

ARTICLE

S-Nitrosohemoglobin Levels and Patient Outcome After Transfusion During Pediatric Bypass Surgery

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Banked blood exhibits impairments in nitric oxide (NO)-based oxygen delivery capability, reflected in rapid depletion of S-nitrosohemoglobin (SNO-Hb). We hypothesized that transfusion of even freshly-stored blood used in pediatric heart surgery would reduce SNO-Hb levels and worsen outcome. In a retrospective review ($n = 29$), the percent of estimated blood volume (% eBV) replaced by transfusion directly correlated with ventilator time and inversely correlated with kidney function; similar results were obtained in a prospective arm ($n = 20$). In addition, an inverse association was identified between SNO-Hb and postoperative increase in Hb (Δ Hb), reflecting the amount of blood retained by the patient. Both SNO-Hb and Δ Hb correlated with the probability of kidney dysfunction and oxygenation-related complications. Further, regression analysis identified SNO-Hb as an inverse predictor of outcome. The findings suggest that SNO-Hb and Δ Hb are prognostic biomarkers following pediatric cardiopulmonary bypass, and that maintenance of red blood cell-derived NO bioactivity might confer therapeutic benefit.

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Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

✔ Pediatric patients who undergo cardiopulmonary bypass can receive large volumes of allogenic red blood cells. Transfusion of banked blood may enhance rather than correct deficits in tissue oxygenation, which may lead to organ dysfunction and worse postoperative outcome. This is because banked blood is depleted of S-nitrosohemoglobin (SNO-Hb), the main regulator of microvascular blood flow.

WHAT QUESTION DID THIS STUDY ADDRESS?

✔ Would transfusion of even freshly-stored blood used in pediatric heart surgery reduce SNO-Hb levels and worsen outcome?

WHAT THIS STUDY ADDS TO OUR KNOWLEDGE

✔ We linked declines in SNO-Hb caused by intraoperative transfusion to reductions in tissue oxygenation, organ dysfunction, and worse outcomes in young cardiac surgery patients.

HOW THIS MIGHT CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE

✔ SNO-Hb was prognostic for outcome, suggesting that it may be used as a biomarker of transfusion efficacy. S-Nitrosylating agents that raise SNO-Hb levels are currently undergoing human testing.

Congenital heart defects are the most frequent birth anomaly, with an occurrence rate close to 1% of all live deliveries.¹ Within this group, at least one-quarter of afflicted individuals will require surgical intervention early in life to correct the lesion. Neonatal and pediatric cardiopulmonary bypass (CPB) equipment and surgical techniques improved in concert with the adult technology during the mid-part of the 20th century. As a result, the current prognosis for children with even the severest congenital defects is greatly improved, with 3–5 year survival rates of >70%.²

Advances in surgical methodology notwithstanding, CPB remains a significant stressor to the young patient and the need to administer banked blood is commonplace. Allogenic red blood cells (RBCs) are utilized to prime the bypass circuit, replace intraoperative blood loss, and maintain hemodynamic stability. As in other anemic settings, the administration of RBCs during CPB is premised on a direct correlation between the oxygen-carrying capacity of blood and the delivery of oxygen to tissues, i.e., it is assumed that transfusion will improve tissue oxygenation.

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However, similar to adult cardiac populations, infants and neonates who receive RBCs have longer recovery periods and higher rates of adverse events than non-transfused cohorts.^{3,4} A possible explanation is that the administration of stored blood may exacerbate rather than correct anemia-induced deficits in tissue oxygenation.⁵

Tissue oxygen delivery is regulated by hypoxic vasodilation, a physiologic mechanism that couples local oxygen requirements to blood flow.⁶ RBCs serve as a principal transducer of this response by mediating the export of S-nitrosothiol (SNO)-based nitric oxide (NO) bioactivity. More specifically, NO is transported in RBCs by the conserved Cys residue at position 93 of the β chain (β Cys93) in hemoglobin in the form of a SNO, i.e., S-nitrosohemoglobin (SNO-Hb).⁷ Low pO_2 in tissues promotes the release of SNO-based vasodilatory activity from RBCs to maintain tissue perfusion. The centrality of β Cys93-derived SNO in maintaining tissue oxygenation has recently been validated by strict genetic criteria,⁸ and is supported further by the demonstration of enhanced myocardial injury and mortality in the absence of β Cys93 across different models of heart disease.⁹ This in turn has led to a reconceptualization of the respiratory cycle as a three-gas system (O_2 /NO/ CO_2).¹⁰ Assessment of NO status provides a basis for understanding why increasing blood oxygen content (e.g., transfusion) can fail to improve tissue oxygenation;¹¹ blood flow, *not* blood oxygen content, is the primary determinant of oxygen delivery under basal physiological conditions.¹²

