A phase I single blind clinical trial of a new oxygen transport agent (MP4), human hemoglobin modified with maleimide-activated polyethylene glycol

Magnus Björkholm
Bengt Fagrell
Robert Przybelski
Nancy Winslow
Mark Young
Robert M. Winslow

Background and Objectives. MP4 (Hemospan®), a hemoglobin-based oxygen carrier, has been designed to deliver oxygen to hypoxic tissues without causing vasoconstriction. A phase I clinical trial of MP4 was undertaken to evaluate whether MP4 elicits the clinical side effects associated with previous hemoglobin-based solutions.

Design and Methods. Twelve volunteers were studied. One cohort (n=4) received 50 mg/kg of MP4, a second (n=4) received 100 mg/kg of MP4, and the third (n=4) received lactated Ringer's solution. Single blind infusions were given at 5 mL/min. Vital signs and symptoms, hematologic parameters, serum chemistry, renal and electrocardiographic measurements were monitored for 15 days after dosing.

Results. Five mild adverse events occurred in the controls and 2 each in the 50 mg/kg and 100 mg/kg MP4 groups. None was severe or judged related to MP4 administration by the principal investigator. There were no clinically significant alterations in blood pressure or heart rate, and there were no gastrointestinal symptoms, abdominal or flank pain, loss of appetite or clinically significant alterations of liver or pancreatic enzymes. In one subject (100 mg/kg of MP4), amylase and lipase were slightly above the upper limit of normal 4 hours after dosing, but without associated symptoms or signs. Pharmacokinetic analysis of plasma hemoglobin (assuming no hemolysis) yielded an estimated half-life ($T_{1/2}$) of 43 hours in the 100 mg/kg MP4 subjects.

Interpretation and Conclusions. MP4 appears to have a favorable safety profile. Subjects in both study groups survived and did no less well than those in the control group.

Key words: MP4, blood substitutes, oxygen therapeutics, hemoglobin, PEG, clinical trials.

Haematologica 2005; 90:505-515

Karolinska Hospital and Institute, Stockholm, Sweden (MB, BF); University of Wisconsin, Madison, Wisconsin (RP); Sangart, Inc., San Diego, California, USA; Sangart, Inc., San Diego, California, USA (NW, MY, RMW); Department of Bioengineering, University of California, San Diego, CA, USA (RMW).

Correspondence:
Robert M. Winslow, MD, Sangart, Inc., 11189 Sorrento Valley Road, Suite 104, San Diego, CA 92121, USA. E-mail: rwinslow@sangart.com

We have recently described human hemoglobin surface-modified with polyethylene glycol (MP4, registered trademark Hemospan®) that does not possess the vasoconstrictive properties which have hampered development of some hemoglobin-based oxygen carriers.[1] Based on *in vitro* studies, we postulated that this lack of vasoconstriction is due to decreased diffusive oxygen transfer in the plasma space as a result of increased molecular size and oxygen affinity.[2,3] Animal data have supported this hypothesis[3-6] and have suggested that MP4 may be of clinical benefit in augmenting oxygen delivery to hypoxic or ischemic tissue. Although phase I clinical trials with earlier polyethylene glycol (PEG)-modified hemoglobins have been performed, the results have not been reported in the literature, and their safety and clinical potential remain undocumented.

Therefore we have initiated clinical trials with MP4 in humans. MP4 is prepared from stroma-free human hemoglobin (SFH), chemically modified with monofunctional PEG activated with a terminal maleimide group.[7] The physical properties of MP4 are presented in Table 1. The product is formulated in physiological lactated Ringer's solution, and is stable for long periods at –20°C. The effectiveness of MP4 in maintaining tissue perfusion and oxygenation is postulated to occur by a combination of properties which avoid engaging autoregulatory vasoconstriction.[8] These properties include large molecular excluded volume, relatively high viscosity, high oncotic pressure and oxygen affinity, properties associated with maintained functional capillary density and acid-base balance, which correlate directly with survival after

severe hemorrhage in a hamster shock model.[9] The concentration of MP4, 4.2 g/dL, in its final formulation, was selected to have an oncotic pressure in the range of a colloidal plasma expander (Pentastan) that is in current clinical use in Europe. In order to support the first administration of MP4 to human volunteers, toxicology studies were performed in pigs, primates and rats. The studies included hemodynamic, serum chemistry and special organ system evaluations with particular reference to the cardiovascular and renal systems. Myocardial lesions, noted for one product,[10] were not found in either pigs or rhesus monkeys. Renal toxicity, seen frequently with free hemoglobin solutions,[11] was not observed after replacement of up to 30% of blood volume in rats and rhesus monkeys. Pharmacokinetic studies were performed in rats, primates and pigs, with the elimination half-life ranging between 4 and 24 hours depending on dose and species (the elimination kinetics of hemoglobin-based oxygen carriers is known to be dose- and species-dependent).[12]

MP4 has been designed as an oxygen-carrying plasma expander and hemodiluent for patients undergoing elective surgical procedures in which two units or more of blood are expected to be used. Based on preliminary animal studies it is estimated that the unit dose for this application will be approximately 10 g of MP4 per adult patient or 150 mg/kg. Preclinical animal data suggest that relatively small volumes (i.e., smaller than the volume of shed blood in hemorrhage experiments) can provide rapid and effective plasma expansion and tissue perfusion.[5,6]

The purpose of the present study was to assess the safety of MP4 in human volunteers, specifically to determine whether administration results in the characteristic effects noted for earlier generation hemoglobin-based products, including hypertension and gastrointestinal symptoms. Such a study can only be performed in a phase I setting in normal volunteers because interpretation of hemodynamic and symptomatic data cannot be interpreted in hospitalized patients of different ages, with different disease states, states of hydration, anesthesia regimens and receipt of different drugs and intravenous solutions. Studies in surgical or trauma patients are complicated further by variable amounts of blood loss and fluid administration. In the case of hemoglobin-based oxygen carriers, this is especially important, since one of the main toxic effects of hemoglobin is its propensity to cause vasoconstriction. Hypertension is commonly taken as the clinical consequence of vasoconstriction, but it is only so when volume and flow status are controlled and when no confounding drugs are being used in the study subjects. Hence, this phase I study is critically important in the development of MP4 for potential clinical approval.

