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Effects of Red Blood Cell Sickling on Right Ventricular Afterload *in vivo*

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Abstract

Background: Hemolysis in sickle cell disease (SCD) releases cell free hemoglobin, which scavenges nitric oxide (NO), leading to pulmonary vascular vasoconstriction, increased pulmonary vascular resistance (PVR), and the development of PH. However, PVR is only one component of right ventricular (RV) afterload. Whether sickled red blood cells increase the total RV afterload, including compliance and wave reflections, is unclear.

Objective: Patients with SCD and pulmonary hypertension (PH) have a significantly increased risk of sudden death compared to patients with SCD alone. Sickled red blood cells (RBCs) are fragile and lyse easily. Here, we sought to determine the acute effects of SCD RBCs and increased cell free hemoglobin on RV afterload.

Methods: Main pulmonary artery pressures and flows were measured in C57BL6 mice before and after exchanges of whole blood (~200 uL, Hct=45%) with an equal volume of SCD RBCs in plasma (Hct=45%) or cell free hemoglobin (Hb^+) in solution. After transfusions, animals were additionally stressed with acute hypoxia (AH; 10% O_2).

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Author Contributions

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1. Designed research
2. Performed research
3. Analyzed data
4. Wrote the paper

This study is in compliance with all relevant ethical standards.

No conflicts of interest, financial or otherwise, are declared by the author(s). The University of Wisconsin-Madison Institutional Animal Care and Use Committee approved all procedures.

Results: SCD RBCs increased PVR only compared to control RBCs; cell free hemoglobin increased PVR and wave reflections. These increases in RV afterload increased further with AH.

Conclusions: The release of cell free hemoglobin from fragile SCD RBCs *in vivo* increases the total RV afterload and may impair RV function more than the SCD RBCs themselves.

Keywords

cardiopulmonary hemodynamics; sickle cell anemia; pulmonary vascular impedance; cell free hemoglobin; pulmonary hypertension; pulsatile hemodynamic; wave reflections

Introduction

With ~275,000 births per year world-wide, sickle cell disease (SCD) is one of the most common heritable monogenetic diseases in the world [1]. SCD is characterized by red blood cells (RBC) that become sickled from the association of hemoglobin inside the RBC. Once the RBCs become irreversibly sickled, they are 10 times more likely to occlude blood flow [2, 3]. In addition, SCD RBCs frequently lyse, releasing cell-free hemoglobin, which leads to vasoconstriction. In the lung, vasoconstriction leads to an increase in pulmonary vascular resistance (PVR) and pressure, which has been observed in SCD patients [4]. In addition, cell free hemoglobin and its byproducts, heme and iron, contribute to oxidative stress and inflammation [5]. The World Health Organization has found that of the chronic cardiopulmonary effects of SCD, pulmonary hypertension (PH) is a major cause of morbidity and mortality. Approximately 10% of patients with SCD develop PH during their lifetime [6–8] and as many as 75% demonstrate evidence of PH in lung tissue at the time of death [9]. Patients with PH and SCD have a significantly increased risk of vaso-occlusive crisis [2], sudden death [11], and shortened lifespan, as high as 50% over 2 years [12].

Patients with SCD associated PH have only moderate elevation of pulmonary arterial pressures compared to patients with PH alone (30-35 mmHg mean pulmonary artery pressure (mPAP) in SCD, compared to 60-90 mmHg mPAP in patients with PH) [13]. Additionally since SCD is associated with chronic anemia, cardiac output is elevated, primarily driven by a left ventricular response [14]. Moderate pressure elevation despite increased cardiac output suggests relatively low PVR in SCD patients with PH, which has been confirmed clinically [13, 15–17] [18], so it is perhaps not surprising that patients with SCD associated PH do not respond to standard treatments for PH [19]. Nevertheless, PH in SCD dramatically increases mortality via mechanisms that are not well understood [20].

The SCD research community has debated whether the release of hemoglobin is the primary driver of PH in SCD [21]. Hemolysis, correlated to an increase in RBC intracellular density [22], releases cell free hemoglobin into the plasma, which binds nitric oxide in solution. The scavenging of nitric oxide, a potent vasodilator, has been postulated as one of the primary causes of PH [2, 21, 23]. The release of cell free hemoglobin increases PVR [2]; cell free hemoglobin may also decrease pulmonary vascular compliance since it decreases aortic compliance [24]. Moreover, loss of pulmonary vascular compliance has been suggested to decrease survival in SCD [25]. In patients with PH alone, decreases in compliance predict morbidity and mortality [26].