A variety of conditions characterized by impairments in tissue oxygenation are associated with decreased bioavailability of RBC SNO,^{13–15} including transfusion.^{16,17} Storage of blood leads to rapid losses in SNO-Hb that are paralleled by declines in the ability of banked RBCs to effect hypoxic vasodilation.¹⁶ Administration of these SNO-Hb-depleted RBCs to anemic animals impairs microvascular blood flow, tissue oxygenation, and organ function (replicating the hypoxic pathophysiology exhibited by β Cys93Ala, i.e., SNO-deficient, mutant mice).^{8,9} Administration of SNO-Hb-replete blood prevents these adverse events.¹⁷

Most practitioners strive to administer the “freshest” blood to their young patients. Yet the pediatric outcome data suggest that receipt of any allogenic RBCs,¹⁸ not expressly older blood,¹⁹ is associated with increased morbidity. Similarly, studies of fresh vs. aged blood in adults have not identified improvements in outcome.^{20–22} The loss of SNO-Hb begins within hours of donation and is 80% depleted within 2 days.^{16,23} Accordingly, administration of SNO-depleted RBCs provides a plausible explanation for how transfusion may worsen outcome. With this in mind, we aimed to first determine if RBC transfusion was associated with adverse events in our population of neonatal congenital heart patients. As the answer was yes, we followed the retrospective review with a prospective study to determine the effects of transfusion on SNO-Hb levels. We predicted that transfusion-induced reductions in SNO-Hb would portend worse outcome, including impaired organ function and increased incidence of oxygenation-related complications.

METHODS

Study overview

This investigation was a single-site observational study. The research protocol was approved by the Institutional Review Board (IRB) of University Hospitals Cleveland Medical Center and written informed consent for study participation in the prospective arm was obtained from the parent(s) or legal guardian(s) of each patient prior to surgery (the IRB waived the need to obtain consent for the retrospective chart review). The target population was neonates and young children (<12 months of age) anticipated to receive packed RBCs during open-heart surgery with CPB for repair of congenital heart disease. Older children and patients undergoing redo procedures were excluded.

The chart review retrieved demographic and perioperative data (RBC transfusion volumes, clinical chemistries, ventilator times, complications, length of stay, etc.) for children operated on between November 2008 and December 2009. The prospective arm enrolled subjects between January 2011 and April 2013.

Procedures

An established bypass procedure (including muscle relaxation with vecuronium and antifibrinolytic therapy with aminocaproic acid) was used for all patients with the surgical techniques dictated by the cardiac anomaly. All CPB surgeries in the prospective study were conducted by the same surgeon (P.C.K.) and perfusion team (J.O. and R.S.) (see **Supplemental Content** for details). A dual-lead INVOS near infrared spectroscopy (NIRS) system (Medtronic, Minneapolis, MN) was used to monitor cerebral and kidney oxygenation.^{24,25} To determine SNO-Hb levels, blood samples (~2 mL) were drawn from the arterial port of the oxygenator during set points of the surgery and from an indwelling arterial line on postoperative Day 1. (Blood sampling and RBC administration did not occur contemporaneously.) No samples were obtained from percutaneous needle sticks, so we do not have preinduction SNO-Hb or blood gas values. The decision to remove the arterial line was made independently of SNO sampling needs (per hospital protocol, this typically occurred on postoperative Day 1), which precluded procurement of additional arterial blood samples for this minimally invasive study. Postoperative patient management was directed by the pediatric ICU staff who were unaware of the study goals and all other blood samplings were done at the direction of the clinical team. RBC SNO-Hb levels were quantified offline and the resultant values were not used to direct any child’s clinical care. Additional patient details and information on postoperative course were collected from the medical record.