Design and Methods

MP4

The production and characteristics of MP4 have been described previously.[1] At the scale of manufacture used for the present studies, production begins with 6 units of outdated, leukodepleted red blood cells, obtained from the San Diego Blood Center. Red cells are washed by diafiltration against 0.9% NaCl, then lysed gently in distilled water, and the membranes are removed by tangential flow filtration (0.16 μm filter cassette, Pall Filtron). The resulting stroma-free hemoglobin (SFH) is filtered through a 500 kDa hollow fiber filter (VirA/Gard, A/G Technologies) for further purification and virus reduction. SFH is reacted with 2-iminothiolane (Traut's reagent, Sigma Aldrich, St. Louis, MO, USA), resulting in thiolation of ε-amino groups of surface lysine residues. The thiolated SFH is then reacted with maleimide-activated monofunctional PEG, (MalPEG, MW 5000±500 kDa, NOF, Tokyo, Japan). All steps are performed at 4°C. The final reaction mixture is purified by tangential flow filtration (70 kDa, Pall Filtron) before formulation in lactated Ringer's solution. MP4 for this study was manufactured in the Sangart facility in San Diego, CA, USA and fill and finish was performed at Matrix Pharmaceuticals, also in San Diego. Both the Sangart and Matrix facilities were inspected by the Swedish Medical Products Agency and certified for cGMP compliance. Production at this scale yields approximately 100 glass vials, each containing approximately 50 mL of MP4 at a final concentration of 4.2 g/dL. The concentration is measured spectrophotometrically using an extinction coefficient for heme; thus, concentration is given in terms of g/dL of hemoglobin, not MalPEG-hemoglobin. The bottles were stored frozen at -20°C and shipped to Sweden on dry ice.

Clinical chemistry interference

To evaluate the effect of plasma MP4 on clinical chemistry, coagulation and hematology measurements, blood was collected from normal volunteers at the Karolinska Hospital Clinical Chemistry Department, Stockholm. MP4 was added in increasing amounts to serum (for the chemistry assays), to citrate plasma (for the coagulation assays), or to heparinized plasma (plasma hemoglobin). An identical set of dilutions was prepared using lactated Ringer's solution. Measurements using the Vitros 950 Clinical Chemistry analyzer were performed in duplicate on blood from each of 2 normal volunteers using the sample appropriate for the particular test (plasma or serum) to determine the analyte concentration in the absence of MP4 (unspiked), and in the

presence of MP4. Identical concentrations of hemoglobin were formulated in lactated Ringer's solution by addition of MP4. A separate study of interference of MP4 on troponin T was performed since troponin T was undetectable in plasma from normal volunteers. Human plasma from normal volunteers was spiked with concentrations of MP4 and troponin T (6×5 matrix) that could potentially be encountered in clinical practice. Spiked plasma samples were analyzed using the Elecsys 2010 immunoanalyzer (Roche Diagnostics, Mannheim, Germany).

For each measurement, the predicted value (X_p) is calculated:

(i) $$X_p = \frac{X_o}{d}$$

where X_o is the observed value, and d is the dilution factor. The error (e_x) due to the presence of MP4 is then

(ii) $$e_x = X_o - X_p$$

Study design

The clinical study was performed according to the principles stated in the Declaration of Helsinki and good clinical practices (GCP) as described by the International Conference on Harmonization (ICH). The protocol and consent forms were approved by the institutional review (Ethics) committee of the Karolinska Hospital, Stockholm, and the study was approved by the Swedish Medical Products Agency. The design of the Case Report Forms, handling of data, statistical analysis and report writing was done by Quintiles AB, Uppsala, Sweden.

The subjects for the study were volunteers, most of whom were students at the Karolinska Institute. They received a small stipend to compensate for routine expenses and inconvenience. The principal investigator or an associate explained orally and in writing the nature of the study and action of the test product to candidate volunteers so they were aware of potential risks. They were informed that they were able to withdraw from the study at any time and signed the consent form in the presence of a witness. Candidate volunteers were assured that if they chose not to participate there would be no adverse consequence or penalty for them.

The design of the study was a single-blind, placebo-controlled, escalating-dose safety and tolerability study involving the first time administration of MP4 to 8 healthy human volunteers. Four additional volunteers received lactated Ringer's solution as controls. The doses of MP4 selected for this study, 50 mg/kg and 100 mg/kg, were based on previous published phase I studies of hemoglobin solutions. The Baxter study of diaspirin cross-linked hemoglobin (DCLHb) investigated 25, 50 and 100 mg/kg,[13] while the Hemosol study of HemolinkTM 14 used doses ranging from 25-600 mg/kg.[14] In both studies, hypertension and gastrointestinal complaints were observed at these doses. In both of these studies lactated Ringer's solution was used as the control (placebo) solution.

Study subjects

The protocol included adult male or surgically sterile female volunteers, preferably with previous clinical trial experience, from 18 to 50 years of age, inclusive. Subjects were screened within 1 to 4 weeks before the start of the study and fasted for 12 hours prior to the screening visit. A medical history and physical examination, including vital signs and 12-lead supine ECG, were obtained. Blood samples for hematology and clinical chemistry testing were collected and analyzed at screening. Any abnormal findings at the pretreatment visit were assessed according to their clinical importance and if considered clinically significant by the principal investigator, the volunteer was not included in the study. Subjects were excluded from the study if they had any acute or chronic renal, cardiovascular, metabolic, gastrointestinal, psychiatric or hepatic condition as assessed by history, physical examination or laboratory screening. Subjects were also rejected if they had a history of drug or alcohol abuse, a positive urine drug screen, taken any investigational drugs within 30 days, used any prescription drug within 2 weeks, donated blood within 6 weeks, or were allergic to iodine-containing contrast materials. Screening tests were done for human immunodeficiency virus and hemoglobinopathies. According to these guidelines, two potential subjects were excluded from the study at screening.

On the day of infusion (day 1) all volunteers were required to drink 250 mL of water every hour for 3 hours prior to infusion to ensure good urine flow during the test period. One of three volunteers was randomly assigned to serve as a control to receive lactated Ringer's solution, and all volunteers were blinded to the assignment. During the infusion of test material or control, the volunteers' arms were shrouded so they could not see the material infused. All other participants in the study, including the investigator and nursing personnel, were aware of the assignment to MP4 and control groups.

After calculation of the total amount of MP4 to be administered to each subject, the requisite bottles were pooled into an administration bottle in a laminar flow hood of the hospital pharmacy using an aseptic technique. Administrations were via a standard intravenous infusion set into an antecubital vein

at 5 mL/min. Volunteers were confined to the clinical study unit one day prior to dosing and remained in the unit for 24 hours following administration of the test article. The volunteers remained in the hospital facility until the morning of day 4 when a physical examination and interim safety laboratory assessments were obtained. The subjects were discharged from the unit on day 4 if the evaluations were normal. They were required to return to the study unit every day for 1 week (to day 8) and again at two weeks (day 15) for continued evaluations.