Pulmonary vascular compliance is a component of the total right ventricular (RV) afterload, or opposition to blood flow faced by the RV. PVR, which is a more commonly used metric of RV afterload, reflects only opposition to steady flow; compliance and also wave reflections reflect opposition to pulsatile flow [27]. We postulate that increases in total RV afterload, specifically decreases in compliance and increases in wave reflections, contribute to PH in SCD. In patients with PH alone, decreased main pulmonary arterial compliance is predictor of early mortality [26]. If true in SCD, measuring total RV afterload or main pulmonary artery compliance could predict increased mortality.

In this study we sought to investigate the impact of SCD RBCs and cell free hemoglobin on pulmonary pulsatile hemodynamics *in vivo* in otherwise healthy mice. Mice offer a consistent and reproducible model to measure resistance and compliance of the pulmonary vasculature, and our lab has extensive experience with these techniques [28–31]. We hypothesized that SCD RBCs administered acutely would increase PVR and wave reflections and decrease compliance, and that cell free hemoglobin would have similar effects. We also tested the hypothesis that RBCs with increased density (with chemical treatment) would increase RV afterload by releasing Hb^+ upon lysis or other mechanisms.

Materials and Methods

Mice

Male C57BL6/J and Berkeley SCD mice, 14 weeks-old, were obtained from Jackson Laboratory (Bar Harbor, ME) and used either as donors for RBCs (C57BL6 and Berkeley) or for *in vivo* experiments (C57BL6). Mice used for *in vivo* experiments were divided into five separate groups: (*i*) injected with control RBCs (CTL RBCs) (n=8), (*ii*) injected with RBCs chemically-treated to have increased density (treated RBCs) (n=8), (*iii*) injected with SCD RBCs (n=8), (*iv*) injected with cell free hemoglobin in solution (Hb+) (n=9) or (*v*) injected with saline solution (n=9). Details of these injections are provided below. The University of Wisconsin-Madison Institutional Animal Care and Use Committee approved all procedures.

Red blood cell preparation

Whole blood was drawn from donor mice (C57BL6 and SCD) and centrifuged at 500g for 15 minutes. Then, the plasma, buffy coat and top layer of cells were removed. RBCs were resuspended to a Hct of ~45%, consistent with normal Hct levels in mice, with osmolality and PH balanced saline and centrifuged three more times to remove all non-RBC components. To create SCD-like increased RBC intracellular density, RBCs were exposed to nystatin (15 μg/ml, Sigma—Aldrich, Germany) for 48 hours per prior studies [32]. These chemically-treated RBCs were centrifuged at 500g for 15 minutes 8 times with isotonic saline and then were resuspended to 45% Hct with isotonic buffered saline. Cell free hemoglobin was purchased (Sigma—Aldrich, Germany) and suspended in solution at two concentrations (100 μM and 200 μM in isotonic buffered saline). The concentrations chosen were based on values observed clinically when SCD patients are exercising or in vaso-occlusive crisis, respectively [33, 34].

Injections and *in vivo* hemodynamic measurements

Mice were instrumented for main pulmonary artery pressure and flow measurements as previously described [30, 29]. In brief, under deep anesthesia with urethane to preserve heart rate (2mg/g body weight), mice were intubated for ventilation and placed on a heated pad to maintain body temperature The chest cavity was then opened to expose the right ventricle and a 1.0F pressure-tip catheter (Millar Instruments, Houston, TX) was inserted through a puncture in the right ventricular free wall and then advanced into the main pulmonary artery (PA). PA pressure was recorded at 5 kHz on a hemodynamic workstation (Cardiovascular Engineering, Norwood, MA) while flow was simultaneously measured via ultrasound (Visualsonics, Toronto, Ontario, Canada) with a 30 MHz probe and recorded with the same hemodynamic workstation (Figure 1). The probe was angled until the maximal velocity signal was obtained. Measurement at this point allows for better detection of the main pulmonary artery inner diameter (MPA ID), which was used to convert the flow velocity signal to volume flow rate (Q) assuming a circular cross-section. The signals were visually checked for quality and recorded for later analysis.

After all measurements were completed at baseline, ~200 μL of blood was extracted from the superior vena cava through the jugular vein (Figure 1) and replaced with an equal volume of blood containing one of the following: (*i*) CTL RBCs, (*ii*) treated RBCs, (*iii*) SCD RBCs, (*iv* Hb^+ in solution, or (*v*) saline solution. After a five-minute stabilization period, all hemodynamic measurements were repeated, and this cycle was repeated once more, for 2 ~200 μL blood replacements followed by exposure to acute hypoxic (AH) ventilation (10% O_2) lasting five minutes, resulting in 4 total sets of pressure and flow measurements. In addition to simulating acute stress, AH was used to replicate the hypoxic consequences of SCD.

