

The Novel Hemoglobin-based Oxygen Carrier HRC 101 Improves Survival in Murine Sick Cell Disease

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Background: Erythrocyte transfusion decreases morbidity in sickle cell disease, but is not without risk. Use of a hemoglobin-based oxygen carrier could offer the benefits of erythrocyte transfusion while reducing related complications. The authors tested the hypothesis that the novel hemoglobin-based oxygen carrier, HRC 101, would improve survival during exposure to acute hypoxia in a murine model of sickle cell disease, the transgenic mouse expressing hemoglobin SAD ($\alpha_2^{\text{human}}\beta_2^{\text{S,Antilles,D-Punjab}}$).

Methods: Wild-type (n = 30) and transgenic SAD (n = 36) mice received 0.02 ml/g HRC 101 (hemoglobin concentration, 10 g/dl) or an equal volume of 5% albumin. Thirty percent or 6% oxygen was administered to spontaneously breathing mice during halothane anesthesia (inspired concentration, 0.5%). The time to cessation of cardiac electrical activity was recorded. Survival was compared using Kaplan-Meier analysis.

Results: Control mice survived the 60-min study period, whether breathing 30% or 6% oxygen. In contrast, all SAD mice given albumin and 6% oxygen died, with a median survival time of 9.0 min (interquartile range, 6.9–11.6 min; $P < 0.0001$). HRC 101 significantly increased survival in SAD mice breathing 6% oxygen. Of 12 SAD mice given HRC 101 and 6% oxygen, 4 survived the entire study period and 8 died, with a median survival time of 48 min (19–60 min; $P < 0.0001$ vs. albumin).

Conclusion: HRC 101 significantly decreased sickle-related mortality during exposure to acute hypoxic stress in transgenic mice expressing hemoglobin SAD. HRC 101 warrants further evaluation as a therapeutic modality in sickle cell disease.

ERYTHROCYTE transfusion decreases morbidity in patients with sickle cell disease,^{1,2} but the benefits of erythrocyte transfusion must be balanced against risks such as increased blood viscosity, transfusion reactions, alloimmunization, immune suppression, and transmission of infection. Hemoglobin-based oxygen carriers (HBOCs) are erythrocyte substitutes that can be used to increase oxygen-carrying capacity and intravascular volume. HBOCs have potential advantages over allogeneic blood in that they can be produced in large volume, can be stored for prolonged periods, can be administered without the need for cross-matching, should not give

rise to alloimmunization, and can be made free of infectious agents. In randomized clinical trials, HBOCs have been shown to reduce the need for allogeneic blood transfusion in patients undergoing cardiac surgery and after major trauma.^{3,4} Early clinical evaluation of HBOCs identified a requirement to minimize side effects associated with extravasation of low-molecular-weight hemoglobin components,⁵⁻⁸ leading to the development of high-molecular-weight polymeric and colloid-modified hemoglobin solutions.

Hemoglobin-based oxygen carriers have been reported anecdotally to be useful in the management of sickle cell disease.⁹⁻¹¹ A salutary effect during a sickle crisis might result from the ability of HBOCs to bypass vasoocclusion caused by sickle erythrocytes, thereby enhancing oxygen delivery to the microcirculation and reducing subsequent reperfusion injury (various HBOCs have diameters ranging from 6 to 60 nm¹²; erythrocyte diameter, 7 μ m). In one report, a small cohort of patients in vasoocclusive or aplastic crisis benefited from administration of a bovine HBOC, demonstrating a rapid reduction in the severity of pain in the absence of any analgesic medications.⁹ However, these products might also be associated with deleterious effects because they have the potential to bind nitric oxide and to steal oxygen from erythrocytes containing sickle hemoglobin. The safety and usefulness of HBOCs in sickle cell disease has not been extensively studied.

Transgenic murine models of sickle cell disease have been developed to help further our understanding of the disease pathogenesis and to assess therapeutic interventions. One such model has been generated by the introduction of three mutations to the β -globin gene, β S, β Antilles, and β D-Punjab (β SAD).¹³⁻¹⁶ Coexpression of β SAD and human α genes results in the production of hemoglobin SAD in murine erythrocytes. This model reproduces both the acute spontaneous vasoocclusive events of human sickle cell disease and the chronic steady state disease.¹⁷ The aim of the current study was to evaluate the efficacy of a novel high-molecular-weight, low-oxygen-affinity HBOC, HRC 101 (Hemosol; Mississauga, Ontario, Canada), in preventing sickle-related mortality in transgenic SAD mice during exposure to acute, severe hypoxic stress. Given its unique colloidal and other physical properties, such as large molecular size, high P_{50} (partial pressure at which hemoglobin is 50% saturated), and high oxygen-carrying capacity, together with its ability to bypass vasoocclusion resulting from erythrocyte sickling and adhesion to the endothe-

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lium, HRC 101 should minimize nitric oxide binding and oxygen steal but enhance oxygen delivery to the microcirculation during vasoocclusive crisis. For these reasons, we tested the hypothesis that HRC 101 would improve the survival of SAD animals during exposure to acute, severe hypoxic stress.

