16	103 ± 28	199 ± 52†‡*	112 ± 55¶
57 ± 16	115 ± 57	251 ± 171†‡*	60 ± 34¶
5.3 ± 1.9	14.3 ± 9.9	62.4 ± 27.3†‡*	33.1 ± 15.1‡¶
16 ± 3	4 ± 3†	5 ± 4†	9 ± 7†
80 ± 6#	38 ± 20†	35 ± 12†	48 ± 20†

volume, and the volume of blood and lactated Ringer's solution infused during shock and resuscitation (table 1). No significant differences were found in mean arterial pressure between any of the groups before, during, and 2 h after HS and resuscitation (data not shown). No differences in arterial blood gas tensions or arterial pH were noted between standard-diet– and HFD-fed mice at 2 h after resuscitation with either fresh or stored erythrocytes (table 1). However, blood lactate levels at 2 h in mice fed an HFD and resuscitated with stored erythrocytes after HS were greater than those of sham mice, of standard-diet–fed mice resuscitated with either fresh or stored erythrocytes, or of HFD-fed mice transfused with fresh erythrocytes (table 1). The increase of plasma lactate levels measured in HFD-fed mice transfused with stored erythrocytes could be prevented by breathing nitric oxide (table 1).

To investigate the effect of resuscitation with fresh or stored erythrocytes on mortality after HS, we measured the short-term (12 h) and long-term (7 days) survival rates after HS and resuscitation (fig. 2). Short-term and long-term survival rates did not significantly differ in standard-diet–fed mice resuscitated with fresh or stored erythrocytes (fig. 2A and 2B). However, the short-term survival was greater in HFD-fed mice resuscitated with fresh erythrocytes than in HFD-fed mice resuscitated with stored erythrocytes ($P < 0.01$, fig. 2C). None of the HFD-fed mice resuscitated with stored erythrocytes after HS survived for 7 days (fig. 2D).

Because nitric oxide attenuated tissue injury, we examined whether breathing 80 ppm nitric oxide could improve the survival rate of HFD-fed mice resuscitated with stored erythrocytes. Nitric oxide inhalation improved the short-term survival rate of HFD-fed mice transfused with stored erythrocytes (80% survival with nitric oxide breathing *vs.* 30% without nitric oxide, $P = 0.014$, fig. 2D). However, there was no significant long-term survival benefit produced by breathing nitric oxide for the first 2 h after HFD-fed mice were resuscitated with stored erythrocytes ($P = 0.093$). Our results suggest that HFD-fed mice are more vulnerable to an early death (within 12 h) after HS and resuscitation with stored erythrocytes, and that the increased early mortality associated with transfusing stored erythrocytes can be prevented by breathing 80 ppm nitric oxide for 2 h.

Effects of Resuscitation with Fresh or Stored Erythrocytes on Oxygen Affinity

We have previously reported that levels of P_{50} and 2,3-DPG are markedly reduced in murine blood stored for 2 weeks.[22] To explore whether resuscitation with stored erythrocytes after HS would impair oxygen delivery to the periphery, we measured 2,3-DPG and P_{50} levels at 2 h after resuscitation. In HFD-fed mice, erythrocyte 2,3-DPG concentrations were greater after resuscitation with fresh erythrocytes than after resuscitation with stored erythrocytes (4.2 ± 0.3 and 2.4 ± 0.2 mM, respectively). P_{50} was greater after transfusion of fresh erythrocytes than after transfusion with stored erythrocytes (44 ± 1 *vs.* 33 ± 2 mmHg, respectively) after HS. Our results suggest that transfusion of 40% of the blood volume with erythrocytes stored for 2 weeks to resuscitate mice from HS produces markedly decreased 2,3-DPG levels and increased erythrocyte oxygen affinity (lower P_{50}).

Inflammatory Response after Resuscitation with Fresh or Stored Erythrocytes

To investigate whether the inflammatory response to HS is greater after resuscitation with stored erythrocyte than after resuscitation with fresh erythrocyte, plasma IL-6 levels were measured at 2 h after resuscitation. There was no

significant difference in IL-6 levels between sham-operated standard-diet–fed mice and sham-operated HFD-fed mice. In standard-diet–fed mice, plasma IL-6 levels were greater in mice resuscitated with fresh erythrocytes than in sham-operated controls. Resuscitation with stored erythrocytes induced a greater increase of plasma IL-6 levels than did resuscitation with fresh erythrocytes ($P < 0.01$, table 3) in standard diet–fed mice. In HFD-fed mice, resuscitation with either fresh or stored erythrocytes increased IL-6 levels compared with sham-operated HFD-fed mice. IL-6 levels were greater in HFD-fed mice resuscitated with stored erythrocytes than in HFD-fed mice resuscitated with fresh erythrocytes ($P < 0.05$). In addition, IL-6 levels were greater in HFD-fed mice resuscitated with either fresh or stored erythrocytes than in standard-diet–fed mice resuscitated with fresh or stored erythrocytes, respectively ($P < 0.05$). Of note, inhaled nitric oxide did not attenuate the increase of IL-6 levels in HFD-fed mice resuscitated with stored erythrocytes. These results suggest that resuscitation from HS with either fresh or stored erythrocytes induces an inflammatory response. Resuscitation with stored erythrocytes induces a greater inflammatory response than does resuscitation with fresh erythrocytes. Furthermore, HFD-fed mice are more sensitive to the inflammatory effects of HS and resuscitation.