RBC SNO-Hb measurement protocol

Photolysis-chemiluminescence was used to quantify SNO-Hb levels.^{16,23,26,27} The method has been well validated for selective determination of RBC HbNO without artificial contamination from other nitrosative species, including nitrite and nitrate.^{28,29} Within 1 h of procurement, the arterial blood samples were spun at low speed, then, after decanting, the pelleted RBCs were washed twice with 0.1 mM diethylene-triaminepentaacetic acid (DTPA)

in phosphate-buffered saline (PBS) at pH 7.4 and then lysed in excess deionized water (1:4 volume ratio x 10 min) containing 0.1 mM DTPA. Lysates were centrifuged (20,000g for 10 min) to separate membranes and cytosol. Membranes were dissolved in Triton X-100 (2%) in PBS, and Hb was desalted by centrifugation (3,000g for 1 min) through Sephadex G-25 spin columns (Pharmacia, Uppsala, Sweden). The Hb samples were then stored at -80°C for batch analysis. The amount of Hb in the eluents was determined spectrophotometrically, adjusted to a final concentration of $400\ \mu\text{M}$, and then incubated with either PBS alone or with sixfold molar excess of mercuric chloride (which selectively cleaves thiol-bound NO groups). Standard curves were generated daily with S-nitrosoglutathione. Concentrations of SNO-Hb and Hb[FeNO] were calculated based on the difference between the amount of NO liberated by UV light in the absence (SNO-Hb plus Hb[FeNO]) vs. presence of mercuric chloride (Hb[FeNO]).^{13,30}

Statistics

Interval and ratio data are expressed as mean \pm standard deviation (SD) and ordinal data are expressed as median \pm interquartile range. To assess kidney function, estimated glomerular filtration rate (eGFR) was calculated from serum chemistries using the formula $\text{eGFR} = 0.45 \times [\text{height}/\text{Scr}]$ with height in centimeters and serum creatinine (Scr) in mg/dl.³¹ Estimated blood volume (eBV) for each patient was determined by body weight and age.³² Where appropriate, log-transformation was used to satisfy conditions of data normality. Standard parametric methods were used to check for differences over time (e.g., repeated-measures analysis of variance (ANOVA) with Dunnett's post-hoc testing), while linear regression was used to test for associations. A logistic stepwise regression model was employed to construct a predictive model for major morbidity (described in detail in the **Supplement**).

RESULTS

Demographic data and general patient outcomes

Data from 29 clinical records were collated in the retrospective study while 20 pediatric heart patients were enrolled in the prospective arm; their demographic and operative data are presented in **Supplemental Table S1**. The two patient populations and the procedural aspects of the surgeries were similar. Importantly, both groups demonstrated negative responses to the intraoperative receipt of RBCs. The amount of blood received (% of eBV replaced) directly correlated with increased postoperative ventilator time (**Supplemental Figure S1A and S1B**; $r = 0.676$, $P = 0.0008$ for the retrospective group and $r = 0.595$, $P = 0.005$ for the prospective cohort) and increased intensive care unit (ICU) stay in the prospective cohort (**Figure S1C**; $r = 0.542$, $P = 0.012$). (Note: information on ICU duration was not recorded in the files of the retrospective group.) Organ function was also impacted negatively by transfusion in the retrospective group, where receipt of more blood was associated with worse kidney function, i.e., declines in eGFR (**Figure S1D**; $r = -0.392$, $P = 0.04$). This functional consequence was explored further in the prospective study.