Evaluations

The safety and tolerance of each dose level were determined by evaluation of clinical examination, vital signs, review of adverse events and clinical laboratory results. All clinical laboratory tests were performed by the Clinical Chemistry Department of the Karolinska Hospital, and the data were transferred to Quintiles, AB, in Uppsala, electronically. Iohexol clearance measurements were performed at baseline and at 24 hours after dosing. An independent committee reviewed the clinical data after the first cohort and approved progression to the next cohort according to predefined stop-go criteria. The safety evaluation committee consisted of senior faculty members of the Karolinska Institute who were not connected with the study or Sangart in any way. Progression from the first cohort to the second required that no serious adverse event was detected in any of the treatment volunteers.

Pharmacokinetic analysis

At the end of the infusion, the infusion bottle was sampled for total hemoglobin concentration. Individual blood samples were drawn into 5 mL heparinized vacutainers using a 19-gauge needle to minimize the risk of hemolysis. The tubes were centrifuged at approximately 2500 rpm for 10 minutes, and the resultant plasma was transferred to a second tube and centrifugation was repeated. At least 1 mL of this final plasma sample was transferred to Sarstedt/storage container. Processing was performed at room temperature, and the final tubes were transferred to the freezer for storage (-20°C) within 60 minutes of collection.

Analysis of plasma hemoglobin concentration was done at the Department of Clinical Chemistry, Karolinska Hospital, Stockholm. The plasma was transferred to disposable cuvettes and assayed by spectrophotometry using the Hemocue B-Hemoglobin system (B-Hemocue, Ängelholm, Sweden), modified for low hemoglobin concentration. The absorbance was measured at two wavelengths (565 and 880 nm) to compensate for turbidity. The calibration range was 0.3-30 g/L, and the lower limit of quantification (LLOQ) of hemoglobin in plasma was 0.3 g/L. This method was validated for MP4 using cyanomethemoglobin standards.

Pharmacokinetic calculations, based on plasma concentrations of hemoglobin (assuming no hemolysis) were performed by means of non-compartmental analysis using model 202 (constant infusion administration) of WinNonlin Professional, version 3.0 (Pharsight Corporation, Mountain View, CA, USA). Individual plasma concentration values from each subject and the matching time points for blood sampling were used throughout the analyses except for the time points prior to infusion (time=0). At this point, time as well as any detected concentrations (due to hemolysis) were set to 0. Samples with plasma hemoglobin concentrations below the LLOQ in the terminal phase were omitted from the analysis.

The maximum observed serum concentration, Cmax, and the corresponding time, tmax, were obtained without interpolation. The area under the plasma concentration-time curve from time 0 to time of last detectable serum concentration AUC0-t(last) was calculated using the log-linear trapezoidal rule. The area under the serum concentration-time curve from time 0 to infinity, AUC0-inf was calculated as AUC(0-t) + Ct/k. Ct is the last measurable concentration, and k is the terminal single exponential elimination rate constant. Elimination half-life ($t_{1/2}$) was calculated as $t_{1/2} = 0.693/k$. The chosen observations for estimation of the terminal single exponential elimination rate constant were based on WinNonlin selections. The WinNonlin output was checked against the printouts from the study database with respect to sampling times and concentrations. The pharmacokinetic analysis was performed at Quintiles AB, Uppsala.

Statistics

The number of subjects was too small for a detailed statistical analysis. Consequently, only descriptive values (mean, standard errors) are reported, and no attempt has been made to determine between-group significance. Analysis of the power required to show efficacy or safety in a statistically convincing way was not possible due to the small size of the study and because potential markers for safety and efficacy have not yet been identified. It is acknowledged that any claim to statistical significance of any of the results of this study is not possible. In the instances where it is appropriate, we have used the notation *clinically significant* to suggest that particular changes would not be considered outside the range of normal observation by any reasonable clinician.

Results

MP4

The physical properties of MP4 are given in Table 1. Note that the viscosity and oncotic pressure of MP4 are higher than those usually accepted as conventional for hemoglobin-based solutions, and the P50 is lower.[11] The oxygen equilibrium curve of MP4 is essentially devoid of cooperativity, as demonstrated by its Hill parameter of approximately 1.2.

Clinical chemistry interference

Results of the interference studies are reported as slopes and intercepts of the equation (equation 2) relating the error resulting from the presence of plasma hemoglobin to MP4 concentration (Table 2). The units of measurement for each analyte are given using the designations commonly used in European countries (e.g., mmol vs mg, and μkat/L vs Units/L). In the case of 12 analytes, the maximum error was found to be less than 10% of the baseline normal value, and no correction was applied. Larger errors were found for 12 additional analytes. All of the regression lines are linear from the origin within the range of MP4 concentration tested (0 to 2 g/dL). The regression line for conjugated bilirubin is linear, thus no correction is necessary below 0.5 mg/dL MP4. The error from MP4 in the assay for troponin T was found to be proportional to both the concentration of MP4 and the concentration of troponin. At concentrations of MP4 above 10 g/L, and concentrations of troponin above the diagnostic cut-off value (0.1 μg/L), a negative interference of approximately -50% was observed. These results indicate that a value for troponin T could be interpreted as falsely negative if MP4 concentration was high. However, at the concentrations of MP4 achieved in the current study (<5.0 g/dL), the interference in the assay for troponin was -4.3%. No corrections could be determined for haptoglobin or troponin T, since there were no detectable levels of either analyte at any concentration of MP4. Thus, for the current observations, interference from MP4 was determined not to be a factor in the interpretation of the troponin data from subjects receiving MP4.

The case of haptoglobin measurement appears to be special. Haptoglobin was detected in the normal samples to which MP4 was not added (mean 92 mg/dL, normal range 60-270 mg/dL). Haptoglobin was not detected in the clinical samples, however, in the presence of MP4 at any concentration. Since the assay method measures total (free + bound) haptoglobin, it is not clear why it is not detected, and this finding warrants further study.

Subjects

All subjects who volunteered for this study were

Table 1. Physical characteristics of MP4.

Test	*Specification*
Hemoglobin concentration (g/dL)*	4.2±0.2
Methemoglobin (%)	< 0.5
pH (20±2°C)	7.4±0.4
Endotoxin (EU/mL)	< 0.05
Viscosity (cP)	2.5±1.0
COP (mmHg)	55±20
P50 (mmHg)	6±2
Hill coefficient (at P50)	1.2±0.5
Bohr effect (ΔlogP50/ΔpH)	-0.24
Sterility	Pass

**Heme basis.*

Table 2. Clinical chemistry interference.