Acute pulmonary inflammation is characterized by neutrophil infiltration, which can be quantified by measuring pulmonary myeloperoxidase activity.[28] To learn whether resuscitation with stored erythrocytes after HS induced an inflammatory response in the lung and whether HFD-fed mice are more sensitive to transfusing stored erythrocytes than are standard-diet–fed mice are, pulmonary myeloperoxidase activity was measured at 2 h after resuscitation (table 3). In standard-diet–fed mice, resuscitation with fresh and stored erythrocytes increased pulmonary myeloperoxidase activity, and the activity of myeloperoxidase was greater in the group resuscitated with stored erythrocytes than in the group resuscitated with fresh erythrocytes. Pulmonary myeloperoxidase activity did not significantly differ in HFD-fed mice subjected to HS and resuscitated with fresh erythrocytes and in sham-operated HFD-fed mice. In contrast, resuscitation with stored erythrocytes increased pulmonary myeloperoxidase activity in HFD-fed mice. Furthermore, pulmonary myeloperoxidase activity after resuscitation with stored erythrocytes was greater in HFD-fed mice than in standard-diet–fed mice. Inhaled nitric oxide reduced the increased pulmonary myeloperoxidase levels after resuscitation with stored erythrocytes in HFD-fed mice. These data suggest that resuscitation with stored erythrocytes induced pulmonary inflammation in both standard-diet– and HFD-fed mice, and that HFD-fed mice are more sensitive to transfusing stored erythrocytes than are standard-diet–fed mice.

Prothrombotic Response after Resuscitation with Fresh or Stored Erythrocytes

To evaluate the prothrombotic response after HS and resuscitation, tissue factor (TF) mRNA levels were measured in lung and liver at 2 h after resuscitation (fig. 3). In standard-diet–fed mice subjected to HS, resuscitation with neither fresh nor stored erythrocytes altered TF mRNA levels in lung and liver. In HFD-fed mice, resuscitation with both fresh and stored erythrocytes increased pulmonary TF mRNA levels, but only resuscitation with stored erythrocytes increased hepatic TF mRNA levels. In addition, pulmonary TF mRNA levels were higher in HFD-fed mice resuscitated with fresh or stored erythrocytes than that in standard-diet–fed mice resuscitated with fresh or stored erythrocytes. Hepatic TF mRNA levels were greater in HFD-fed mice receiving stored erythrocytes after HS than levels in standard-diet–fed mice resuscitated with stored erythrocytes. Inhaled nitric oxide did not reduce the increase of pulmonary or hepatic TF mRNA levels in HFD-fed mice resuscitated with stored erythrocytes. These data suggest that resuscitation with stored erythrocytes after HS induces a prothrombotic response in HFD-fed mice.

Oxidative Damage after Resuscitation with Fresh or Stored Erythrocytes

To test whether resuscitation from HS with fresh or stored erythrocytes induces oxidative lipid peroxidation and DNA damage, the levels of 4-HNE histidine protein adducts and 8-OHdG were measured in lung, liver, and kidney. In standard-diet–fed mice, resuscitation with either fresh or stored erythrocytes did not increase tissue levels of 4-HNE adducts or 8-OHdG (table 3). In HFD-fed mice, resuscitation with fresh erythrocytes did not alter the tissue levels of 4-HNE adducts or 8-OHdG. In contrast, resuscitation of HFD-fed mice with stored erythrocytes increased the levels of both 4-HNE adducts and 8-OHdG in the lung, liver, and kidney. It is noteworthy that tissue 4-HNE and 8-OHdG levels were greater in HFD-fed mice resuscitated with stored erythrocytes than the levels in standard-diet–fed mice resuscitated with stored erythrocytes. Breathing nitric oxide prevented the increase of the levels of 4-HNE adducts and 8-OHdG induced by transfusing stored erythrocytes after HS in HFD-fed mice. These data indicate that HFD-fed mice are more vulnerable to oxidative damage, including lipid peroxidation and oxidative DNA damage, induced by resuscitation with stored erythrocytes than are standard-diet–fed mice and that breathing nitric oxide can prevent this oxidative stress.

Plasma Hemoglobin Levels after Resuscitation with Fresh or Stored Erythrocytes

Previously, we and others have reported that murine erythrocytes stored for 2 weeks undergo hemolysis during storage and after transfusion.[22,29] To assess alterations of hematologic variables after HS and resuscitation, we measured plasma hemoglobin levels at 2 h after resuscitation with fresh or stored erythrocytes. Plasma hemoglobin levels did not differ in sham-operated standard-diet–fed mice and sham-operated HFD-fed mice. Plasma hemoglobin levels were greater in standard-diet–fed mice subjected to HS and resuscitated