Materials and Methods

Animals

The study was approved by the Animal Care Committee of the Hospital for Sick Children, Toronto, Canada. Sixty-six pathogen-free wild-type ($n = 30$) and transgenic SAD mice ($n = 36$) aged 6–13 weeks and weighing 17–31 g were studied. SAD mice were derived from a breeding pair imported from Marie Trudel, D.Sc. (Professor, Institut de Recherches Cliniques de Montreal, University of Montreal, Montreal, Quebec, Canada).^{13–17} Wild-type controls consisted of C57BL/6 littermates. The mice were housed in a 12-h light-dark cycle, with free access to feed and water.

Hemoglobin Solution

HRC 101 is a high-molecular-weight HBOC comprised of a covalent conjugate of highly purified human hemoglobin and oxidized hydroxyethyl starch. The covalent conjugate is prepared by reaction of hemoglobin amino groups with aldehydes of the oxidized hydroxyethyl starch, to form Schiff base linkages that are subsequently reduced to irreversible imine linkages. HRC 101 contains less than 10% of hemoglobin-hydroxyethyl starch components that are less than 192 kd, the remainder comprising high-molecular-weight hemoglobin-hydroxyethyl starch conjugates. It is iso-oncotic with plasma and has a hemoglobin concentration of 10 g/dl. The P_{50} , determined by direct galvanometric titration, is approximately 70 mmHg, and oxygen binding is noncooperative (Hill coefficient, $n = 1.1$). HRC 101 has an oxygen off-loading capability of 3.5 ml O_2 /dl over the oxygen tension (P_{O_2}) range of 100 to 40 mmHg. It is formulated in lactated Ringer's solution at physiologic pH. The HRC 101 half-life is 17 h in rats after a 50% isovolemic exchange transfusion. To prevent oxidation to the methemoglobin form, HRC 101 was stored at -80°C and thawed immediately before use according to the manufacturer's recommendations.

Hemoglobin Electrophoresis

All SAD mice were screened for transgenic expression of the human genes. Screening was performed by hemoglobin electrophoresis using cellulose acetate in alkaline buffer for qualitative and quantitative determination of abnormal hemoglobin, as previously described.¹⁴

Experimental Protocol

Wild-type and SAD mice were gently restrained and assigned at random to receive 0.02 ml/g body weight

HRC 101 or an equal volume of 5% albumin administered *via* a 26-gauge catheter over 2 min into a tail vein. After fluid administration, the animals were placed in a custom-built plastic chamber and observed for 20 min while breathing room air. Anesthesia was induced with halothane administered in 30% oxygen in nitrogen using a calibrated vaporizer and a continuous fresh gas flow rate of 5 l/min. Lead II of the electrocardiogram was monitored using needle electrodes and data acquisition software (AcqKnowledge version 3, MP100 Work Station for Macintosh; Harvard Apparatus, Quebec, Canada). During halothane anesthesia (inspired concentration, 0.5%) and spontaneous ventilation, either 30% or 6% oxygen was administered. The inspired concentrations of oxygen and halothane were monitored continuously using a calibrated oxygen analyzer and an infrared analyzer (Capnomac Ultima; Datex-Ohmeda, Helsinki, Finland), respectively. Rectal temperature was monitored, and normothermia was maintained using a radiant heating lamp. Heart rate, inspired concentrations of oxygen and halothane, and rectal temperature were recorded every 5 min. Time to cessation of cardiac electrical activity was recorded. At the time of death (cessation of cardiac electrical activity) or at the end of 1 h, blood was sampled *via* cardiac puncture for determination of blood gases and lactate concentration.

Statistical Analysis

For the estimation of sample size, the mean survival time of SAD mice exposed to 6% oxygen was assumed to be 8.2 ± 1.2 min.¹⁸ Further assuming a 10% difference in survival time, we estimated *a priori* that six mice would be needed per group for a two-tailed α of 0.05 and β of 0.2. To increase accuracy in the estimation of percentage survival the sample size was increased for groups receiving 6% oxygen. Data are expressed as median and interquartile range or mean \pm SD, as appropriate. Kaplan-Meier analysis, a distribution-free method, and the log-rank test were used for the analysis of survival time. Two-way repeated-measures analysis of variance and the Student-Newman-Keuls *post hoc* test were used for statistical analysis of heart rate. One-way analysis of variance and the Student-Newman-Keuls *post hoc* test were used to examine intergroup differences in pH, blood gases, and lactate concentration. $P < 0.05$ was considered statistically significant.