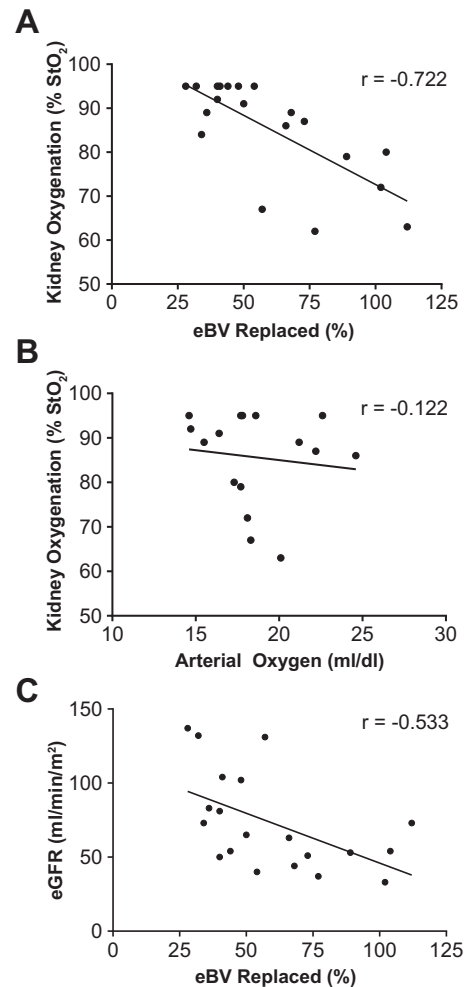


Figure 1 Transfusion, tissue oxygenation, and organ function. (a) The % eBV replaced by intraoperative transfusion was inversely correlated with kidney StO_2 at the end of surgery ($n = 20$; $r = -0.722$, $P = 0.0003$). (b) There was no correlation between kidney StO_2 and arterial blood oxygen content ($r = -0.122$, $P = 0.651$). (c) Scatterplot depicts the inverse correlation between eGFR and % eBV replaced ($r = -0.533$, $P = 0.015$).

Intraoperative transfusion and tissue oxygenation

We predicted that receipt of SNO-Hb-depleted RBCs would adversely impact tissue oxygenation and support for this hypothesis was demonstrated in the prospective arm. First, the % of eBV replaced was inversely correlated with kidney tissue oxygenation (StO_2) at the end of surgery (**Figure 1a**; $r = -0.722$, $P = 0.0003$); a similar albeit weaker inverse association with brain StO_2 was also observed ($r = -0.317$, $P = 0.172$). Second, transfusion increased RBC mass but no correlation was found between kidney StO_2 and arterial oxygen content (**Figure 1b**; $r = -0.122$, $P = 0.651$). Third, as with the retrospective analysis, the amount of blood transfused (% of eBV replaced) was inversely correlated with eGFR (**Figure 1c**; $r = -0.533$, $P = 0.015$), reflecting the importance of microvascular flow on tissue oxygenation. Taken together, these data demonstrate the negative effect of transfusion on postoperative renal function as a consequence of decreased intraoperative kidney StO_2 .