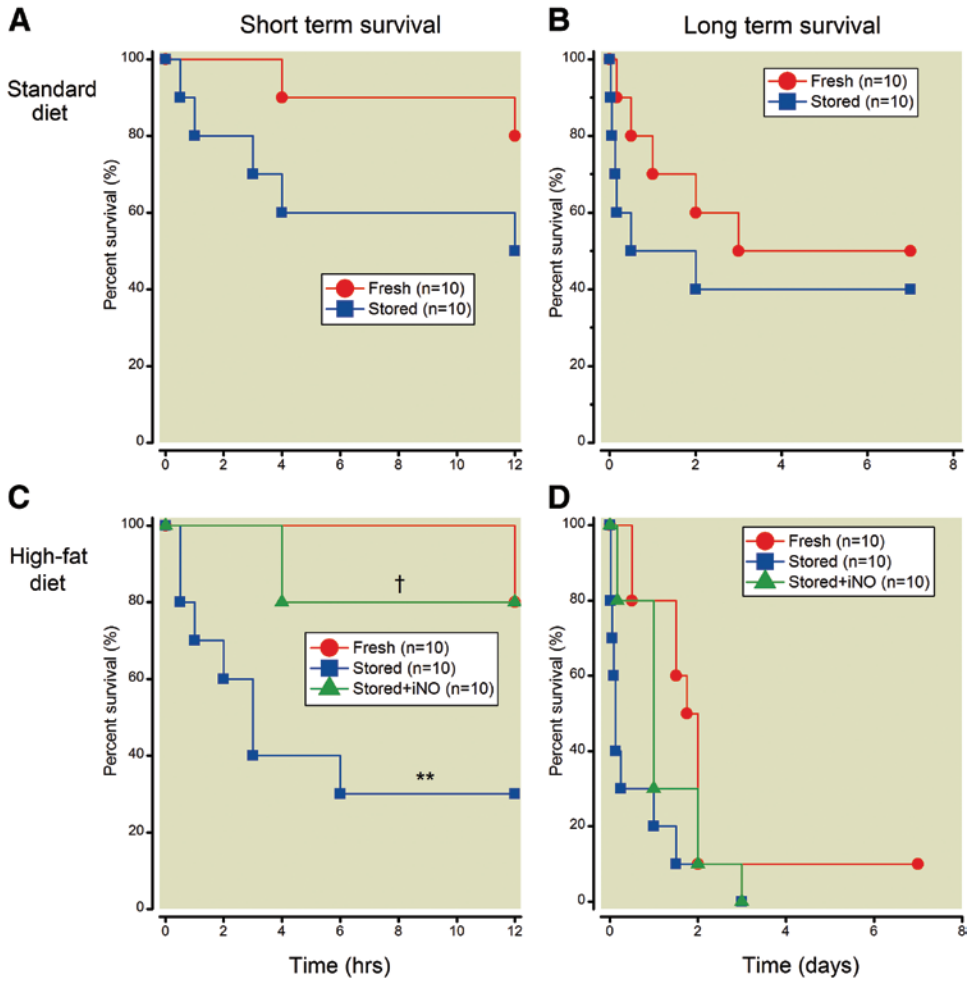


Fig. 2. Resuscitation with stored erythrocytes is associated with a reduced survival rate in high-fat diet–fed mice. Standard-diet–fed (*A, B*) or high-fat diet–fed (*C, D*) mice were subjected to 90 min of hemorrhagic shock and resuscitated with either fresh erythrocytes (n = 10, stored for <24 h) or stored erythrocytes (n = 10, stored for 2 weeks). An additional group of high-fat diet–fed mice (n = 10) was resuscitated with stored erythrocytes, while breathing 80 parts per million nitric oxide from 10 min before resuscitation until 2 h after resuscitation. Data are expressed as percent survival (%). $^{**}P < 0.01$ differs *versus* fresh erythrocytes, $\dagger P < 0.05$ differs *versus* stored erythrocytes. iNO = inhaled nitric oxide (80 parts per million).

with fresh erythrocytes than in sham-operated mice. Plasma hemoglobin levels were greater in standard-diet–fed mice resuscitated with stored erythrocytes than in similar mice resuscitated with fresh erythrocytes ($P < 0.01$, table 3). In HFD-fed mice, resuscitation with fresh erythrocytes did not significantly increase plasma hemoglobin levels above those measured in sham-operated mice. Plasma hemoglobin levels were greater in HFD-fed mice resuscitated with stored erythrocytes than in HFD-fed mice resuscitated with fresh erythrocytes ($P < 0.01$). Furthermore, plasma hemoglobin levels were much greater in HFD-fed mice receiving stored erythrocytes than the levels in standard-diet–fed mice resuscitated with stored erythrocytes. To study the effect of breathing nitric oxide on hemolysis of erythrocytes after transfusion, we examined whether nitric oxide breathing would attenuate the increase of plasma hemoglobin levels in HFD-fed mice resuscitated with stored erythrocytes. We found that inhaled nitric oxide reduced the increase of plasma hemoglobin levels in HFD-fed mice resuscitated with stored erythrocytes ($P < 0.01$, table 3). In addition, plasma hemoglobin levels were correlated with many indices of tissue injury in many organs (*e.g.*, plasma BUN $R^2 = 0.3$, $P < 0.0001$), pulmonary myeloperoxidase activity ($R^2 = 0.4$, $P < 0.0001$), and pulmonary and hepatic oxidative stress as measured by 4-HNE ($R^2 = 0.3$, $P = 0.0008$) and 8-OHdG ($R^2 = 0.4$, $P < 0.0001$). These results suggest that increased plasma hemoglobin levels likely contribute to the adverse effects induced by HS and resuscitation with stored erythrocytes.

Effects of Resuscitation with Fresh or Stored Erythrocytes on Plasma Levels of Haptoglobin and Hemopexin and Pulmonary and Hepatic HO-1 mRNA Levels

Mammals produce proteins to neutralize plasma hemoglobin (*e.g.*, haptoglobin) and heme (*e.g.*, hemopexin) and, thereby, prevent inflammatory damage and vasoconstriction.[30,31] There was no significant difference in plasma hemopexin