Hematocrit was measured after each 200-μL blood extraction. Before euthanasia, a 500-μL sample of blood was taken for either viscosity analysis or blood gas measurement. Blood gas levels were measured with a portable analyzer and CG4+ cartridge immediately after extraction (I-STAT, Abbott Laboratories, Princeton, NJ). Viscosity analysis was performed with samples drawn into a closed tube for oscillatory tube viscometry (Vilastic-3 Viscoelasticity Analyzer under normoxic conditions as described previously [29]. In brief, with a small sample volume (~1 ml), a wide range of strain rates (0.1 to 10000/second) can be applied to the fluid in a temperature-controlled environment (1 to 95 °C). The resulting shear stress-strain rate relationships are automatically curve-fit to pre-established non-Newtonian rheological models to enable quantification of fluid properties.

In vivo hemodynamic calculations

Pressure (P) and flow (Q) measurements were analyzed as previously described [30, 29] using a custom software and hemodynamic workstation (Cardiovascular Engineering, Norwood, MA). Total PVR was calculated as mean PA pressure divided by Q averaged over the cardiac cycle (i.e., CO). Total pulmonary arterial compliance was calculated from an exponential fit to the PA pressure decay during diastole [36]. Characteristic impedance (Z_C) was calculated from the ratio of the change in pressure to the change in flow in early ejection. That is, $Zc = \frac{dP}{dQ}$, where dP and dQ are taken during the linear portion when

plotting P and Q. An assumption inherent in this calculation is that the system is free from reflections because the reflected waves do not have time to return to the proximal bed so early in the cardiac cycle [36]. We calculated pulse wave velocity (PWV) as $PWV = \frac{Zc * A}{\rho}$ assuming the density of blood ρ=1060 kg/m^3 and cross-sectional area $A = \frac{\pi}{4}(MPAID)^2$.

Finally, the pulmonary arterial pressure waveform was separated into forward (P_f) and backward (P_b) traveling components using the linear wave separation method [37]. The index of global wave reflections (RQ) was calculated as the ratio of the amplitude of P_b to P_f.

Statistical analysis

The significances of the overall changes in parameters with blood replacement were assessed using a one-way analysis of variance (ANOVA) for condition (CTL RBCs, treated RBCs, SCD RBCs, Hb$^+$ in solution, or saline solution) or generalized least squares for repeated measurements (with subsequent blood replacements). When the ANOVA reached statistical significance, Tukey multiple comparisons were used for post hoc analysis. Data were considered significant for P-values less than 0.05. All data are presented in terms of means ± standard error. Statistical analysis was performed using R software (Foundation for Statistical Computing, USA, version 2.14.0).

Results

Body weight and hematocrit

The average body weight for mice in each experimental group was not different (Supplemental data Table 1 and 2). Acute injection of CTL RBCs, treated RBCs, SCD RBCs, Hb$^+$ and saline did not alter hematocrit (Supplemental data Table 1 and 2).

Effects of blood replacements on pulmonary pressure, systemic pressure, and heart rate

Mean pulmonary artery pressure (mPAP) did not changes under any conditions with normoxic ventilation (Supplemental data Table 1 and 2) but decreased significantly in the SCD RBC group after exposure to AH. Systolic pulmonary arterial pressure (sPAP) and diastolic pulmonary arterial pressure (dPAP) changed in the same way as mPAP, only decreasing in the SCD AH data (data not shown). Systemic pressure did not change in any of the 5 groups with normoxic ventilation or AH. Heart rate decreased significantly after AH exposure in both the Hb+ group and the saline solution group (Supplemental data Table 1 and 2).

Effects of blood replacements on resistance and cardiac output

While mPAP did not change, cardiac output (CO) decreased after the second blood replacements in the SCD RBC group; with AH, CO decreased in the Hb$^+$ group and decreased further in the SCD group (Figure 2). CO did not decrease with blood replacement using control RBCs, treated RBCs, or saline solution (Figure 2; Supplemental data Table 1 and 2). Calculated as the ratio of mPAP to CO, total pulmonary vascular resistance (PVR) increased after the second blood replacements in the SCD RBC and Hb$^+$ groups and with

AH remained elevated in these two groups but did not significantly increase further (Figure 2; Supplemental data Table 1 and 2).

Effects of blood replacements on wave reflections, compliance and blood measurements

Wave reflections (R_Q) significantly increased in the Hb^+ group but did not change for any other group (Figure 4; Supplemental data Table 1 and 2). Characteristic impedance (Z_C) did not change significantly in any of the 5 groups. Pulse wave velocity (PWV), main pulmonary artery diameter, pulmonary pulse pressure and pulmonary arterial compliance did not change after any blood replacement using either RBCs or solutions (Supplemental data Table 1 and 2).