Results

Survival

The inspired concentrations of oxygen and halothane remained stable at the target levels for the 60-min study period. Survival data are summarized in table 1. Kaplan-Meier curves showing the percentage of animals surviving the 60-min study period are pre-

Table 1. Survival Time

	Wild Type		SAD	
	30% Oxygen	6% Oxygen	30% Oxygen	6% Oxygen
5% Albumin	60 (60–60) (n = 6)	60 (60–60) (n = 8)	60 (60–60) (n = 6)	9.0 (6.9–11.6) (n = 12)
HRC 101	60 (60–60) (n = 6)	60 (60–60) (n = 10)	60 (60–60) (n = 6)	48 (19–60) (n = 12)

Survival times in minutes are expressed as median (interquartile range).

n = number of animals in each group; SAD = transgenic mice expressing hemoglobin SAD ($\alpha_2^{\text{human}}\beta_2^{\text{S, Antiles, D-Punjab}}$).

sented in figure 1. Analysis of the curves indicated significant differences between groups. All wild-type mice given 5% albumin survived for the entire 60-min study period, whether breathing 30% or 6% oxygen. In SAD groups given 5% albumin, all mice breathing 30% oxygen survived for the entire 60-min study period, whereas all those breathing 6% oxygen died, with a median survival time of 9.0 min (6.9–11.6 min; $P < 0.0001$ vs. 30% oxygen). All wild-type mice given HRC 101 survived for the entire 60-min study period, whether breathing 30% or 6% oxygen. Similarly, all SAD mice given HRC 101 and 30% oxygen survived for the entire 60-min study period. Administration of HRC 101 significantly increased the median survival time in SAD mice breathing 6% oxygen ($P < 0.0001$ vs. 5% albumin). Of the SAD mice given HRC 101 and 6% oxygen, four survived the entire 60-min study period and eight died, with a fivefold increase in median survival time to 48 min (19–60 min).

Heart Rate, Blood Gases, and Lactate Concentration

Mean heart rate was significantly greater in groups breathing 6% oxygen compared with those breathing

30% oxygen ($P < 0.01$) (fig. 2). Compared with 5% albumin, administration of HRC 101 resulted in a significant reduction in heart rate, whether breathing 30% ($P < 0.01$) or 6% oxygen ($P < 0.05$) (fig. 2).

Determination of the pH of blood sampled by intracardiac puncture revealed that SAD mice breathing 6% oxygen were significantly more acidotic compared with all other groups ($P < 0.001$) (table 2). Among SAD groups breathing 6% oxygen, mice given HRC 101 were significantly less acidotic compared with those given 5% albumin ($P < 0.001$). With the exception of SAD mice given 5% albumin, groups breathing 6% oxygen were hypocapnic compared with those breathing 30% oxygen (table 2), indicating hyperventilation in hypoxic animals. For SAD groups breathing 6% oxygen, carbon dioxide tension in mice given HRC 101 was decreased compared with mice given 5% albumin ($P < 0.001$). Bicarbonate concentration in groups breathing 6% oxygen was significantly decreased, whereas lactate concentration was significantly increased, compared with groups breathing 30% oxygen ($P < 0.01$) (table 2). For SAD animals given HRC 101 and 6% oxygen, the mean lactate concentration for survivors (10.3 ± 0.6 mM; n = 4) was significantly less than that for nonsurvivors (19.9 ± 4.8 mM; n = 8) ($P < 0.01$). The mean rectal temperature for the 60-min study period was $36.6^\circ \pm 0.04^\circ\text{C}$, with no significant differences between groups.

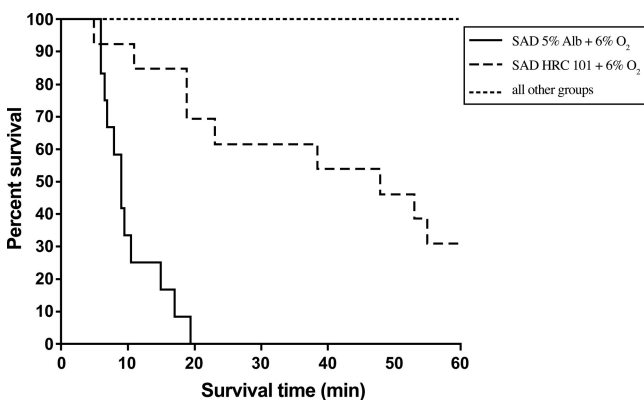


Fig. 1. Kaplan-Meier curves showing the percentage of animals surviving the 60-min study period. In control groups, all mice survived the 60-min study period, whether breathing 30% or 6% oxygen. In SAD groups given 5% albumin, all mice breathing 30% oxygen survived for the entire 60-min study period, whereas all those breathing 6% oxygen died, with a median survival time of 9.0 min (6.9–11.6 min; $P < 0.0001$ vs. 30% oxygen). Administration of 0.02 ml/g HRC 101 (hemoglobin concentration, 10 g/dl) significantly increased the median survival time in SAD mice breathing 6% oxygen ($P < 0.0001$ vs. 5% albumin). Alb = albumin; SAD = transgenic mice expressing hemoglobin SAD.