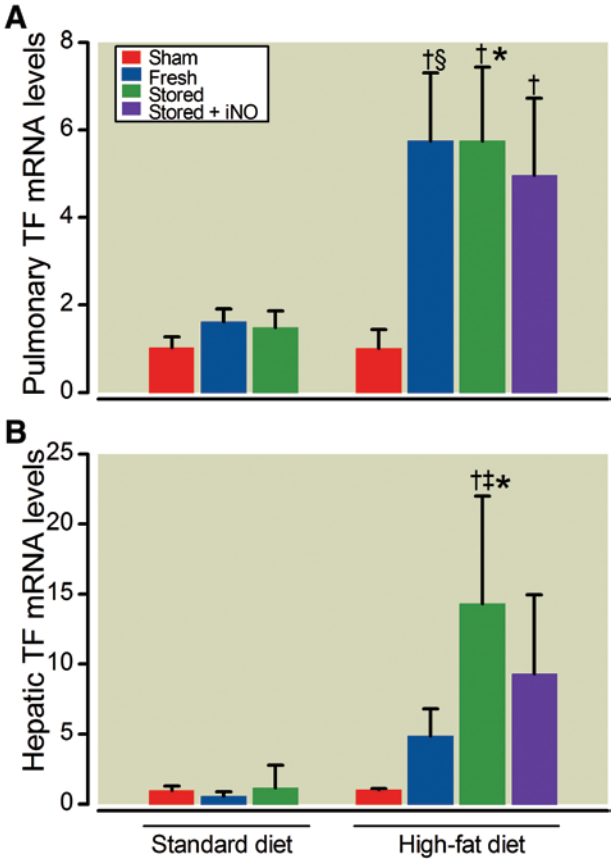


Fig. 3. TF mRNA levels were increased in lung and liver after resuscitation with stored erythrocytes in high-fat diet–fed (HFD-fed) mice. Standard-diet– and HFD-fed mice were subjected to 90 min of hemorrhagic shock and then resuscitated with fresh or stored erythrocytes. An additional group of HFD-fed mice was resuscitated with stored erythrocytes combined with 80 parts per million nitric oxide inhalation. Pulmonary (*A*) and hepatic (*B*) TF mRNA levels were determined at 2 h after resuscitation. Sham, no hemorrhagic shock and resuscitation (n = 6); Fresh, resuscitated with fresh erythrocytes stored less than 24 h (n = 6); Stored, resuscitated with erythrocytes stored for 2 weeks (n = 6). Stored + iNO, resuscitation with stored erythrocytes and breathed 80 parts per million nitric oxide (n = 6). Data are expressed as mean ± SD. †P < 0.01 differs *versus* corresponding sham, ‡P < 0.05 differs *versus* corresponding fresh erythrocytes, §P < 0.05 differs *versus* standard diet–fed mice resuscitated with fresh erythrocytes, *P < 0.05 differs *versus* standard-diet–fed mice resuscitated with stored erythrocytes. iNO = inhaled nitric oxide (80 parts per million); mRNA = messenger ribonucleic acid; TF = tissue factor.

levels between sham-operated standard diet–fed mice and sham-operated HFD-fed mice. In contrast, greater baseline levels of haptoglobin were measured in HFD-fed mice compared with the haptoglobin baseline of standard-diet–fed mice. Hemopexin levels were less in standard-diet–fed mice resuscitated with fresh erythrocytes than in sham-operated mice, whereas haptoglobin levels did not significantly differ (table 3). Both haptoglobin and hemopexin levels were less in standard-diet–fed mice resuscitated with stored erythrocytes than in mice resuscitated with fresh erythrocytes or in sham-operated mice (P < 0.01 for both). In HFD-fed mice, resuscitation with either fresh or stored erythrocytes reduced plasma haptoglobin and hemopexin levels compared with sham-operated mice. There were no significant differences of haptoglobin levels between standard-diet– or HFD-fed mice resuscitated with either fresh or stored erythrocytes. Similarly, no significant differences were measured of hemopexin levels in standard diet– or HFD-fed mice resuscitated with fresh or stored erythrocytes. Breathing nitric oxide did not attenuate the decrease of haptoglobin and hemopexin levels after resuscitation with stored erythrocytes. Our data suggest that resuscitation with stored erythrocytes released greater amounts of hemoglobin into the plasma and, thereby, lead to increased plasma hemoglobin and heme levels, which were bound by haptoglobin and hemopexin, respectively.

Complexes of hemoglobin–haptoglobin or heme–hemopexin are transported to monocytes/macrophages and/or hepatic parenchymal cells, where the heme is metabolized by heme oxygenase-1 (HO-1). Our recent study has shown that transfusion with stored erythrocytes increased hepatic HO-1 mRNA levels.[22] In the current study, we noted that pulmonary and hepatic HO-1 mRNA levels were not different in sham-operated mice between standard-diet– and HFD-fed mice (fig. 4). In standard-diet–fed mice, pulmonary and hepatic HO-1 mRNA levels were higher in mice resuscitated with fresh erythrocytes than in sham-operated mice (P < 0.01). At 2 h after transfusion, pulmonary and hepatic HO-1 mRNA levels were greater in standard-diet–fed mice that were resuscitated with stored erythrocytes than in the sham group, and hepatic HO-1 mRNA levels were much higher in mice resuscitated with stored erythrocytes than in mice resuscitated with fresh erythrocytes. In HFD-fed mice, HO-1 mRNA levels in lung and liver were higher in mice resuscitated with fresh and stored erythrocytes than in sham-operated mice. Resuscitation with stored erythrocytes induced HO-1 gene expression to a greater extent in lung and liver than did resuscitation with fresh erythrocytes. In addition, pulmonary HO-1 gene expression was greater in HFD-fed mice resuscitated with stored erythrocytes than in standard-diet–fed mice resuscitated with stored erythrocytes (P < 0.05). Breathing nitric oxide during resuscitation with stored erythrocytes in HFD-fed mice did not alter the increase of HO-1 mRNA levels in lung and liver. Our results indicate that the increase of pulmonary and hepatic HO-1 mRNA levels may occur *via* other pathways than the reduction of nitric oxide bioavailability. These data suggest that hemolysis after resuscitation with stored erythrocytes induced greater levels of pulmonary and hepatic HO-1 gene expression in both standard-diet– and HFD-fed mice.

Discussion

In this study, we examined resuscitation with either fresh or stored erythrocytes in a murine model of severe HS. We report that resuscitation of HS with stored erythrocytes is worse than