Blood viscosity was not changed in solutions containing CTL RBCs, treated RBCs or SCD RBCs; the solutions for treated RBCs and SCD RBCs contained 10% of each type with CTL RBCs (400 μL of injected blood at 45% hematocrit compared to total blood volume of mouse of 1.8 ml) comprising the rest (data not shown). Blood gas levels did not change in mice injected with CTL RBCs, treated RBCs, or SCD RBCs (data not shown).

Discussion

To investigate the blood cell factors that contribute to PH in SCD, we quantified the impact of SCD RBCs and cell free hemoglobin on pulmonary pulsatile hemodynamics *in vivo* in otherwise healthy mice. Our results demonstrate that both SCD RBCs and cell free hemoglobin increase opposition to blood flow by increasing PVR (Figure 3). Interestingly, instead of increasing pressures, the increased PVR led to a decrease in cardiac output in these animals. Acute administration of cell free hemoglobin also increased wave reflections measured in the main pulmonary artery. While the increase in resistance from cell free hemoglobin administration is expected from distal vasoconstriction, the increase in wave reflections and thus RV afterload is a novel finding.

An important component of the RV afterload is the resistance to (steady) blood flow. Infusion of either SCD RBCs or cell free hemoglobin in solution increased the resistance to flow in healthy mouse lungs. Instead of leading to PH, however, the increase in PVR led to a decrease in CO. Our group has previously observed this phenomenon in mice exposed chronic hypoxia and/or blood exchanges [28, 29]. If it also occurs in humans, this finding is potentially clinically significant because it suggests that increases in mPAP with SCD progression may dramatically underestimate increases in PVR. Indeed, many patients with PH-SCD present with only moderate increases in pressure [8] but have higher mortality than SCD patients [18]. Greater RV overload in PH-SCD may be a critical factor.

RV afterload depends on resistance, compliance and the magnitude of wave reflection in the pulmonary circulation [27]. While SCD RBCs had no effect on the pulsatile components of the RV afterload in healthy mice, infusion of cell free hemoglobin increased wave reflections to the RV. In canine studies using acute hypoxia to induce vasoconstriction, there was an increase in the backward reflected wave, resulting in increased RV afterload [38, 39]. Another study *in utero* in fetal lambs demonstrated that wave reflections were increased in the highly constricted pulmonary vasculature, and the combination of increased resistance

and wave reflections contributed to the low blood flow profiles to fetal lungs [40]. Therefore, the increase in wave reflections with Hb^+ infusion may have contributed to the drop in CO in these conditions, but it was not as dramatic as the drop in CO with SCD RBC infusion. Moreover, since neither arterial compliance nor characteristic impedance were altered by Hb^+ infusion, it is unclear what the impact would be on morbidity or mortality. Increased wave reflections have been linked to a decrease in proximal vascular compliance [41] but the decreases in compliance in patients with PH alone predict morbidity and mortality [26].

Increased RBC intracellular density due to frequent sickling and unsickling cycles [31] is thought to contribute to hemolysis and subsequent increases in cell free hemoglobin concentration [42, 43]. While the impact of intracellular RBC density on flow has been tested *in vitro* [44–46], no study to date has investigated the impact of RBC density *in vivo*. Our results suggest that increased RBC density alone is insufficient to either increase hemolysis or increase RV afterload.

The impact whole SCD blood on flow also has been tested *in vitro* [44–48], but we are the first to measure the impact of SCD RBCs on flow in healthy mouse lungs. To do so, we used multiple controls including CTL RBCs and saline solution. We designed this study to maintain hematocrit under all conditions for all groups and there were no observed changes in hematocrit. Whole blood viscosity also did not change in samples representative of the mixtures infused into animals when measured under normoxic conditions. While whole blood viscosity is modified in SCD, and more so under hypoxic conditions, our blood samples contained a mixture of healthy and SCD RBCs, with a higher proportion of healthy RBCs. Despite the relatively low proportion of SCD RBCs, RV afterload was dramatically increased after infusion and increased further with AH.

In SCD, the blood cell abnormalities investigated independently here combine to increase the steady and pulsatile components of RV afterload. Our results suggest that release of cell free hemoglobin due to SCD RBC lysis is a major contributor to both components of the RV afterload, likely by causing vasoconstriction throughout the vasculature. In contrast, the sickling defect in SCD RBCs contributes only to the steady component of the RV afterload, PVR, likely by becoming lodged in the microcirculation. Also, in SCD, chronic abnormalities in the lung vasculature may contribute to the development and progression of PH either on their own or by augmenting the effects of Hb+ and SCD RBCs found here in healthy lungs.