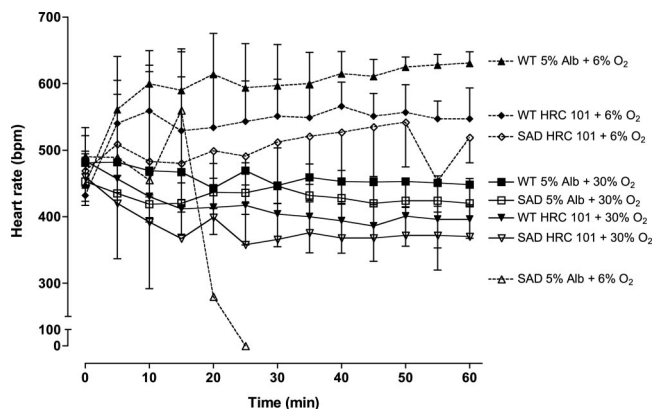


Fig. 2. Heart rate during the 60-min study period. Pooled mean heart rate in groups breathing 6% oxygen was significantly greater than that in groups breathing 30% oxygen ($P < 0.01$). Administration of 0.02 ml/g HRC 101 attenuated the hypoxia-induced increase in heart rate. Data are mean \pm SD. Alb = albumin; SAD = transgenic mice expressing hemoglobin SAD; WT = wild-type.

Table 2. Blood Gases and Lactate Concentration

	pH	Pco ₂ , mmHg	Po ₂ , mmHg	HCO ₃ ⁻¹ , mM	Lactate, mM
WT 5% Alb + 30% O ₂	7.30 ± 0.04	46 ± 4.5	67 ± 43	22 ± 2.7	1.7 ± 0.5
WT 5% Alb + 6% O ₂	7.53 ± 0.05	19 ± 4.3*	21 ± 6.5†	15 ± 2.7†	7.3 ± 2.2†
WT HRC 101 + 30% O ₂	7.31 ± 0.10	48 ± 12	80 ± 44	25 ± 6.5	2.2 ± 1.3
WT HRC 101 + 6% O ₂	7.41 ± 0.10	21 ± 5.5*	32 ± 9.5†	11 ± 3.4†	9.1 ± 1.3†
SAD 5% Alb + 30% O ₂	7.39 ± 0.01	39 ± 3.3	107 ± 32	22 ± 2.3	1.9 ± 0.9
SAD 5% Alb + 6% O ₂	6.90 ± 0.07‡	52 ± 17	19 ± 18†	10 ± 2.8†	18 ± 4.3†
SAD HRC 101 + 30% O ₂	7.32 ± 0.05	44 ± 12	72 ± 32	22 ± 5.9	2.2 ± 1.8
SAD HRC 101 + 6% O ₂	7.11 ± 0.20‡	31 ± 11§	17 ± 6.8†	9 ± 2.1†	18 ± 6.0†

Data are mean ± SD.

* $P < 0.01$ vs. corresponding groups given 30% oxygen. † $P < 0.01$ vs. corresponding groups given 30% oxygen. ‡ $P < 0.001$ vs. all other groups. § $P < 0.05$ vs. corresponding groups given 30% oxygen and $P < 0.001$ vs. SAD 5% Alb + 6% oxygen.

Alb = albumin; HCO₃⁻¹ = bicarbonate; Pco₂ = carbon dioxide tension; Po₂ = oxygen tension; SAD = transgenic mice expressing hemoglobin SAD (α_2 human β_2 S_{Antiles,D-Punjab}); WT = wild type.

Discussion

The results show that administration of HRC 101 significantly decreased sickle-related mortality during exposure to acute, severe hypoxic stress in transgenic SAD mice. Control SAD animals given 5% albumin all died during exposure to an environment containing 6% oxygen, whereas 33% of those given HRC 101 survived in the same environment for the entire 60-min study period, with an overall fivefold increase in median survival time (9 min vs. 48 min, respectively; $P < 0.0001$). These findings, which are consistent with the hypothesis that HRC 101 augments oxygen delivery to the microcirculation, likely have a multifactorial origin including an increase in the oxygen-carrying capacity of blood, a decrease in erythrocyte sickling through a reduction in erythrocyte deoxy-HbSAD concentration, and/or the ability of HRC 101 to bypass hypoxia-induced vasoocclusion resulting from erythrocyte sickling and adhesion to the endothelium.