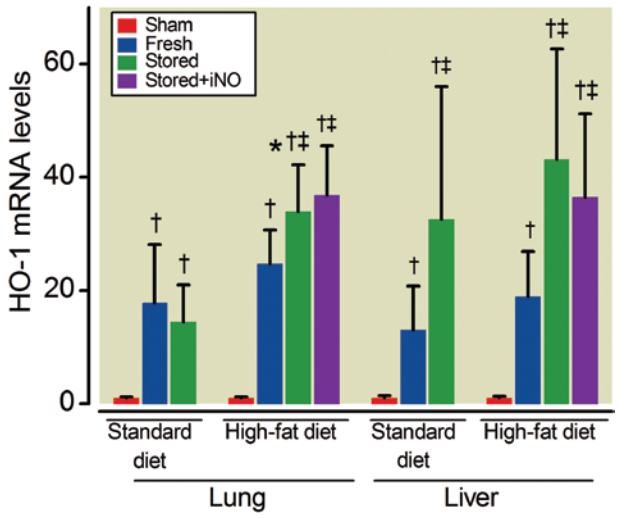


Fig. 4. Resuscitation with stored erythrocytes increased pulmonary and hepatic HO-1 mRNA levels. Standard-diet– and high-fat diet–fed mice were subjected to 90 min of hemorrhagic shock, and resuscitation was completed with fresh or stored erythrocytes with or without breathing 80 parts per million nitric oxide. Pulmonary and hepatic HO-1 mRNA levels were determined at 2 h after resuscitation. Sham, no hemorrhagic shock and resuscitation (n = 6); Fresh, resuscitated with fresh erythrocytes stored for less than 24 h (n = 6); Stored, resuscitated with erythrocytes stored for 2 weeks (n = 6). Stored + iNO, resuscitated with stored erythrocytes and breathed 80 parts per million nitric oxide (n = 6). Data are expressed as mean ± SD. †P < 0.01 differs *versus* corresponding sham, ‡P < 0.05 differs *versus* corresponding fresh erythrocytes, *P < 0.05 differs *versus* standard-diet–fed mice resuscitated with stored erythrocytes. HO-1 = heme oxygenase-1; iNO = inhaled nitric oxide (80 parts per million); messenger ribonucleic acid = mRNA.

resuscitation with fresh erythrocytes. HFD-fed mice (with endothelial dysfunction) are more sensitive to transfusion than are standard-diet–fed mice. Inhaled nitric oxide reduces some, but not all, of the injuries associated with transfusing stored blood into HFD-fed mice. We also report that recipient factors, and the duration of erythrocyte storage, can play major roles modulating the adverse effects of transfusing stored blood.

The toxicity of transfusing stored blood is likely related to the amount of blood transfused and its duration of storage. A recent clinical study reported that the risk of acute kidney injury was in direct proportion to the number of units of erythrocytes transfused in patients undergoing cardiac surgery.[32] In our murine model of severe HS (90 min at 40 mmHg mean arterial pressure), we transfused approximately 40% of total blood volume with either fresh or stored syngeneic erythrocytes. We report that after HS resuscitation with either fresh or stored erythrocytes induced tissue injury in all of the resuscitated groups. Tissue injury induced by resuscitation with either fresh or stored erythrocytes was greater in HFD-fed mice than in standard-diet–fed mice.

In this study, we measured a decrease of both circulating 2,3-DPG and P_{50} levels in HFD-fed mice resuscitated with stored erythrocytes, but there was no change of these variables from normal levels in HFD-fed mice resuscitated with fresh erythrocytes. Reduced P_{50} impairs the ability of blood to provide oxygen to tissues. Others have found that transfusing blood stored for 28 days fails to improve intestinal microvascular oxygenation in a rat HS model.[33] It is conceivable that the tissue injury and early mortality seen in HFD-fed mice resuscitated with stored erythrocytes may be partly attributable to this reduction in P_{50}.

Gladwin and Kim-Shapiro[34] have proposed that several of the adverse events (vasoconstriction, inflammation, and thrombosis) due to stored erythrocyte transfusion are attributable to a reduction of nitric oxide bioavailability, due to an imbalance between nitric oxide destruction and production. When human erythrocytes are stored for 42 days, hemolysis increases extracellular hemoglobin levels to 25 μM.[35] Recently, Berra *et al.*[36] transfused 1 unit of autologous erythrocytes stored for 40 days into healthy volunteers. They reported that plasma hemoglobin levels reached 25 μM at 2 h after transfusion. In the current study, we found that plasma hemoglobin levels were greater in HFD-fed mice subjected to HS and resuscitated with stored erythrocytes than in standard-diet–fed mice resuscitated with stored erythrocytes. Furthermore, the increased levels of extracellular hemoglobin positively correlated with tissue injury, inflammation, and oxidative stress. Our data also suggest this as a possible mechanism, the reduced nitric oxide bioavailability produced by plasma hemoglobin scavenging is likely to contribute to the adverse effects we measured after transfusion of murine erythrocytes stored for prolonged periods.

Haptoglobin and hemopexin are two major protective proteins against the toxicities produced by free hemoglobin and heme, respectively.[37,38] In this study, consistent with the increased plasma hemoglobin and heme levels after transfusion, we measured a decrease of haptoglobin and hemopexin levels in all groups resuscitated with stored erythrocytes. Haptoglobin and hemopexin may attenuate the toxicity associated with increased plasma levels of hemoglobin or heme. Baek *et al.*[39] have recently demonstrated that coinfusion of haptoglobin attenuates the intravascular hemolysis, acute hypertension, vascular injury, and kidney dysfunction induced by transfusing 28-day-old blood into guinea pigs. In addition, HO-1, a stress-inducible protein with potential antiinflammatory effect, can be induced by increased heme levels.[40,41] We consistently measured an increase of pulmonary and hepatic HO-1 mRNA levels in all groups resuscitated with fresh or stored erythrocytes.

In the current study, we measured an increased plasma IL-6 concentration and pulmonary myeloperoxidase activity in standard-diet–fed mice resuscitated with stored erythrocytes than in mice receiving fresh erythrocytes, with an exacerbated inflammatory response in HFD-fed mice. The increased plasma IL-6 levels measured in HFD-fed mice in the current HS model are consistent with our recent study, where transfusion with stored erythrocytes increased plasma

IL-6 levels in HFD-fed mice.[22] Furthermore, Hod *et al.*[42] have demonstrated that transfusion of murine erythrocytes stored for 14 days led to increased plasma proinflammatory cytokine levels. In addition, in HFD-fed mice, we measured increased prothrombotic effects after HS and resuscitation with stored erythrocytes. Together, our data suggest that HS and resuscitation with stored erythrocytes activate the inflammatory and coagulation cascades and contribute to the development of tissue injury and multiorgan dysfunction.