There are a number of limitations to consider in these experimental results. First, we did not observe a typical hypoxic pulmonary vasoconstriction (HPV) after five minutes of 10% hypoxic ventilation. We have observed HPV in previous studies of mouse cardiovascular physiology [29], but we suspect that the stress of multiple injections before acute hypoxia may have complicated the typical vasoconstriction response. Alternatively, a longer duration of hypoxia exposure may be required under conditions of multiple injections. Second, we measured neither right ventricular stroke volume nor contractility in our experiments, which could contribute to the reported changes PVR and CO and could suggest cardiotoxic events. Differential effects of SCD RBCs and Hb+ on the heart and pulmonary vasculature could have confounded our interpretation of the data. Third, since SCD patients are exposed to

additive effects of cell free hemoglobin and sickled red blood cells, an impactful study in the future would elucidate the additive effects of each.

In conclusion, while SCD RBCs increased PVR only, cell free hemoglobin increased PVR and wave reflections. Therefore, the release of cell free hemoglobin from lysis of fragile SCD RBCs *in vivo* may impair RV function more than the SCD RBCs themselves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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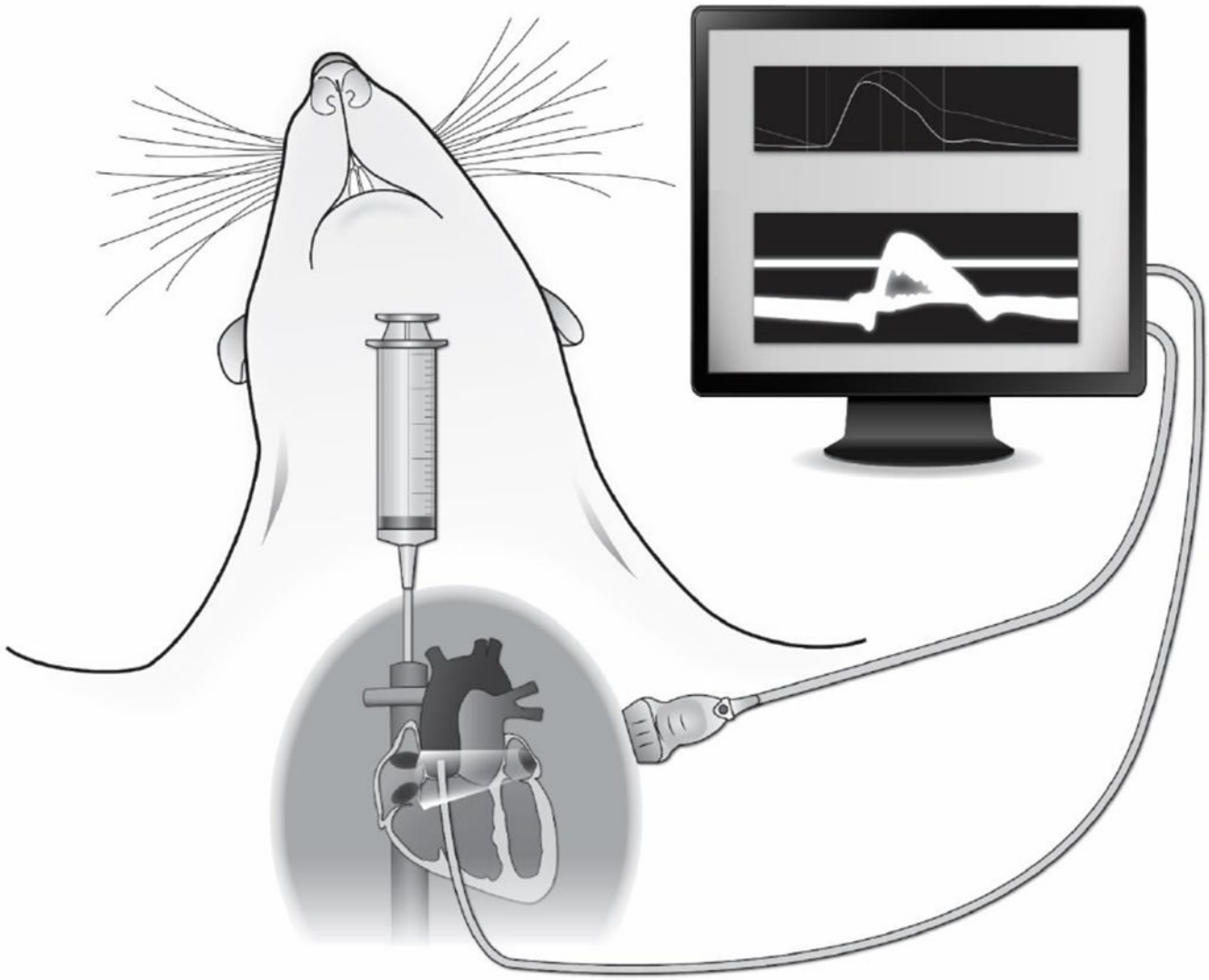