Analyte	*Instrument*	*Units*	**Slope*	R^2	*Comments*
Na	Vitros	mEq/L	-0.06	-0.07	No correction
K	Vitros	mEq/L	-0.10	0.95	No correction
Cl	VItros	mEq/L	0.07	-0.69	No correction
Osmolarity		mOsm/L	-5.10	0.89	No correction
Glucose	Vitros	mmol/L	0.20	0.55	No correction
Urea	Vitros	mmol/L	0.02	-0.93	No correction
Creatinine	Vitros	mmol/L	-1.68	-1.47	No correction
Total protein	Vitros	g/L	28.85	0.99	
Albumin	Vitros	g/L	13.08	0.99	
Total bilirubin	Vitros	mmol/L	48.5	0.99	
Conjugated bilirubin	Vitros	mmol/L	9.15	0.99	Correct when MP4 > 0.5 g/dL
AST	Vitros	mkat/L	0.35	0.99	
ALT	Vitros	mkat/L	-0.03	0.65	No correction
Alk Phos	Vitros	mkat/L	-1.70	0.70	
GGT	Vitros	mkat/L	0.08	0.95	
Cholesterol	Vitros	mmol/L	-0.20	0.92	No correction
Amylase	Vitros	mkat/L	0.03	0.00	No correction
Lipase	Vitros	mkat/L	0.13	-0.14	No correction
LDH	Vitros	mkat/L	15.11	0.98	
CK	Vitros	mkat/L	0.21	0.86	
CK MB	Elecsys	mkat/L	0.38	0.99	
Troponin T	Elecsys	mg/L			Below detection
Uric acid	Vitros	mmol/L	-11.00	0.88	No correction
Fibrinogen	Thrombolyzer	g/L	0.04	0.38	
PT	Thrombolyzer	INR	-0.33	0.96	
APTT	Thrombolyzer	Seconds	-9.00	0.99	
D-dimer	Hitachi	mg/L	0.01	0.50	
Haptoglobin	Immage	g/L			Below detection
C3	Immage	g/L	0.00	-0.36	No correction
C5a	Elisa/DRG International	mg/L	0.04	0.98	

**slope units are g/dL of MP4.*

Table 3. Study subjects.

Cohort A 50 mg/kg	Article	Weight (kg)	Height (cm)	Age (yr)	Sex
1	MP4	72.0	182	21	M
2	MP4	60.5	176	25	M
3	LR	77.5	174	25	M
4	LR	81.0	188	22	M
5	MP4	68.5	184	24	M
6	MP4	84.7	181	23	M
Cohort B 100 mg/kg	Article	Weight (kg)	Height (cm)	Age (yr)	Sex
7	LR	80.5	184	19	M
8	MP4	79.5	178	19	M
9	MP4	67.0	171	19	M
10	MP4	84.0	185	30	M
11	LR	86.0	187	23	M
12	MP4	77.0	177	26	M
50 mg/kg mean±sd		71.8±10.0	180.8±3.4	23±2	
100 mg/kg mean±sd		76.8±7.1	177.8±5.7	24±5	
LR±sd		80.8±3.8	183.3±6.4	22±2	

Table 4. Adverse events (AEs). The days on which each AE occurred are shown in parentheses.

	MP4 50 mg/kg n	MP4 100 mg/kg n	Control n	Total AE n
Nausea	–	1 (14)	2 (3,4)	3
Pharyngolaryngeal pain	–	1 (2)	–	1
Toothache	–	1 (6)	–	1
Vomiting	1 (14)	–	–	1
Hypersensitivity	–	–	1 (11)	1
Dizziness	1 (5)			1
Headache	–	–	2 (4,11)	2

males (Table 3). They were well matched with regard to height and age, but the body weight of the control (LR) group was somewhat greater than that of either of the two MP4 groups. None of the between-group differences in mean values (Table 3) is statistically significant, however. The average dose of MP4 in cohort A was 90.3 mL and in cohort B 192.7 mL.

No abnormalities were noted in physical examinations at any point in the study in any group. In particular, no jaundice, skin discoloration or red urine was noted. Electrocardiograms in some subjects were interpreted as showing sinus bradycardia, but this was felt to be attributable to their state of physical conditioning, since there was no relation to drug administration and no associated symptoms.

Adverse events

There were no serious adverse events determined by the principal investigator or the Safety Evaluation Committee (Table 4). No subject reported loss of appetite, chest, abdominal or costovertebral pain. There were 5 adverse events, considered to be mild, in the control group, and 2 and 3 adverse events in each of the 50 mg/kg and 100 mg/kg groups; all were mild and unrelated to MP4 administration. Mild nausea was reported in 2 of the control volunteers (days 3 and 4) and in one of the MP4 volunteers. In the latter case, nausea and vomiting occurred on day 14, and was considered to be due to excessive ingestion of candy and energy bars. One of the control subjects reported allergy symptoms (which he had had on prior occasions) on day 11. A volunteer in the 50 mg/kg MP4 group reported a mild 10-minute episode of dizziness on day 5, which resolved spontaneously. The subject had a history of similar events in the past. A subject in the 100 mg/kg MP4 cohort reported a mild sore throat on day 2, and underwent a planned dental extraction on day 6. None of the adverse events was judged to be related to MP4 administration.

Pharmacokinetics

The control group received an intravenous infusion of lactated Ringer's solution, and hemoglobin should normally not be present in their plasma. Of the 88 plasma samples from the control group, 37 (42%) had measurable hemoglobin, albeit at very low concentrations (median 0.4 g/L; minimum 0.3 g/L, maximum 1.5 g/L); this was possibly the result of hemolysis. Most of the hemolysed samples had been collected at night when processing was somewhat delayed. The total plasma concentration of hemoglobin after receiving MP4 is presented in Figure 1. The calculated $t_{1/2}$ for MP4 (50 mg/kg) was 66.2 hours, and the $t_{1/2}$ for MP4 (100 mg/kg) was 42.8 hours. Although the AUC did not increase in proportion to dose, the Cmax describes a linear relationship between dose and exposure. The t_{max} would be expected to occur simultaneously with the end of the infusion or at the next sampling. The t_{max} was expected at approximately 20 minutes for the MP4 50 mg/kg group, but the observed t_{max} occurred at 7 hours, approximately 6 hours after the end of the infusion. The t_{max} occurred at 0.86 hours (52 minutes) for the MP4 100 mg/kg group which is close to the expected value of approximately 40 minutes.

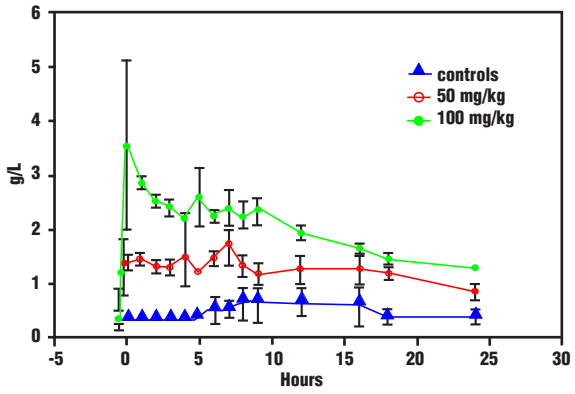

Figure 1. Mean plasma hemoglobin concentration (± sd) of MP4 after administration in controls (triangles), 50 mg/kg (open circles) or 100 mg/kg (closed circles) cohorts.