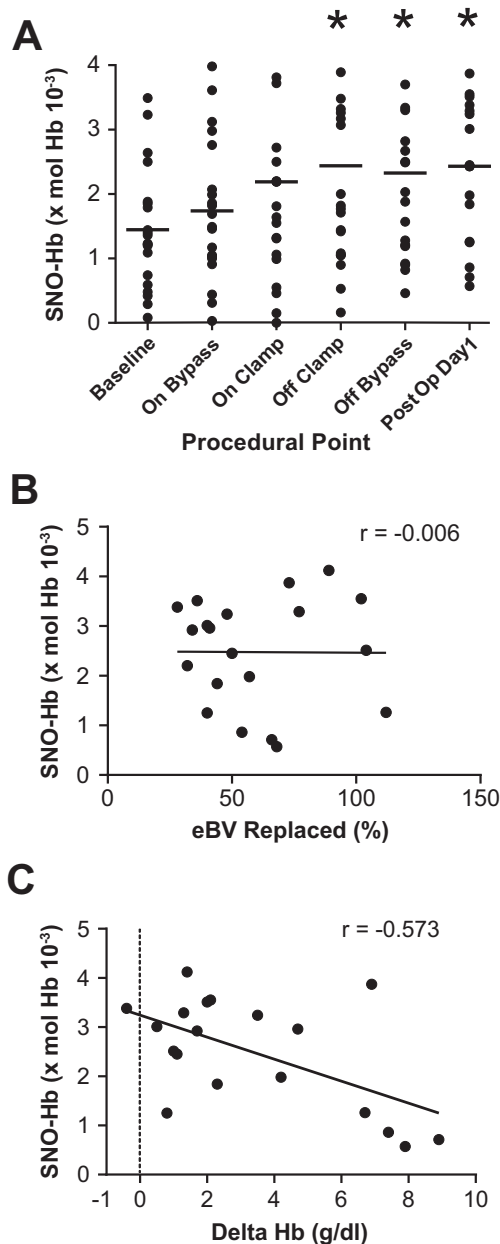


Figure 2 SNO-Hb, CPB, and transfusion. (a) Circulating RBC SNO-Hb concentrations in pediatric patients ($n = 20$; group means are designated by the bars) at various procedural points before, during, and after CPB. SNO-Hb levels increased after going on bypass and continued to rise into the postoperative period. *Significant difference compared with baseline, $P < 0.05$, as determined by repeated-measures ANOVA followed by Dunnet's test. (b) There was no relationship between SNO-Hb levels and % eBV replaced. (c) The increase in SNO-Hb correlated inversely with the magnitude of the pre-to-posttransfusion increase in Hb (Δ Hb) ($r = -0.573$, $P = 0.010$). Retention of more blood (i.e., greater increase in postop Hb) was thus associated with lower SNO-Hb.

SNO-Hb, transfusion, and outcome

The negative association between intraoperative transfusion and StO_2 suggested a role for SNO-Hb, which we tracked prospectively. Initiating bypass alone resulted in a rise in arterial SNO-Hb levels (Figure 2a). Specifically, SNO-Hb

increased by more than 20% (from 1.45 ± 0.95 to 1.74 ± 1.09 moles SNO-Hb per moles Hb $\times 10^{-3}$) after patients were put on pump, then continued to rise over the ensuing 24 h, peaking at 2.43 ± 1.08 per Hb $\times 10^{-3}$ (before removal of arterial access). However, this trend showed significant interpatient variability. To assess whether the variation in SNO-Hb was related to transfusion volume we first sought a correlation between SNO-Hb and % eBV replaced, but none was found (Figure 2b; $r = -0.006$, $P = 0.979$). Rather, postop SNO-Hb concentrations were in fact strongly correlated with amounts of transfused RBCs *retained* by the subject as measured by the increase between pre- and posttransfusion Hb (Δ Hb) (Figure 2c; $r = -0.573$, $P = 0.010$). Thus, individuals whose blood was diluted the least by allogenic RBCs had the highest levels of postoperative SNO-Hb and *vice versa*. In addition, there was no correlation between the RACHS score (Risk Adjustment for Congenital Heart Surgery)³³ and transfusion volume, implying that the impact of RBC administration on outcome was independent of the neonates' preoperative condition or surgical complexity.

Next we sought a relationship between SNO-Hb and patient outcomes. Notably, a positive linear relationship was found between eGFR and SNO-Hb levels ($r = 0.464$, $P = 0.039$), consistent with the idea that SNO-Hb maintains kidney oxygenation and with the finding that transfusion, which lowered SNO-Hb concentration, was associated with a decline in kidney StO_2 (Figure 1a). We compiled a list of postoperative adverse events that may be linked directly to disruptions in oxygen delivery (Figure 3a) and found a strong positive correlation with Δ Hb (Figure 3c; $r = 0.587$, $P = 0.008$) and a strong inverse correlation with SNO-Hb (Figure 3D; $r = -0.695$, $P = 0.0007$). In contrast, we found only weak correlations between complication risk and % of eBV replaced (Figure 3b; $r = 0.392$, $P = 0.08$). Furthermore, a logistic regression model (defined in Supplemental Tables S2 and S3) identified an inverse correlation between SNO-Hb levels and postoperative complications, including all-cause morbidity (Table 1). Thus, postoperative SNO-Hb was a strong prognostic biomarker following intraoperative transfusion.