Figure 1.
Schematic of subject instrumentation with an invasive pressure catheter in the right ventricle and non-invasive ultrasound probe used to measure pulmonary artery diameter and blood flow with signals sent to a single data capture system (on the left), and invasive catheter in the superior vena cava for blood exchanges and injections (on the right).

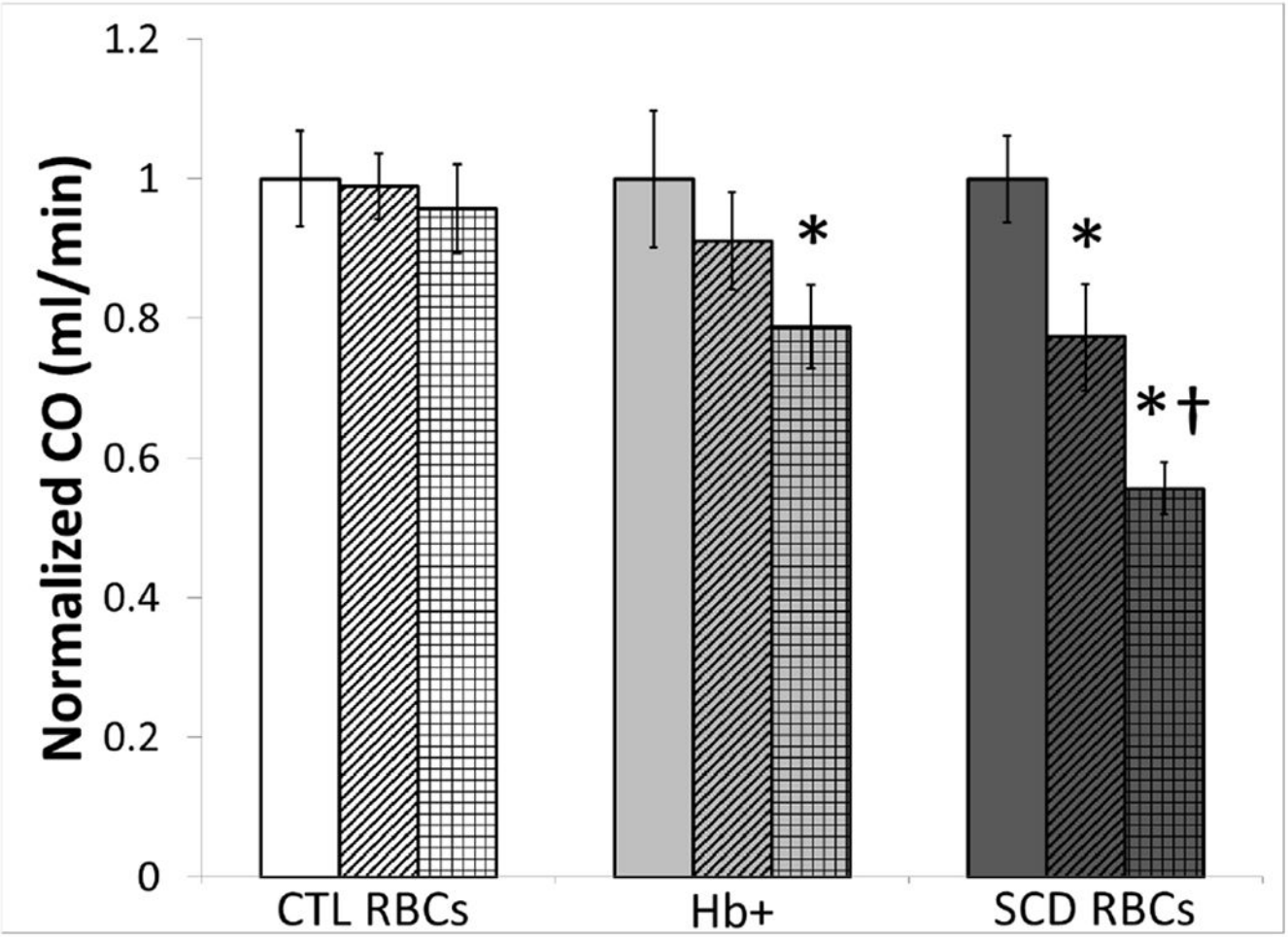

Figure 2.