Vital signs

No alterations in tympanic membrane temperature, respiration rate or pulse oximeter values were observed in any subject. The blood pressure and heart rate data are shown in Figure 2. There were no clinically significant differences, either between the cohorts or between either cohort and controls in systolic blood pressure, except for a single point at which systolic pressure was slightly lower in cohort A than in controls at 60 minutes after dosing. This single point is apparently due to an elevation in the controls, rather than to a drop in cohort A.

Some of the diastolic blood pressure measurements were elevated compared to either baseline or controls, in the 50 mg/kg and 100 mg/kg cohorts, but none occurred until more than 2 hours post-dosing. Diastolic blood pressure was slightly higher in the 50 mg/kg cohort at 150 minutes post-dosing compared to baseline. However, in the 100 mg/kg cohort, diastolic blood pressure was greater than baseline at 105 and 195 minutes and at 4 hours. The diastolic blood pressure was greater than that in the controls at 150 minutes. The heart rate for both the 50 mg/kg and 100 mg/kg cohorts decreased somewhat compared to baseline for most of the 4-hour observation period. All of the subjects were very fit, and had resting heart rates that were quite low. In general, the heart rates were faster at screening and check-in than at pre-dosing, which was assumed to be due to greater level of activity. In general, the bradycardia was most pronounced in those subjects who displayed bradycardia at screening and baseline.

Continuous electrocardiographic monitoring was carried out for 4 hours post-dosing, and rhythm strips were recorded on days 2, 3 and 4. No abnormalities were noted except for occasional sinus bradycardia which did not differ from baseline.

Renal function

There were no alterations noted in creatinine, β_2-microglobulin, N-acetyl glucosamine (NAG) or iohexol clearance, and there were no significant differences in creatinine clearance between the control and MP4 groups during any measurement period (Figure 3). Although all urine collections were free of any red color, individual urine analyses post-dosing showed a 1+ to 3+ test for hemoglobin using the Dip Stix method.

Hematology

Hematologic measurements included hematocrit, hemoglobin, red cell indices, leukocyte counts with differential, blood smears, coagulation tests including platelet counts, activated partial thromboplastin time (APTT), International Normalized Ratio (INR), fibrinogen and D-dimer. No abnormalities were observed in any of the treatment or control subjects. Plasma haptoglobin levels were markedly reduced 2 hours after dosing in volunteers who received MP4 (Figure 4), and remained so until 4 days after dosing. The recovery was slightly faster in the 50 mg/kg dose group than in the 100 mg/kg group.

Clinical chemistry

Bilirubin (total and conjugated), alkaline phosphatase, aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (GGT) were all normal throughout the study. Bilirubin rose slightly in the MP4 groups, but there were no other abnormalities. Troponin T, creatine kinase (CK) and cholesterol also remained within normal limits. Serum amylase and lipase remained within the normal limits for all subjects, except for subject 10, who received 100 mg/kg of MP4. In this subject, amylase level at 4 hours after dosing reached 1.88 μmol/L (normal range, 0.5–1.55 μmol/L) and his lipase was 12.2 μmol/L (normal range, 0.4–5 μmol/L). Both enzymes were within the normal range at the next sampling time, 18 hours. There were no associated gastrointestinal symptoms, including nausea, loss of appetite or pain. These elevations did not constitute an adverse event, having been noted by the principal investigator and the study Safety Committee and regarded as having no clinical significance.

Complement activation

Both C3 and C5a were measured and remained normal in all subjects, regardless of 0.

Discussion

Our selection of the doses to be studied was based on the earlier published studies with modified hemo-

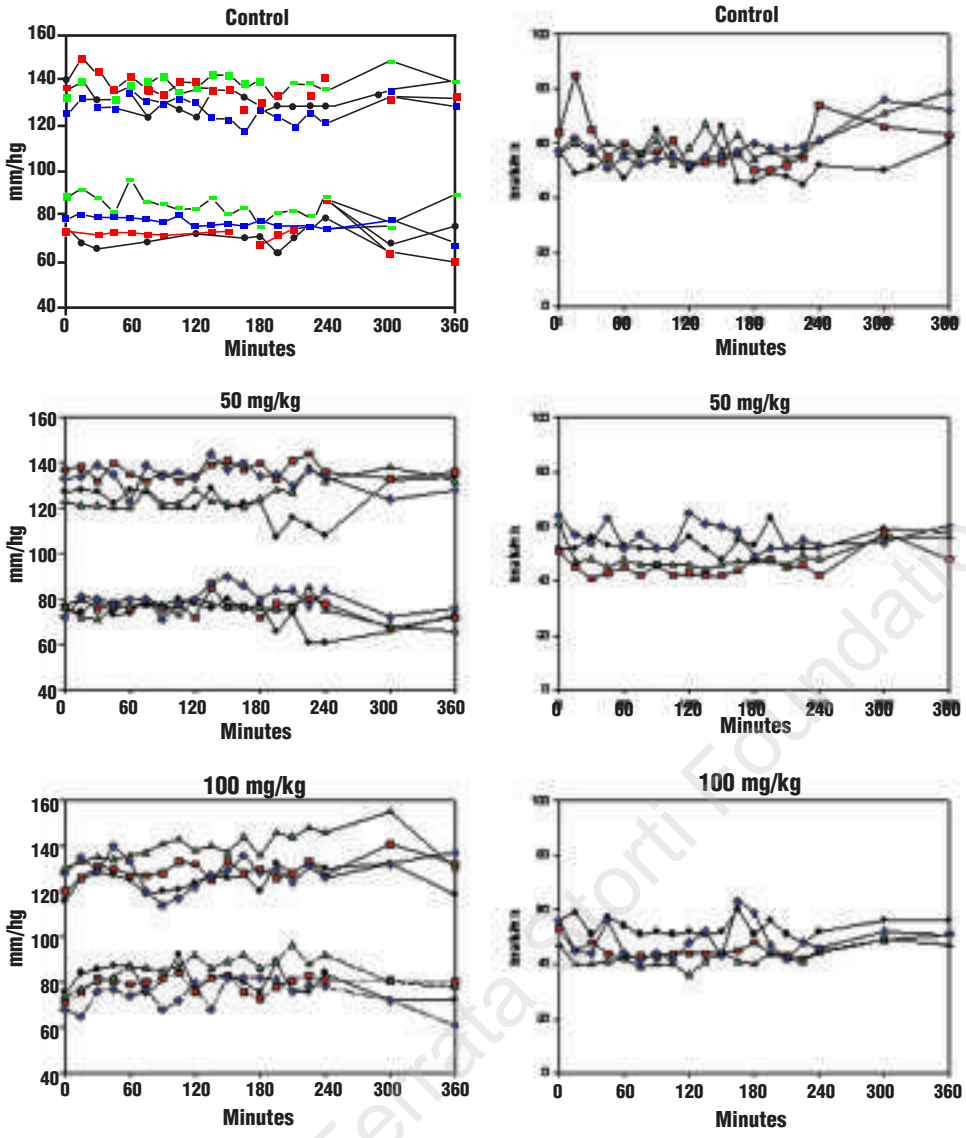

Figure 2. Blood pressure (left) and heart rate (right) after infusion of lactated Ringer's solution (control, top panel), MP4 at 50 mg/kg (middle panel), or MP4 at 100 mg/kg (bottom panel). Each line represents a single subject.