DISCUSSION

Donated RBCs undergo progressive time-dependent changes in RBC integrity and function, including loss of SNO-Hb, which impairs the ability of RBCs to oxygenate tissues.^{8,17} The loss of SNO-Hb begins within hours of donation,^{16,23} which is consistent with reports that even freshly-processed blood may negatively impact tissue oxygenation³⁴ and patient outcomes.²⁰⁻²² Deficiency in SNO-Hb is causally linked to renal injury,^{17,35} a major adverse consequence of intraoperative blood transfusion.³⁶ The current findings add to this perspective by suggesting a mechanistic basis for transfusion-related tissue injury: lower SNO-Hb levels reduce kidney StO_2 and portend adverse patient outcomes, including reductions in eGFR.

We had anticipated that neonatal bypass would result in a net decline in SNO-Hb because SNO-depleted RBCs were used in the priming solution; however, the opposite was in fact observed. As a group, the subjects exhibited an

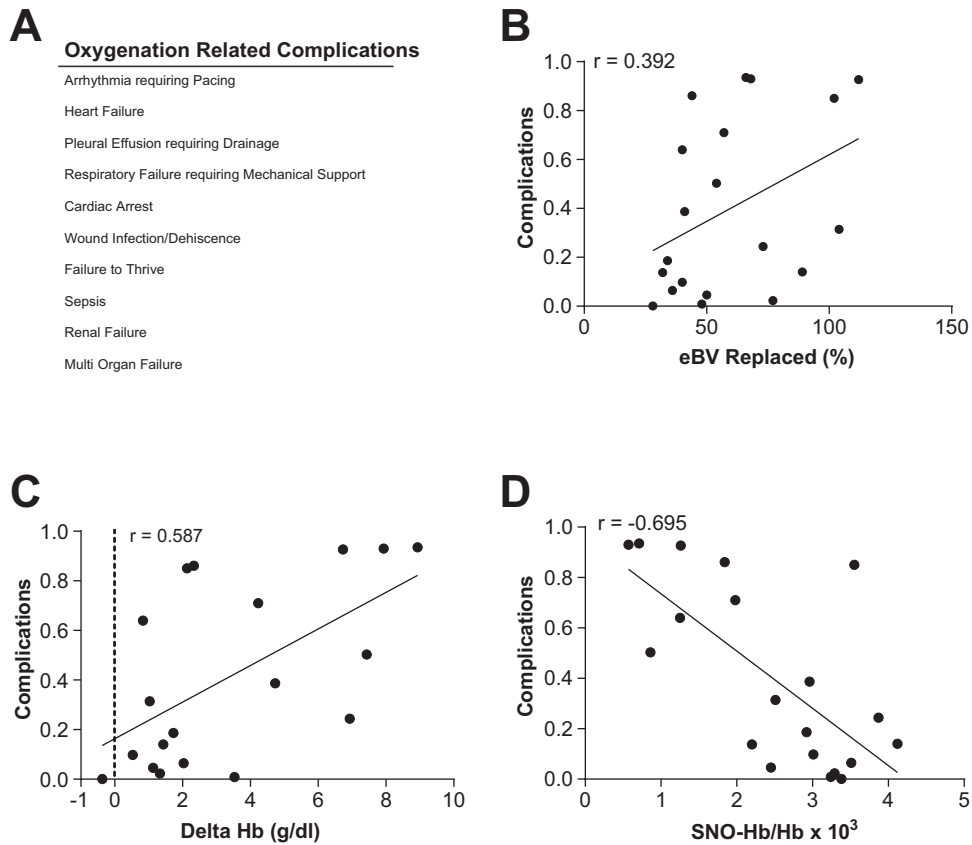


Figure 3 Oxygenation-related complications. (a) Table enumerating the oxygenation-related complications. (b) A scatterplot depicting the weak correlation between % eBV replaced and probability of complications ($n = 20$, $r = 0.392$, $P = 0.08$). (c) Δ Hb demonstrates a positive correlation with probability of complications ($n = 20$, $r = 0.587$, $P = 0.008$). (d) SNO-Hb has a negative correlation with probability of complications ($n = 20$, $r = -0.695$, $P = 0.0007$).

Table 1 Logistical regression analysis between subject factors

Major morbidity ^a	β	SE	Wald (χ^2)	df	P	e^β (odds ratio)
Postop SNO-Hb ^b	-1.437	0.693	4.293	1	0.03	0.238
Constant	12.297	5.658	4.723	1	0.30	
Goodness of fit test	R^2		χ^2	df	P	
Hosmer and Lemeshow test			8.088	8	0.425	
Cox and Snell	0.445					
Nagelkerke	0.602					

^aMajor morbidity was coded as yes or no.