The oxidation of extracellular hemoglobin to methemoglobin and the production of free heme can initiate oxidative damage to lipids, nucleic acids, and proteins,[43] all of which could contribute to the tissue injury associated with increased plasma hemoglobin levels.[44] Measurements of 4-HNE modified protein adducts and 8-OHdG are reliable markers of *in vivo* oxidative stress.[25,45] In our study, resuscitating HFD-fed mice with stored erythrocytes produced markedly increased levels of both 4-HNE adducts and 8-OHdG. These data suggest that oxidative stress after resuscitation from HS contributes to the adverse effects after transfusion of stored erythrocytes.

Exposing circulating extracellular hemoglobin (in the absence of methemoglobin reductase outside of erythrocytes) to a high concentration of inhaled nitric oxide rapidly converts it to ferric hemoglobin species, which do not scavenge nitric oxide.[46] Breathing nitric oxide leads to a rapid accumulation of a variety of nitric oxide metabolites, which are transported to the periphery where they can be converted back to nitric oxide.[47] Increased tissue levels of nitric oxide may reduce tissue injury, inflammation, thrombosis, oxidative stress, and metabolic acidosis (reduced lactate levels). Furthermore, inhaled nitric oxide interacts with leukocytes and platelets as they transit the lungs impairing their ability to be activated in the periphery. Inhaled nitric oxide reduced plasma hemoglobin levels; this may be due to increased renal excretion of hemoglobin during nitric oxide breathing.[48] The antiinflammatory effects and vascular dilation function of nitric oxide may have contributed to the improved short-term survival rate of HFD-fed mice resuscitated with stored erythrocytes. However, breathing nitric oxide did not attenuate all the adverse effects. It is conceivable that transfusion with stored erythrocytes may induce inflammation *via* other mechanisms, such as induction by lipid or other components of transfused erythrocytes other than extracellular hemoglobin.

Host factors can sensitize individuals to transfusion with blood stored for prolonged periods. Recently, Vlaar *et al.*[49] reported that transfusion of erythrocytes stored for 14 days induced mild lung injury in healthy rats, and to a greater extent in lipopolysaccharide-pretreated rats. We have shown that HFD-fed mice exhibit endothelial dysfunction. Deleterious events after transfusion of stored erythrocytes in healthy patients seem to be exacerbated in recipients compromised by preexisting disorders.[11] If our findings in mice can be extrapolated to patients with endothelial dysfunction, then the complications after stored blood transfusion in patients with HS who suffer from diabetes or atherosclerosis may be similar to the effect of transfusing stored erythrocytes in mice with endothelial dysfunction after HS. Care should be taken when transfusing stored erythrocytes into patients with endothelial dysfunction. Transfusion of "younger" erythrocytes should be considered when they are available, especially for patients in HS who have endothelial dysfunction. Breathing nitric oxide during the transfusion of "older" erythrocytes should be studied as it may prevent some of the deleterious effects of transfusing stored erythrocytes. However, because murine blood differs markedly from human blood (*e.g.*, shorter erythrocyte half-life, reduced oxygen affinity, lower plasma haptoglobin levels), the conclusions drawn from our murine studies warrant further clinical study in humans.

In conclusion, transfusion of syngeneic murine blood stored for prolonged periods adversely impacts outcome in a mouse model of HS. Endothelial dysfunction sensitizes mice to the adverse effects of transfusing stored erythrocytes. Inhaled nitric oxide can ameliorate many but not all of the adverse effects of resuscitating mice after HS with stored erythrocytes.

The authors thank Hui Zheng, Ph.D., Assistant Professor in Medicine at Harvard Medical School, Massachusetts General Hospital Biostatistics Center, Boston, Massachusetts, and Rajeev Malhotra, M.D., Instructor in Medicine, Cardiology Division, Massachusetts General Hospital, Boston, Massachusetts, for statistical assistance and help with data analysis. The authors also thank Aranya Bagchi, M.D., Clinical and Research Fellow, Department of Anesthesia, Critical Care and Pain Medicine, Massachusetts General Hospital, Boston, Massachusetts, and Miss Sarah R. Hayton, B.Sc., Research Technician in the Department of Anesthesia, Critical Care, and Pain Medicine, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts, for their kind assistance in measuring tissue myeloperoxidase activity and 8-hydroxy-2-deoxy guanosine concentrations.

References

1. Hess JR: Red cell storage. J Proteomics 2010; 73:368–73
2. Tinmouth A, Fergusson D, Yee IC, Hébert PC; ABLE Investigators; Canadian Critical Care Trials Group: Clinical consequences of red cell storage in the critically ill. Transfusion 2006; 46:2014–27
3. Yoshida T, Shevkoplyas SS: Anaerobic storage of red blood cells. Blood Transfus 2010; 8:220–36
4. Offner PJ, Moore EE, Biffl WL, Johnson JL, Silliman CC: Increased rate of infection associated with transfusion of old blood after severe injury. Arch Surg 2002; 137:711–6; discussion 716–7
5. Leal-Noval SR, Muñoz-Gómez M, Arellano-Orden V, Marín-Caballos A, Amaya-Villar R, Marín A, Puppo-Moreno A, Ferrándiz-Millón C, Flores-Cordero JM, Murillo-Cabezas F: Impact of age of transfused blood on cerebral oxygenation in male patients with severe traumatic brain injury. Crit Care Med 2008; 36:1290–6
6. Keller ME, Jean R, LaMorte WW, Millham F, Hirsch E: Effects of age of transfused blood on length of stay in trauma patients: A preliminary report. J Trauma 2002; 53:1023–5
7. Weinberg JA, McGwin G Jr, Griffin RL, Huynh VQ, Cherry SA 3rd, Marques MB, Reiff DA, Kerby JD, Rue LW 3rd: Age of transfused blood: An independent predictor of mortality despite universal leukoreduction. J Trauma 2008; 65:279–82; discussion 282–4