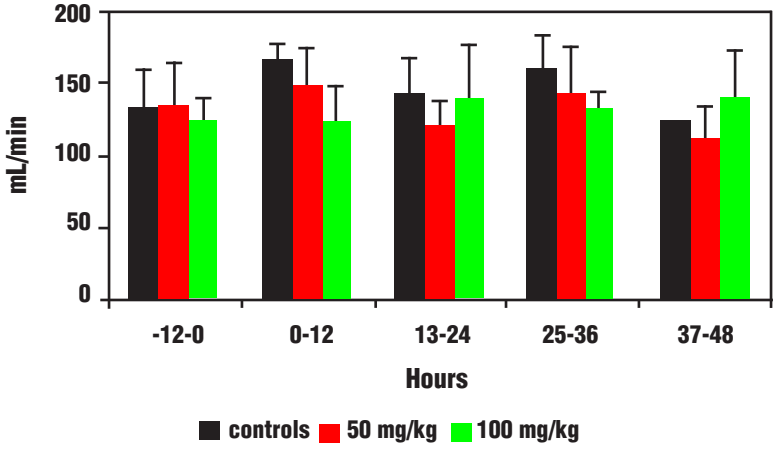

Figure 3. Creatinine clearance, calculated at 12-hour intervals in controls (black bars), after administration of MP4 at 50 mg/kg (red bars) or 100 mg/kg (green bars).

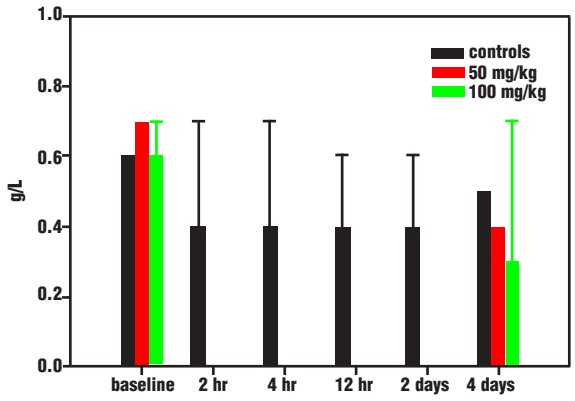

Figure 4. Serum haptoglobin concentration in controls (black bars), after administration of MP4 at 50 mg/kg (red bars) or 100 mg/kg (green bars).

globin solutions, one by Przybelski *et al.*,[13] in which the doses were 25, 50 and 100 mg/kg. In a study by Carmichael *et al.*,[14] the doses ranged from 25 to 600 mg/kg. In both studies, lactated Ringer's solution was the control, and in both studies the *typical* side effects characteristic of cell-free hemoglobin were noted, including hypertension, bradycardia and gastrointestinal symptoms. Based on these earlier studies, we felt the absence of these side effects, even in a relatively small study, would indicate improved safety of MP4. In regard to the choice of the control material (lactated Ringer's solution), a colloid may have been more useful in some ways, since an oncotically matched colloid would have expanded the blood volume to the same extent as MP4, making the hemodynamic comparisons more meaningful. However, again, we chose the crystalloid control in order to be comparable with the two previously published studies.

The principal findings of this study are that no clinically significant elevations of blood pressure or reductions in heart rate were observed in any of the study volunteers, although the low number of volunteers precludes any definitive statistical analysis. Furthermore, none of the subjects manifested any gastrointestinal symptoms or signs. There was no indication of pancreatic, liver or renal impairment, and no complement activation. No adverse events were attributable to MP4 administration. Taken together, these findings are consistent with the lack of vasoconstriction observed in animal studies with MP4. MP4 administration to normal volunteers in this study may have resulted in a slightly (but not significantly) elevated diastolic blood pressure and decrease in heart rate, effects that could be attributed to volume expansion. This is very likely, in view of the increased oncotic pressure of MP4 (Table 1). Furthermore, published phase I[14-16] and animal studies[4] suggest that hypertension would occur immediately after dosing. However, a specific effect of cell-free hemoglobin, apart from volume expansion, cannot be ruled out, and only further dose escalation will determine the extent of the effect. There are significant differences in properties such as viscosity and COP between lactated Ringer's solution and MP4. However, for this phase I study, lactated Ringer's solution was thought to be the safer choice, since side effects have been reported for the available colloids including the starches, which might be matched in COP. The difference in plasma expanding potential for lactated Ringer's solution and MP4 could well influence the interpretation of the blood pressure measurements, but in a way that would exaggerate the MP4 effect, not mask any possible hypertensive effect.

The hallmark of cell-free hemoglobin administration to animals and humans is vasoconstriction, manifested by hypertension.[11] Additional recurrent side effects have included gastrointestinal symptoms, regardless of whether the products are crosslinked or polymerized.[17,18] Our working hypothesis is that those (and possibly other) side effects have occurred as a consequence of oxygen-mediated arteriolar vasoconstriction, a normal autoregulatory mechanism.[19] We have postulated that this phenomenon can be attenuated by decreasing the diffusion of cell-free hemoglobin by increasing its molecular volume, oxygen affinity and viscosity. MP4 is based on these design parameters.[2,8]

Phase III studies with DBBF-crosslinked human hemoglobin (DCLHb) were halted because of increased mortality.[20,21] Our view is that this unfortunate result was predicted by preclinical animal studies.[19] The autoregulatory theory[2] predicts that because of increased molecular radius, polymerized hemoglobin should be less vasoactive than low molecular weight hemoglobins such as DCLHb. This theory is further supported by the observation that polymerization of DBBF-crosslinked human hemoglobin decreases its hypertensive effect.[22]

Phase I studies of hemoglobin-based products are critically important because of the nature of the potential toxicity of such products, including hypertension and vasoconstriction, both of which are presumed to be mediated by smooth muscle stimulation. Such effects can be masked in patients who receive anesthetic agents, are actively bleeding or who are receiving intravenous fluids.