The transgenic SAD mouse reproduces not only the acute spontaneous vasoocclusive events of human sickle cell disease but also the pathophysiologic manifestations of the chronic steady state disease.¹³⁻¹⁵ In this model, the most common chronic organ lesions are congestive splenomegaly and renal glomerulopathy, which affect approximately 80% of animals by 10 months of age. In the kidneys, focal or global glomerulosclerosis, fibrosis, and papillary necrosis are associated with elevation in blood urea nitrogen, proteinuria, and chronic renal failure. The lungs demonstrate congestion of capillaries, thrombosis, infarction, and hemorrhage, and the liver demonstrates hyperplasia of Kupffer cells and portal macrophages. The mean life span of transgenic SAD mice is reduced by 40% when compared with that of nontransgenic littermates. Because of the difficulties in evaluating erythrocyte sickling in acute studies, survival has been used to evaluate tolerance of SAD mice to varying levels of hypoxic stress.^{13,18} In one study, all mice survived prolonged exposure (> 17 h) to an atmosphere containing an oxygen tension of 70 mmHg and

3-h exposure to 49 mmHg.¹³ When atmospheric oxygen tension was further decreased to 46 mmHg, SAD mice died within 20 min, whereas control mice tolerated these low atmospheric oxygen tensions without obvious adverse effects, which is consistent with our results. Autopsy of animals exposed to hypoxic environment revealed extensive sickling and microvascular and visceral congestion. Pretreatment with the antisickling compound BW12C79, which increases hemoglobin oxygen affinity, improved the survival of SAD mice exposed to hypoxia.¹³ In another study, mean survival time was 8.2 ± 1.2 min in control SAD mice exposed to 6% oxygen, which is consistent with our findings, and threefold greater during inhalation of nitric oxide,¹⁸ further demonstrating the suitability of this murine model for the evaluation of treatment modalities for sickle cell disease.

To facilitate oxygen off-loading, the oxygen affinity of an HBOC should be less than that of native hemoglobin (higher P₅₀).¹⁹ Gould *et al.*²⁰ measured regional arterial and venous oxygen content after transfusion of a polymerized human hemoglobin solution in nonsickle patients with acute blood loss due to trauma or surgery. Calculation of oxygen extraction ratios using the Fick relation revealed a preferential use of oxygen from the HBOC (P₅₀ reported as 28-30 mmHg) compared with erythrocyte hemoglobin (P₅₀, 26.5 mmHg). For patients with sickle cell disease, preferential unloading of oxygen from an HBOC could attenuate the deoxygenation of sickle hemoglobin, thereby reducing deoxy-HbSAD erythrocyte concentration and the propensity of sickle hemoglobin-containing erythrocytes to sickle. The oxy-hemoglobin curve is right-shifted in sickle cell disease, and there is considerable variability, with P₅₀s as high as 50 mmHg.²¹ Therefore, a concern with the use of HBOCs in sickle cell disease is the potential for HBOCs to steal oxygen from erythrocytes containing sickle hemoglobin causing these cells to sickle. However, HRC 101 is calculated to have a lower affinity for oxygen (P₅₀, approximately 70 mmHg) compared with the right-

shifted sickle hemoglobin at oxygen tensions as low as 40 mmHg (fig. 3), which should confer protection against oxygen steal from sickle hemoglobin.

The oxygen equilibrium curves for HRC 101, normal mouse hemoglobin (P_{50} , 39 mmHg), and SAD mouse hemoglobin (P_{50} , 44 mmHg) are compared in figure 3. In contrast to HRC 101, which exhibits noncooperative oxygen binding (Hill coefficient, approximately 1.1), the mouse hemoglobins exhibit cooperative oxygen binding, and a Hill coefficient of 2.8 was used to construct their curves. The oxygen equilibrium curve for HRC 101 indicates that HRC 101 is partially saturated (60–70%) by oxygen at arterial P_{O_2} and remains 20% saturated at tissue P_{O_2} (20 mmHg). Therefore, HRC 101 has a higher fractional saturation in the low P_{O_2} range compared with SAD hemoglobin (10% saturated at P_{O_2} of 20 mmHg) and would be expected to carry more oxygen per mole of hemoglobin to hypoxic tissue.

The concentration of oxygen carried by the blood-HRC 101 mixture in this 0.02-ml/kg top-load model can be calculated from the above oxygen-binding parameters using the Hill equation, the estimated postinfusion erythrocyte hemoglobin and HRC 101 concentrations, and the expected dissolved oxygen content of the acellular phase (appendix). HRC 101 was calculated to increase blood oxygen content by approximately 20% over the P_{O_2} range of 150 to 30 mmHg and by 1.25 to greater than 10-fold below this range, indicating that the contribution by HRC 101 to the total blood oxygen content increases as the overall oxygen content decreases at low P_{O_2} . At the low fraction of inspired oxygen (approximately 20 mmHg) used in this study, and assuming no significant change in blood volume, SAD blood would carry approximately 2.5 ml O_2 /dl after HRC 101 infusion, compared with approximately 2.0 ml O_2 /dl in the absence of HRC 101. Although an increase in oxygen content of this magnitude may not entirely account for the improved survival in the HRC 101-treated group, oxygen delivery to hypoxic tissue will be dictated by various factors directly and indirectly related to the HBOC, including facilitated diffusion of oxygen in the plasma phase and