8. Murrell Z, Haukoos JS, Putnam B, Klein SR: The effect of older blood on mortality, need for ICU care, and the length of ICU stay after major trauma. Am Surg 2005; 71:781–5
9. Spinella PC, Carroll CL, Staff I, Gross R, Mc Quay J, Keibel L, Wade CE, Holcomb JB: Duration of red blood cell storage is associated with increased incidence of deep vein thrombosis and in hospital mortality in patients with traumatic injuries. Crit Care 2009; 13:R151
10. Koch CG, Li L, Sessler DI, Figueroa P, Hoeltge GA, Mihaljevic T, Blackstone EH: Duration of red-cell storage and complications after cardiac surgery. N Engl J Med 2008; 358:1229–39
11. Triulzi DJ, Yazer MH: Clinical studies of the effect of blood storage on patient outcomes. Transfus Apher Sci 2010; 43:95–106
12. van de Watering L, Lorinser J, Versteegh M, Westendord R, Brand A: Effects of storage time of red blood cell transfusions on the prognosis of coronary artery bypass graft patients. Transfusion 2006; 46:1712–8
13. Yap CH, Lau L, Krishnaswamy M, Gaskell M, Yii M: Age of transfused red cells and early outcomes after cardiac surgery. Ann Thorac Surg 2008; 86:554–9
14. Walsh TS, McArdle F, McLellan SA, Maciver C, Maginnis M, Prescott RJ, McClelland DB: Does the storage time of transfused red blood cells influence regional or global indexes of tissue oxygenation in anemic critically ill patients? Crit Care Med 2004; 32:364–71
15. Hébert PC, Chin-Yee I, Fergusson D, Blajchman M, Martineau R, Clinch J, Olberg B: A pilot trial evaluating the clinical effects of prolonged storage of red cells. Anesth Analg 2005; 100:1433–8
16. Donadee C, Raat NJ, Kanias T, Tejero J, Lee JS, Kelley EE, Zhao X, Liu C, Reynolds H, Azarov I, Frizzell S, Meyer EM, Donnenberg AD, Qu L, Triulzi D, Kim-Shapiro DB, Gladwin MT: Nitric oxide scavenging by red blood cell microparticles and cell-free hemoglobin as a mechanism for the red cell storage lesion. Circulation 2011; 124:465–76
17. Kim-Shapiro DB, Schechter AN, Gladwin MT: Unraveling the reactions of nitric oxide, nitrite, and hemoglobin in physiology and therapeutics. Arterioscler Thromb Vasc Biol 2006; 26:697–705
18. Yu B, Raher MJ, Volpato GP, Bloch KD, Ichinose F, Zapol WM: Inhaled nitric oxide enables artificial blood transfusion without hypertension. Circulation 2008; 117:1982–90
19. Gladwin MT, Crawford JH, Patel RP: The biochemistry of nitric oxide, nitrite, and hemoglobin: Role in blood flow regulation. Free Radic Biol Med 2004; 36:707–17
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; 293:1653–62
21. Yu B, Shahid M, Egorina EM, Sovershaev MA, Raher MJ, Lei C, Wu MX, Bloch KD, Zapol WM: Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. Anesthesiology 2010; 112:586–94
22. Yu B, Lei C, Baron DM, Steinbicker AU, Bloch KD, Zapol WM: Diabetes augments and inhaled nitric oxide prevents the adverse hemodynamic effects of transfusing syngeneic stored blood in mice. Transfusion 2012; 52:1410–22
23. Vaporidi K, Francis RC, Bloch KD, Zapol WM: Nitric oxide synthase 3 contributes to ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 2010; 299:L150–9
24. Butt OI, Buehler PW, D'Agnillo F: Blood-brain barrier disruption and oxidative stress in guinea pig after systemic exposure to modified cell-free hemoglobin. Am J Pathol 2011; 178:1316–28
25. Valavanidis A, Vlachogianni T, Fiotakis C: 8-hydroxy-2' -deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 2009; 27:120–39
26. Atochin DN, Wang A, Liu VW, Critchlow JD, Dantas AP, Looft-Wilson R, Murata T, Salomone S, Shin HK, Ayata C, Moskowitz MA, Michel T, Sessa WC, Huang PL: The phosphorylation state of eNOS modulates vascular reactivity and outcome of cerebral ischemia in vivo. J Clin Invest 2007; 117:1961–7
27. Raher MJ, Thibault HB, Buys ES, Kuruppu D, Shimizu N, Brownell AL, Blake SL, Rieusset J, Kaneki M, Derumeaux G, Picard MH, Bloch KD, Scherrer-Crosbie M: A short duration of high-fat diet induces insulin resistance and predisposes to adverse left ventricular remodeling after pressure overload. Am J Physiol Heart Circ Physiol 2008; 295:H2495–502
28. Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, Moore PK: Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 2005; 19:1196–8
29. Gilson CR, Kraus TS, Hod EA, Hendrickson JE, Spitalnik SL, Hillyer CD, Shaz BH, Zimring JC: A novel mouse model of red blood cell storage and posttransfusion in vivo survival. Transfusion 2009; 49:1546–53
30. Boretti FS, Buehler PW, D'Agnillo F, Kluge K, Glaus T, Butt OI, Jia Y, Goede J, Pereira CP, Maggiorini M, Schoedon G, Alayash AI, Schaer DJ: Sequestration of extracellular hemoglobin within a haptoglobin complex decreases its hypertensive and oxidative effects in dogs and guinea pigs. J Clin Invest 2009; 119:2271–80
31. Balla J, Vercellotti GM, Jeney V, Yachie A, Varga Z, Jacob HS, Eaton JW, Balla G: Heme, heme oxygenase, and ferritin: How the vascular endothelium survives (and dies) in an iron-rich environment. Antioxid Redox Signal 2007; 9:2119–37
32. Karkouti K, Wijeysundera DN, Yau TM, McCluskey SA, Chan CT, Wong PY, Beattie WS: Influence of erythrocyte transfusion on the risk of acute kidney injury after cardiac surgery differs in anemic and nonanemic patients. Anesthesiology 2011; 115:523–30
33. van Bommel J, de Korte D, Lind A, Siegemund M, Trouwborst A, Verhoeven AJ, Ince C, Henny CP: The effect of the transfusion of stored RBCs on intestinal microvascular oxygenation in the rat. Transfusion 2001; 41:1515–23
34. Gladwin MT, Kim-Shapiro DB: Storage lesion in banked blood due to hemolysis-dependent disruption of nitric oxide homeostasis. Curr Opin Hematol 2009; 16:515–23
35. Bennett-Guerrero E, Veldman TH, Doctor A, Telen MJ, Ortel TL, Reid TS, Mulherin MA, Zhu H, Buck RD, Califf RM, McMahon TJ: Evolution of adverse changes in stored RBCs. Proc Natl Acad Sci USA 2007; 104:17063–8
36. Berra L, Coppadoro A, Yu B, Lei C, Spagnolli E, Steinbicker AU, Bloch KD, Lin T, Sammy FY, Warren HS, Fernandez BO, Feelisch M, Dzik WH, Stowell CP, Zapol WM: Transfusion of stored autologous blood does not alter reactive hyperemia index in healthy volunteers. Anesthesiology 2012; 117: 56–63
37. Buehler PW, Karnaukhova E, Gelderman MP, Alayash AI: Blood aging, safety, and transfusion: Capturing the "radical" menace. Antioxid Redox Signal 2011; 14:1713–28
38. Larsen R, Gozzelino R, Jeney V, Tokaji L, Bozza FA, Japiassú AM, Bonaparte D, Cavalcante MM, Chora A, Ferreira A, Marguti I, Cardoso S, Sepúlveda N, Smith A, Soares MP: A central role for free heme in the pathogenesis of severe sepsis. Sci Transl Med 2010; 2:51ra71
39. Baek JH, D'Agnillo F, Vallelian F, Pereira CP, Williams MC, Jia Y, Schaer DJ, Buehler PW: 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; 122:1444–58
40. Nielsen MJ, Moestrup SK: Receptor targeting of hemoglobin mediated by the haptoglobins: Roles beyond heme scavenging. Blood 2009; 114:764–71