Studies that have been published show evidence of vasoconstriction. In an early phase I clinical trial using purified (but not modified) stroma-free hemoglobin,[15] diastolic blood pressure rose to 25 mmHg above baseline in normal volunteers given approximately 200 mg/kg. This effect was maximal at about 1 hour after initiation of infusion and persisted for about 2 additional hours. At the same time, the heart rate slowed

significantly (90 to 53 bpm), reaching a nadir at 1 hour and lasting about 2 hours. Savitsky showed significant but transient reductions in urine flow and creatinine clearance, which returned to normal by 24 hours after dosing.[15] That study also reported significant gastrointestinal symptoms ranging from mild nausea and loss of appetite to severe stomach, chest and costovertebral angle pain, as well as prolongation of the APTT by 57% of the baseline value.

In a phase I study of DCLHb,[16] the hemoglobin concentration in the infused solution was somewhat higher (10.4 g/dL), but the solution stimulated significant elevations of diastolic blood pressure even at 25 mg/kg. Maximal elevation of approximately 10 mmHg occurred at 50 mg/kg, but blood pressure did not increase further with dose escalation to 100 mg/kg. The findings in a study of o-raffinose cross-linked human hemoglobin[14] were very similar, showing a maximal blood pressure effect at 100 mg/kg with no further increase at higher doses. In contrast, none of these effects was observed in the present study at 100 mg/kg. A second objective of the present study was to estimate the pharmacokinetics of MP4. The plasma $t_{1/2}$ of 66.2 hours, calculated in the 50 mg/kg MP4 subjects, is probably unreliable, due to the very low plasma hemoglobin concentrations observed in these subjects. In some instances we found similar levels, near the lower limit of detection, in the control volunteers. We conclude that some degree of hemolysis occurred in the samples drawn at night, a consequence of increased processing time. This is also the presumed explanation for t_{max} occurring 7 hours after dosing in the 50 mg/kg volunteers. The t_{max} at 52 minutes in the 100 mg/kg cohort is close to the expected value of 40 minutes. The $t_{1/2}$ of 42.8 hours is in good agreement with the value observed in rats using ^{14}C-labeled MP4.[1] The plasma retention times of modified hemoglobins have been shown to be dependent on both species and dose.[12] Thus, a 43-hour half-life would be expected to be the minimal value for MP4, and it is likely that the half-life will be longer at higher doses. The concentrations of plasma hemoglobin were not high enough for reliable measurements of methemoglobin in this study.

Gastrointestinal side effects, including nausea, vomiting, and loss of appetite, as well as chest and abdominal pain, have been a feature of all published studies with hemoglobin solutions, whether modified or not. The mechanism of these side effects has been attributed to spasm of the lower esophageal sphincter.[18] None of the volunteers in the present study experienced loss of appetite, and there were only mild, limited episodes of nausea reported in both control and active subjects. The reduction in serum haptoglobin is not considered an untoward effect, since haptoglobin binds plasma free hemoglobin and may be expected to disappear after dosing of MP4.

Elevations in the pancreatic enzymes, lipase and amylase, are commonly attributed to infusions of hemoglobin-based products, but we are unaware of published clinical studies that document this phenomenon with the exception of a phase I trial with o-raffinose cross-linked hemoglobin,[14] which also reported elevations in LDH, AST, AST and CK. In that study, 2/33 and 21/22 normal volunteers had elevated amylase and lipase values, respectively. The elevations were not strongly dose-dependent, peaked at 24 hours post infusion, and returned toward baseline (but were still abnormal) at 48 hours after infusion. The report does not discuss any correlations with gastrointestinal symptoms, which were commonly observed. The etiology of these enzyme elevations is not clear, but there is a suggestion that hemoglobin solutions could affect smooth muscle contraction in the gastrointestinal tract.[23] In animals dosed with DCLHb, in which elevations of amylase and lipase were prominent, direct visualization of the microcirculation failed to show any compromise in blood flow or local tissue perfusion.[24] Thus, while the etiology of these enzyme elevations remains unexplained, association with pathological events has not been established.

While the results reported in this study are consistent with the absence of vasoconstriction, and generally support the oxygen-induced autoregulatory mechanism, final assessment of its role in hemoglobin-induced toxicity reported in the literature is still not established. It is known that hemoglobin is an avid scavenger of nitric oxide (NO) based on *in vitro* experiments with aortic rings,[25] and hemoglobin mutants with reduced heme affinity for NO have a reduced hypertensive effect.[26] However, while correlation of hypertension and vasoconstriction is usually assumed, direct demonstration of this correlation is very difficult in animal models and impossible, so far, in humans.

Alternatively, modified hemoglobin molecules with large excluded volume may extravasate less readily than smaller molecules and may therefore scavenge NO less effectively. Evidence correlating molecular size, extravasation and hypertension has been presented by Matheson *et al.*[27] According to this theory, scavenging of NO by hemoglobin is more effective if hemoglobin is in the interstitial space, rather than the vascular space, and the theory predicts that reduced extravasation would lead to reduced vasoconstriction. This theory cannot be firmly established either, because the large molecules described by Matheson[27] also share many of the properties of MP4, including increased viscosity and oxygen affinity. Yet another theory to explain vasoconstriction is that maintained plasma viscosity

is critical to maintain the shear-dependent synthesis of NO in vascular endothelium.[28]

Regardless of which of these explanations (or a combination of them) proves correct, this phase I study in humans appears to confirm preclinical animal studies showing that at doses up to 100 mg/kg, MP4 does not cause hypertension, nor does it lead to any detectable renal, liver or pancreatic dysfunction, coagulation abnormalities, complement activation or any hematologic or cardiac abnormalities.

MB: responsibility for clinical trial conduct, screening volunteers, and collecting data; BF: Sweden co-ordinator of all Sangart Clinical Trials, maintained liaison between Sangart and the clinical trial site; RP: participated in design of the clinical trial, company monitoring and interpretation of data; NW: co-ordinated activities of clinical nurses with Sangart scientists; MY: measured interference of hemoglobin-containing materials with standard clinical chemistry tests; RMW: overall responsibilities for clinical trial design and preparation of manuscript.