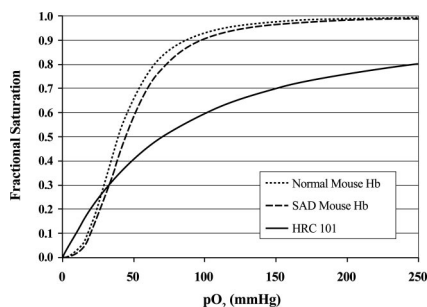


Fig. 3. Oxygen equilibrium curves for HRC 101 (derived from a P_{50} and Hill coefficient of 70 mmHg and 1.1, respectively) (solid line), SAD hemoglobin (Hb) (P_{50} , 44 mmHg; Hill coefficient, 2.8) (dashed line) and normal mouse hemoglobin (P_{50} , 39 mmHg; Hill coefficient, 2.8) (dotted line). P_{O_2} = oxygen tension.

blood flow rate, which are influenced by the viscosity and vasopressor activity of the acellular hemoglobin and autoregulatory control of flow in response to P_{O_2} .^{22,23} Because HRC 101 is comprised of acellular modified hemoglobin, the improved survival may be a result of increased oxygen delivery by the plasma phase, which could bypass vessel occlusions caused by sickled SAD erythrocytes to deliver oxygen to partially ischemic tissue. In comparison with erythrocytes, the oxygen content of plasma is normally low because of the low solubility of oxygen. The presence of HRC 101 in the plasma phase at the dose used in this model is predicted to increase the oxygen content of plasma by approximately 15-fold in the low P_{O_2} range (< 40 mmHg). At a P_{O_2} of 20 mmHg, the oxygen content of plasma would increase from less than 0.1 ml/dl to approximately 1.0 ml/dl in the presence of HRC 101. At a P_{O_2} less than 20 mmHg, the oxygen content of the plasma phase of animals receiving HRC 101 is calculated to be similar to or greater than that of SAD blood, suggesting that HRC 101 in the plasma phase may match or exceed the oxygen delivery capability normally provided by SAD erythrocytes.

Another concern with the use of HBOCs in sickle cell disease is that free hemoglobin tetramer binds nitric oxide,^{24,25} an endogenous vasodilator that may serve to stabilize the HbS structure, possibly in the R state, and thereby reduce polymerization.²⁶ A potential vasoconstrictive effect has been linked to extravasation of low-molecular-weight hemoglobin and subsequent sequestration of endothelial-derived nitric oxide away from smooth muscle cells in the extravascular space.⁵ This effect is decreased through chemical modifications that increase molecular size, such as polymerization or attachment of hemoglobin to macromolecules.¹⁹ In the case of HRC 101, purified human hemoglobin is conjugated to hydroxyethyl starch to yield a high-molecular-weight HBOC that contains less than 10% of hemoglobin components that are less than 192 kd, compared with 64 kd for tetrameric and 32 kd for non-cross-linked, dissociable hemoglobin. Competition for nitric oxide by HBOC and HbS is dependent on their relative affinities for this ligand and the dose of HBOC administered. Theoretically, decreased availability of nitric oxide through HBOC binding could increase the risk of vasoconstriction, sickle cell polymerization, and vasoocclusion. In healthy subjects, nitric oxide scavenging by stroma-free hemoglobin is associated with dose-dependent clinical sequelae, including hemoglobinuria, gastrointestinal dystonias and pain, and systolic and diastolic hypertension.^{27–29} These side effects correlate with the content of low-molecular-weight hemoglobin components in HBOC products, and would be expected to be reduced for HRC 101 owing to its reduced content of low-molecular-weight hemoglobin. In sickle cell disease, a compensatory up-regulation of non-nitric oxide vaso-

dilators,³⁰ such as prostacyclin and endothelium-derived hyperpolarizing factor, could attenuate clinical sequelae resulting from the binding of nitric oxide by HBOCs. It would seem from the current findings that high-molecular-weight tetramer-free HBOCs such as HRC 101 confer a salutary effect in sickle cell disease.

Relative bradycardia and hypertension after HBOC administration have been reported in other species with and without sickle cell disease and are generally attenuated by anesthesia.³¹⁻³⁴ In the absence of arterial pressure measurements in this study, attenuation of the increase in heart rate in the HRC 101-treated groups is presumed to be partly a baroreflex response to an elevation in arterial blood pressure. However, the relative contributions of an increase in mean arterial pressure, enhanced oxygen delivery, and other HBOC-related effects on reducing heart rate require further investigation.

A limitation of the current study is that for ethical reasons all animals were anesthetized during exposure to the hypoxic atmosphere. Anesthesia could have conferred organ protection,³⁵ and therefore, the results may not apply directly to the conscious state. Although the possibility of anesthetic organ protection cannot be ruled out, animals were lightly anesthetized and this state was imposed equally on all groups. A second limitation is the administration of only a single dose of HRC 101, 0.02 ml/g (corresponding to approximately 25% of blood volume). A similar volume of bovine hemoglobin solution was found to be clinically beneficial in a small cohort of sickle patients at the time of vasoocclusive or aplastic crisis.⁹ The minimum dose of HRC 101 needed for a salutary effect in sickle cell disease remains to be established.