Cardiac output normalized to baseline (same group) values for control (CTL RBCs), infusion of cell free hemoglobin (Hb^{+}), and SCD RBCs. No pattern used for baseline measurements, diagonal pattern for measurements after 2nd blood replacement, and square pattern for measurements after AH. *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. 2nd blood replacement.

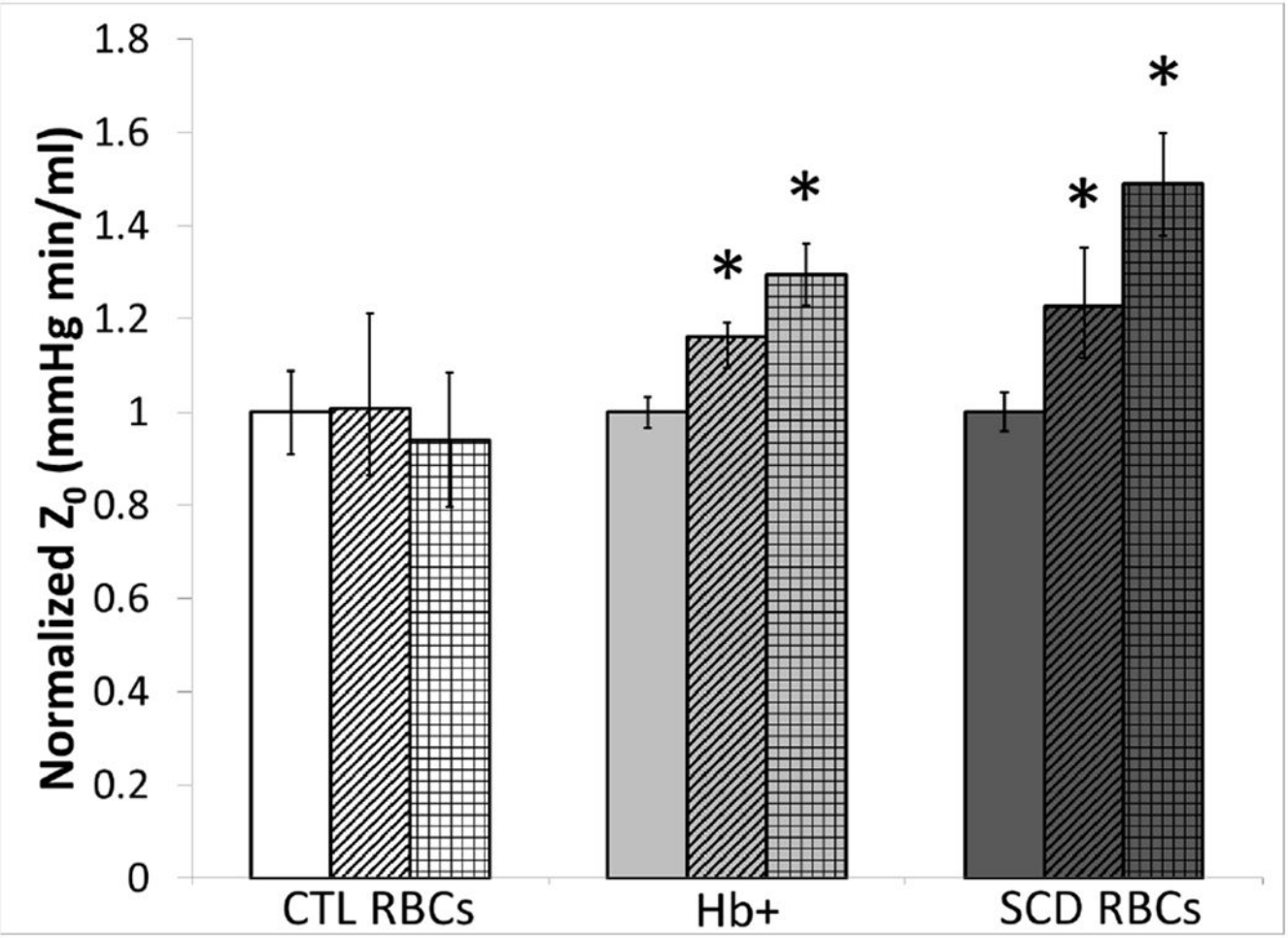

Figure 3.

Total pulmonary vascular resistance (PVR) calculated as mPAP/CO normalized to baseline (same group) for control (CTL RBCs), infusion of cell free hemoglobin (Hb^+), and SCD RBCs. No pattern used for baseline measurements, diagonal pattern for measurements after 2nd blood replacement, and square pattern for measurements after AH. $*P < 0.05$ vs. baseline; $\dagger P < 0.05$ vs. 2nd blood replacement.