^bPostoperative S-nitrosohemoglobin.

early increase in SNO-Hb concentration. We attribute this rise to initial improvements in blood oxygenation as a result of going on pump (especially in the cyanotic babies); oxygenated Hb favors the production of SNO-Hb.^{7,12} At the same time, changes in SNO-Hb concentrations were related to the amounts of allogenic blood received: receipt and retention of higher volumes of stored RBCs was associated with lower SNO-Hb.

The administration and retention of RBCs deficient in SNO-Hb (% of eBV replaced and Δ Hb, respectively) provide a mechanistic explanation for posttransfusion complications.⁵ This can be understood by appreciating that erythrocytes pass through the microcirculation in line.³⁷ Banked RBCs,

unable to elicit vasodilation, will get stuck and thus impede flow to adversely influence oxygen delivery—an interpretation supported also by prior findings that administration of even small volumes of SNO-Hb-depleted RBCs can decrease organ blood flow and tissue pO_2 .^{16,17} This concept of microvascular “plugging” is also consistent with empiric evidence that SNO-deficient RBCs can adhere to endothelial linings to impair oxygenation.³⁸

Subjects with lower SNO-Hb levels had higher postoperative Hb (i.e., administration of SNO-depleted Hb diluted the SNO-replete Hb), and both low SNO-Hb and high Hb predicted worse outcomes. While it is unclear if individuals might have tolerated a more conservative transfusion

strategy (transfusion triggers in pediatric surgical patients are ill-defined) there is accumulating evidence that restricting transfusion might be beneficial in some patients. In a trial of noncyanotic pediatric bypass subjects, dropping the transfusion threshold to 8.0 g/dl (from 10.8) reduced total hospital length of stay.¹⁸ Conceivably, restrictive strategies act to preserve SNO-Hb levels. To this end, clinical¹³ and pre-clinical trials^{35,39,40} have demonstrated therapeutic benefits of Hb renitrosylation, which, in the setting of transfusion, act to enhance oxygen delivery.¹⁷ As such, the current findings support a follow-on clinical trial to determine if perioperative renitrosylation therapy during neonatal heart surgery could improve outcomes.

We recognize that the correlative analysis of the prospectively collected data is a potential weakness of this study, and the multivariable analysis is weakened by the event sample size. However, the matching findings from both the retrospective chart review (and other bypass studies) and the preclinical studies^{8,9} somewhat render moot these concerns. In addition, we note that the amount of blood transfused matched well with other pediatric trials,¹⁸ StO₂ was tracked in real time (NIRS has been used frequently to noninvasively monitor kidney StO₂ and predict renal injury in young cardiac patients),^{24,25} and our transfusion-related morbidities matched literature reports in this patient population.^{3,4,41} Moreover, the identification of SNO-Hb as the determinant of outcome is consistent with a growing body of literature connecting deficits in RBC SNO-Hb to pathologies of oxygenation.⁴²

In summary, we have linked dysregulated SNO homeostasis caused by large-volume intraoperative transfusion to reductions in tissue oxygenation and adverse events. We have also shown that SNO-Hb levels are inversely correlated with kidney function and all-cause morbidity, suggesting its utility as both a prognostic biomarker and target for therapeutic intervention. Together, our findings provide clinical support for the postulate that defects in the oxygen-delivery function of stored RBCs, reflected in impairments in SNO-Hb based vasoregulation, contribute to the deleterious effects of allogenic blood transfusion.

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Conflict of Interest/Disclosure. Dr. Stamler has a financial interest in Nivalis Therapeutics, Adamas Pharma, and Vindica Pharm. Dr. Reynolds has a financial interest in Miach Medical Innovations. Drs. Stamler and Reynolds hold patents related to renitrosylation of blood, some of which have been licensed for commercial development. The institution is aware of these conflicts and appropriate management plans are in place. The other authors have no conflicts of interest relevant to this article to disclose.

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