In conclusion, the results show that HRC 101 protects sickle mice from the lethal effects of acute, severe hypoxia. Further studies are needed to define the mechanisms responsible for this effect. HRC 101's colloidal and other physical properties, including large molecular size, high P₅₀, high oxygen-carrying capacity, and near-blood viscosity could have contributed to the improved outcome of SAD animals. These data suggest that HRC 101 warrants further evaluation as a therapeutic modality in sickle cell disease.

References

1. Styles LA, Vichinsky E: Effects of a long-term transfusion regimen on sickle-cell related illness. *J Pediatr* 1994; 125:909-11
2. Miller ST, Wright E, Abboud M, Berman B, Files B, Scher CD, Styles L, Adams RJ: Impact of chronic transfusion on incidence of pain and acute chest syndrome during the Stroke Prevention Trial (STOP) in sickle cell anemia. *J Pediatr* 2001; 139:785-9
3. Gould SA, Moore EE, Hoyt DB, Burch JM, Haenel JB, Garcia J, DeWoskin R, Moss GS: The first randomized trial of human polymerized hemoglobin as a blood substitute in acute trauma and emergent surgery. *J Am Coll Surg* 1998; 187: 113-20
4. Levy JH, Goodnough LT, Greilich PE, Parr GVS, Stewart RW, Gratz I, Wahr J, Williams J, Comunale ME, Doblzar D, Silvey G, Cohen M, Jahr JS, Vlahakes GJ: Polymerized bovine hemoglobin solution as a replacement for allogeneic red

- blood cell transfusion after cardiac surgery: Results of a randomized double-blind trial. *J Thorac Cardiovasc Surg* 2002; 124:35-40
5. 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
6. Olson JS, Foley EW, Rogge C, Tsai AL, Doyle MP, Lemon DD: No scavenging and the hypertensive effect of hemoglobin-based blood substitutes. *Free Radic Biol Med* 2004; 36:685-97
7. Winslow RM: Current status of blood substitute research: Towards a new paradigm. *J Intern Med* 2003; 253:508-17
8. Yu B, Liu Z, Chang TM: Polyhemoglobin with different percentage of tetrameric hemoglobin and effects on vasoactivity and electrocardiogram. *Artif Cells Blood Substit Immobil Biotechnol* 2006; 34:159-73
9. Feola M, Simoni J, Angelillo R, Lühruma Z, Kabakele M, Manzombi M, Kaluila M: Clinical trail of a hemoglobin based blood substitute in patients with sickle cell anemia. *Surg Gynecol Obstet* 1992; 174:379-86
10. Raff JP, Dobson CE, Tsai HM: Transfusion of a polymerized human hemoglobin in a patient with severe sickle cell anaemia. *Lancet* 2002; 26:464-6
11. Lanzkron S, Moliterno AR, Norris EJ, Gould SA, Segal J, Nuermberger EL, Ness PM: Polymerized human Hb use in acute chest syndrome: A case report. *Transfusion* 2002; 42:1422-7
12. Vandegriff KD, McCarthy M, Rohlfis RJ, Winslow RM: Colloid osmotic properties of modified hemoglobins: Chemically cross-linked *versus* polyethylene glycol surface-conjugated. *Biophys Chem* 1997; 69:23-30
13. Trudel M, De Paepe ME, Chretien N, Saadane N, Jacmain J, Sorette M, Hoang T, Beuzard Y: Sickle cell disease of SAD transgenic mice. *Blood* 1994; 84:3189-97
14. Trudel M, Saadane N, Garel MC, Bardakdjian-Michau J, Blouquit Y, Guerin-Kern JL, Rouyer-Fessard P, Vidaud D, Pachnis A, Roméo PH, Beuzard Y, Costantini F: Towards a transgenic mouse model of sickle cell disease: Hemoglobin SAD. *EMBO J* 1991; 10:3157-65
15. Blouin MJ, De Paepe ME, Trudel M: Altered hematopoiesis in murine sickle cell disease. *Blood* 1999; 94:1451-9
16. De Franceschi L, Saadane N, Trudel M, Alper SL, Brugnara C, Beuzard Y: Treatment with oral clotrimazole blocks Ca²⁺-activated K⁺ transport and reverses electrolyte dehydration in transgenic SAD mice. *J Clin Invest* 1994; 93:1670-6
17. De Paepe ME, Trudel M: The transgenic SAD mouse: A model of human sickle cell glomerulopathy. *Kidney Int* 1994; 46:1337-45
18. Martinez-Ruiz R, Montero-Huerta P, Hromi J, Head CA: Inhaled nitric oxide improves survival rates during hypoxia in a sickle cell (SAD) mouse model. *ANESTHESIOLOGY* 2001; 94:1113-8
19. Greenburg AG, Kim HW: Civilian uses of hemoglobin-based oxygen carriers. *Artif Organs* 2004; 28:795-9
20. Gould SA, Moore EE, Moore FA, Haenel JB, Burch JM, Sehgal H, Sehgal L, DeWoskin R, Moss G: Clinical utility of polymerized hemoglobin as a blood substitute after acute trauma and urgent surgery. *J Trauma* 1997; 43:325-31
21. Seakins M, Gibbs WN, Milner PF, Bertles JF: Erythrocyte Hb-S concentration: an important factor in the low oxygen affinity of blood in sickle cell anemia. *J Clin Invest* 1973; 52:422-32
22. McCarthy MR, Vandegriff KD, Winslow RM: The role of facilitated diffusion in oxygen transport by cell-free hemoglobins: implications for the design of hemoglobin-based oxygen-based carriers. *Biophys Chem* 2001; 92:103-17
23. Budhiraja V, Hellums JD: Effect of hemoglobin polymerization on oxygen transport in hemoglobin solutions. *Microvasc Res* 2002; 64:220-33
24. Carmichael FJL: Recent developments in hemoglobin-based oxygen carriers: An update on clinical trials. *Transfus Apher Sci* 2001; 24:17-21
25. Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, Schechter AN, Gladman MT: Cell-free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 2002; 8:383-9
26. Bonaventura C, Ferruzzi G, Tesh S, Stevens RD: Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. *J Biol Chem* 1999; 274:24742-8
27. Przybelski RJ, Daily EK, Kisicki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA: Phase 1 study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. *Crit Care Med* 1996; 24:1993-2000
28. Carmichael FJ, Ali AC, Campbell JA, Langlois SF, Biro GP, Willan AR, Pierce CH, Greenberg AG: A phase I study of oxidized raffinose cross-linked human hemoglobin. *Crit Care Med* 2000; 28:2283-92
29. Viele MK, Weiskopf RB, Fisher D: Recombinant human hemoglobin does not affect renal function in humans: Analysis of safety and pharmacokinetics. *ANESTHESIOLOGY* 1997; 86:848-58
30. Kaul DK, Liu XD, Chang HY, Nagel RL, Fabry ME: Effect of fetal hemoglobin on microvascular regulation in sickle transgenic-knockout mice. *J Clin Invest* 2004; 114:1136-45
31. Ning J, Wong LT, Christoff B, Carmichael FJ, Biro GP: Haemodynamic response following a 10% topload infusion of Hemolink™ in conscious, anaesthetized and treated spontaneously hypertensive rats. *Transfus Med* 2000; 10: 13-22
32. Gonzales P, Hackney AC, Jones S, Strayhorn D, Horrmann EB, Hughes G, Jacobs EE, Orringer EP: A phase I/II study of polymerized bovine hemoglobin in