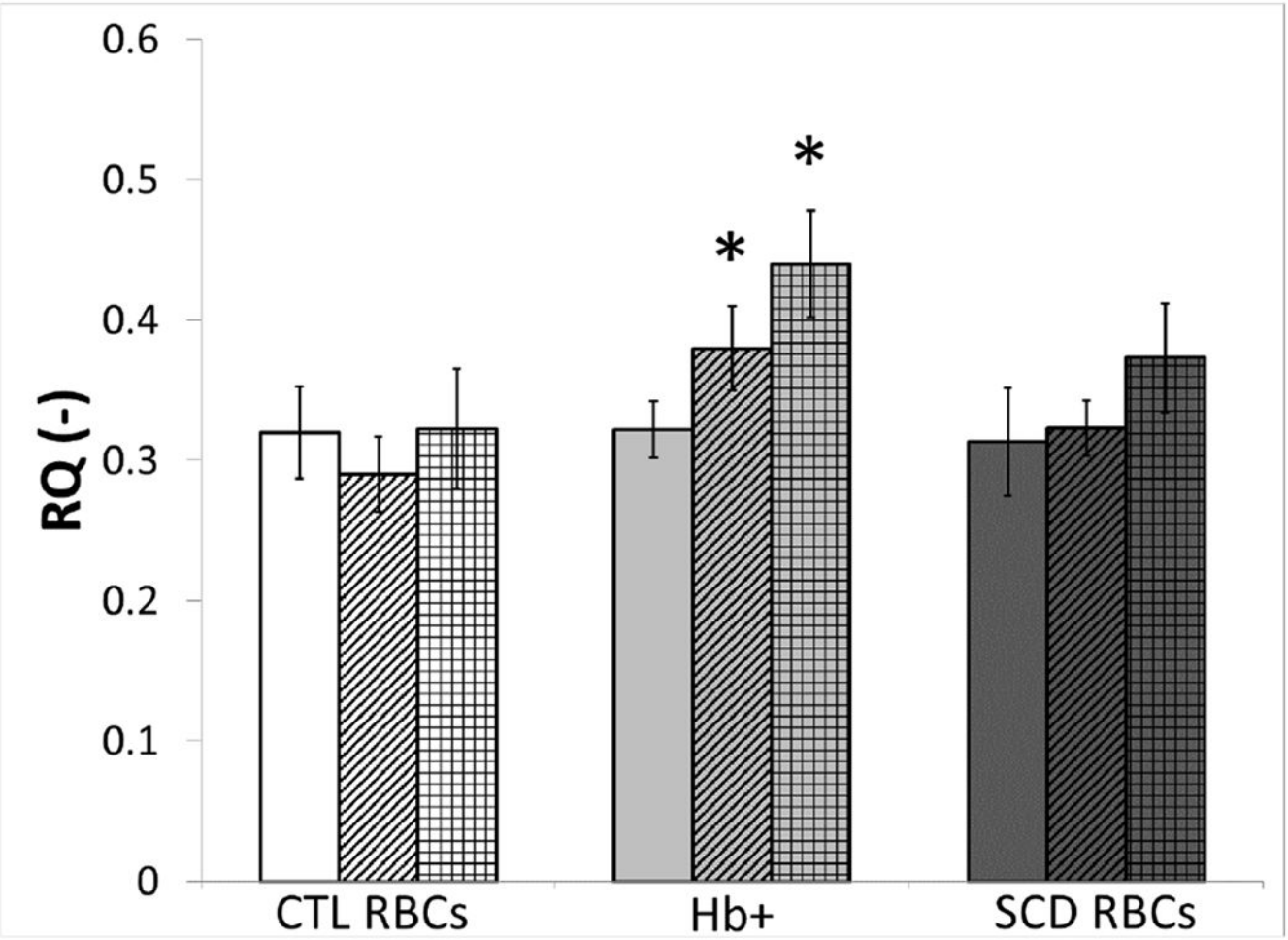

Figure 4.

Pulmonary arterial wave reflections (RQ) calculated as the ratio of the amplitude of backward traveling components (P_b) to forward traveling components (P_f) for control (CTL RBCs), infusion of cell free hemoglobin (Hb^+), as well as and SCD RBCs. No pattern used for baseline measurements, diagonal pattern for measurements after 2nd blood replacement, and square pattern for measurements after AH. *$P < 0.05$ vs. baseline; †$P < 0.05$ vs. 2nd blood replacement.