41. Tolosano E, Fagoonee S, Morello N, Vinchi F, Fiorito V: Heme scavenging and the other facets of hemopexin. Antioxid Redox Signal 2010; 12:305–20
42. Hod EA, Zhang N, Sokol SA, Wojczyk BS, Francis RO, Ansaldi D, Francis KP, Della-Latta P, Whittier S, Sheth S, Hendrickson JE, Zimring JC, Brittenham GM, Spitalnik SL: Transfusion of red blood cells after prolonged storage produces harmful effects that are mediated by iron and inflammation. Blood 2010; 115:4284–92
43. Buehler PW, D'Agnillo F, Schaer DJ: Hemoglobin-based oxygen carriers: From mechanisms of toxicity and clearance to rational drug design. Trends Mol Med 2010; 16:447–57
44. Buehler PW, D'Agnillo F: Toxicological consequences of extracellular hemoglobin: Biochemical and physiological perspectives. Antioxid Redox Signal 2010; 12:275–91
45. Poli G, Schaur RJ, Siems WG, Leonarduzzi G: 4-hydroxynonenal: A membrane lipid oxidation product of medicinal interest. Med Res Rev 2008; 28:569–631
46. Yu B, Bloch KD, Zapol WM: Hemoglobin-based red blood cell substitutes and nitric oxide. Trends Cardiovasc Med 2009; 19:103–7
47. Nagasaka Y, Fernandez BO, Garcia-Saura MF, Petersen B, Ichinose F, Bloch KD, Feelisch M, Zapol WM: Brief periods of nitric oxide inhalation protect against myocardial ischemia-reperfusion injury. Anesthesiology 2008; 109: 675–82
48. Minneci PC, Deans KJ, Zhi H, Yuen PS, Star RA, Banks SM, Schechter AN, Natanson C, Gladwin MT, Solomon SB: Hemolysis-associated endothelial dysfunction mediated by accelerated NO inactivation by decompartmentalized oxyhemoglobin. J Clin Invest 2005; 115:3409–17
49. Vlaar AP, Hofstra JJ, Levi M, Kulik W, Nieuwland R, Tool AT, Schultz MJ, de Korte D, Juffermans NP: Supernatant of aged erythrocytes causes lung inflammation and coagulopathy in a "two-hit" in vivo syngeneic transfusion model. Anesthesiology 2010; 113:92–103

ANESTHESIOLOGY REFLECTIONS FROM THE PIERRE VIARS MUSEUM

Radium and Thorium Applications for the General Public: Unexpected Consequences of the Discovery from Pierre and Marie Curie

After the discoveries of ionizing radiation (by von Röntgen and Becquerel) and radium and thorium by Pierre and Marie Curie in France, there was real enthusiasm for these radioactive elements and the general public alike. Radium was considered beneficial at low dose. Many applications were proposed for healthy and hygienic purposes: radium and thorium were introduced in face cream against wrinkles (were faces fluorescent in the night?), in lipstick (for hot lips?), in drugs for bronchitis. Manufacturers even produced a domestic fountain providing radioactive water to drink and a radium-containing coffeepot with great commercial success.

Jean-Bernard Cazalaà, M.D., President of Club d'Histoire de l'Anesthésie et de la Réanimation (French Association for the History of Anesthesiology and Critical Care), France (www.char-fr.net), and Musée Viars, CHU Pitié-Salpêtrière, Paris, France.