adult patients with sickle cell disease not in crisis at the time of study. *J Investig Med* 1997; 45:258-64

33. Caron A, Mayer JC, Menu P, Alayash A, Marie PY, Vigneron C: Measurement of blood volume after haemodilution with haemoglobin-based oxygen carriers by a radiolabelled-albumin method. *Transfus Med* 2000; 11:433-42

34. Yu B, Liu Z, Chang TM: Polyhemoglobin with different percentage of tetrameric hemoglobin and effects on vasoactivity and electrocardiogram. *Artif Cells Blood Substit Immobil Biotechnol* 2006; 34:159-73

35. Statler KD, Alexander H, Vagni V, Holubkov R, Dixon CE, Clark RS, Jenkins L, Kochanek PM: Isoflurane exerts neuroprotective actions at or near the time of severe traumatic brain injury. *Brain Res* 2006; 1076:216-24

Appendix

Oxygen Transport

The total amount of oxygen in blood was calculated as the sum of the oxygen bound to hemoglobin and that dissolved in plasma. The contributions of oxygen bound to erythrocyte hemoglobin and HRC 101 were calculated separately, using the corresponding

hemoglobin concentrations (Hb; g/dl), P_{50} values (mmHg), and Hill coefficients:

$$\text{Bound oxygen (ml/dl)} = \frac{\text{Hb}}{64,492} \times 22,414 \times 4 \times Y,$$

where 64,492 g/mole is the molecular weight of hemoglobin, 22,414 ml is the molar volume of oxygen at standard temperature and pressure, 4 is the number of oxygen molecules per molecule of hemoglobin, and Y is the fractional saturation of hemoglobin, given by the Hill equation:

$$Y = \frac{P_{O_2}^n}{P_{O_2}^n + P_{50}^n},$$

where P_{O_2} is the partial pressure of oxygen (mmHg), P_{50} is the partial pressure of oxygen at which hemoglobin is 50% saturated (mmHg), and n is the Hill coefficient.

$$\text{Dissolved oxygen (ml/dl)} = 0.003 \times P_{O_2}$$