The authors wish to acknowledge the Sangart production team which includes Edvin Agadzanov, Jay Belke, Keith Chapman, Stephanie Melton, Ann Pimental, Luke Park, Terri Schoeneman, Shawn Snell and Jerod Wooldridge, as well as the Sangart research team, including Jeff Lohman, Ashok Malavalli and Kim Vandegriff. Special thanks are also given to the able members of the Clinical Research Unit at the Karolinska Hospital, Stockholm, headed by Professor John Wahren, including Karin Ekberg. Eva-Lena Forsberg, Alice Skogholm and Agnata Reinholdsson. The authors also wish to thank Ms. Therese Anehall and Ms. Nina Stålhammar of Quintiles AB, Uppsala, and the Safety Committee, whose members were Assoc. Prof. Jan Östergren, Prof. Ulf de Faire, Assoc. Prof. Fredrik Celsing and Assoc. Prof. Bo-Lennart Johansson. The authors are especially grateful for the enthusiastic co-operation of the San Diego Blood Center.

The study was funded by Sangart, Inc. Robert M. Winslow is the founder, CEO, President and Board Chairman of Sangart, Inc. Bengt Fagrell and Robert Przybelski are consultants to Sangart, Inc. Nancy Winslow and Mark Young are Sangart employees. Magnus Björkholm has no business connection with Sangart.

Manuscript received July 1, 2004. Accepted February 22, 2005.

References

1. Vandegriff KD, Malavalli A, Wooldridge J, Lohman J, Winslow RM. MP4, a new non-vasoactive polyethytlene glycol-hemoglobin conjugate. Transfusion 2003;43:509-16.
2. 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 carriers. Biophys Chem 2001;92:103-7.
3. Tsai A, Vandegriff K, Intaglietta M, Winslow R. Targeted O_2 delivery by cell-free hemoglobin: a new basis for oxygen therapeutics. Am J Physiol 2003; 285:H1411-H9.
4. Winslow RM, Gonzales A, Gonzales M, Magde M, McCarthy M, Rohlfs RJ, et al. Vascular resistance and the efficacy of red cell substitutes in a rat hemorrhage model. J Appl Physiol 1998;85:993-1003.
5. Drobin D, Kjellstrom B, Malm E, Malavalli A, Lohman J, Vandegriff KD, et al. Hemodynamic response and oxygen transport in pigs resuscitated with maleimide-polyethylene glycol-modified hemoglobin (MP4). J Appl Physiol 2004;96:1843-53.
6. Wettstein R, Tsai A, Erni D, Winslow R, Intagietta M. Resuscitiation with polyethylene glycol-modified human hemoglobin improves microcirculatory blood flow and tissue oxygenation after hemorrhagic shock in awake hamsters. Crit Care Med 2003;31:1824-30.
7. Acharya A, Manjula B, Smith P. Albert Einstein College of Medicine of Yeshiva University, ed. Hemoglobin crosslinkers. New York patent 1996; 5:585,484.
8. Winslow RM. Current status of blood substitute research: toward a new paradigm. J Intern Med 2003;253:508-17.
9. Kerger H, Tsai AG, Saltzman DJ, Winslow RM, Intaglietta M. Fluid resuscitation with O2 vs. non-O2 carriers after 2h of hemorrhagic shock in conscious hamsters. Am J Physiol 1997; 272: H525-H37.
10. Burhop KE, Estep TE. Hemoglobin-induced myocardial lesions. Artif Cells Blood Substit Immobil Biotechnol 2001; 29:101.
11. Winslow R. Hemoglobin-based red cell substitutes. Baltimore: Johns Hopkins University Press; 1992.
12. Hess J, Fadare S, Tolentino L, Bangal N, Winslow R. The intravascular persistence of crosslinked human hemoglobin. In: The Red Cell: Seventh Ann Arbor Conference. Brewer G, ed. New York: Alan R. Liss; 1989. p. 351-60.
13. Przybelski RJ, Daily EK, Kisicki JC, Mattia-Goldberg C, Bounds MJ, Colburn WA. Phase I study of the safety and pharmacologic effects of diaspirin cross-linked hemoglobin solution. Crit Care Med 1996;24:1993-2000.
14. Carmichael FJ, Ali AC, Campbell JA, Langlois SF, Biro GP, Willan AR, et al. A phase I study of oxidized raffinose cross-linked human hemoglobin. Crit Care Med 2000;28:2283-92.
15. Savitsky JP, Doczi J, Black J, Arnold JD. A clinical safety trial of stroma-free hemoglobin. Clin Pharmacol Ther 1978;23:73-80.
16. Przybelski R, Kisicki J, Dailey E, Mattia-Goldberg C, Bounds MJ, Colburn WA, et al. Diaspirin cross-linked hemoglobin (DCLHb): Phase I clinical safety assessment in normal healthy volunteers (abstract). Crit Care Med 1994;22:231.
17. Noone RBJ, Mythen MG, Vaslef SN. Effect of αα-cross-linked hemoglobin and pyridoxalated hemoglobin polyoxyethylene conjugate solutions on gastrointestinal regional perfusion in hemorrhagic shock. J Trauma 1998; 45:457-69.
18. Conover CD, Lejeune L, Shum K, Shorr RG. The influence of polyethylene glycol conjugation on bovine hemoglobin's intrinsic effects on the gastrointestinal system of the rat. Life Sci 1996;59:1861-9.
19. Winslow RM. αα-crosslinked hemoglobin: was failure predicted by preclinical testing? Vox Sang 2000;79:1-20.
20. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, et al. Controlled safety study of a hemoglobin-based oxygen carrier, DCLHb, in acute ischemic stroke. Stroke 1999; 30: 993-6.
21. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, et al. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock. A randomized controlled efficacy trial. JAMA 1999; 282:1857-64.
22. Abassi Z, Kotob S, Pieruzzi F, Abouassali M, Keiser HR, Fratantoni JC, et al. Effects of polymerization on the hypertensive action of diaspirin cross- linked hemoglobin in rats. J Lab Clin Med 1997; 129: 603-10.
23. Rattan S, Rosenthal GJ, Chakder S. Human recombinant hemoglobin (rHb1.1) inhibits nonadrenergic noncholinergic (NANC) nerve-mediated relaxation of internal anal sphincter. J Pharmacol Exp Ther 1995;272:1211-6.
24. von Dobschuetz E, Hoffman T, Engelschalk C, Messmer K. Effect of diaspirin cross-linked hemoglobin on normal and postischemic microcirculation of the rat pancreas. Am J Physiol (Gastrointestinal and Liver Physiology). 1999;276:G1507-G14.
25. Moncada S, Palmer R, Higgs E. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol Rev 1991; 43: 109-42.
26. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, et al. Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol 1998;16:672-6.
27. Matheson B, Kwansa H, Bucci E, Rebel A, Koehler R. Appearance of dissociable and cross-linked hemoglobins in the renal hilar lymph. J Lab Clin Med 2000; 135:464.
28. Kuchan M, Frangos J. Shear stress regulates endothelin-1 release via protein kinase C and cGMP in cultured endothelial cells. Am J Physiol 1993;264:H150-H6.