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**SYSTEMIC AND PULMONARY  
HYPERTENSION AFTER RESUSCITATION  
WITH CELL-FREE HEMOGLOBIN**

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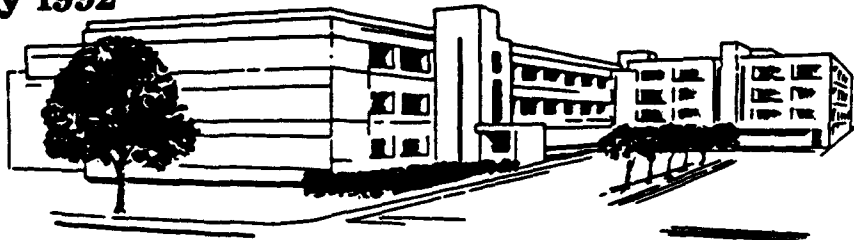
**John R. Hess  
Victor W. Macdonald  
and William W. Brinkley**

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**Blood Research Division  
and Military Trauma Research Division**

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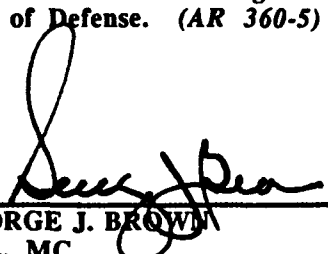
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**GEORGE J. BROWN**  
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blood substitute; bis(3,5-dibromosalicyl)fumarate cross-linked hemoglobin, vasoconstriction, swine, toxicity, renal failure

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## **SYSTEMIC AND PULMONARY HYPERTENSION AFTER RESUSCITATION WITH CELL-FREE HEMOGLOBIN**

**John R. Hess, Victor W. Macdonald, and William W. Brinkley**

The ability of cell-free hemoglobin (Hb) to support mammalian life in the absence of erythrocytes (RBCs) has been demonstrated repeatedly over the last sixty years (32). However, the effects of contamination by RBC stroma and bacterial endotoxin have confounded evaluations of the physiologic effects and toxicity of the Hb molecule itself (7,30). Advances in manufacturing techniques have allowed us to produce solutions of native and chemically modified Hb of high purity, and we have used these solutions to measure the physiologic responses of animals during administration of Hb solution under conditions that mimic their anticipated field use as resuscitation solutions (33).

Renal injury is the most important toxicity observed with the administration of unmodified hemoglobin-saline solutions to humans (22). Hb renal toxicity is more severe in the face of water deprivation (13). Limitation of the renal injury by chemical modification of Hb to prevent tetramer dissociation has been the major approach used to develop less toxic solutions (32). However, persistent uncertainty about the safety of Hb solutions has led to the recommendation that they be tested by large-volume administration to water-deprived, hypotensive animals (3).

This report describes a test of the hypothesis that Hb cross-linked between the  $\alpha$ -chains with bis(3,5-dibromosalicyl)fumarate ( $\alpha\alpha$ Hb) does not cause the acute renal injury associated with administration of unmodified Hb. As a consequence of this test, this report also describes a marked apparent vasoconstrictor response that was observed with all Hb solutions administered in this study. The vasoconstriction is severe in that it markedly raises blood pressure, reduces cardiac output, offsets the potential advantage of the increased blood oxygen content, and was associated with the death of two of the treated animals.

## MATERIALS AND METHODS

**Experimental design.** These experiments were conducted as a randomized four-arm trial of the safety of resuscitation with different solutions in water-deprived hemorrhaged swine. The effects of resuscitation with unmodified human Hb and  $\alpha\alpha$ Hb solutions in volumes equal to the amount of blood removed were compared to resuscitation with human serum albumin (HSA) solution of equal volume and colloid osmotic pressure and resuscitation with three times the shed blood volume of Ringer's lactate (RL) solution. Outcome was determined by comparing hemodynamic function, oxygen transport, and the concentrations of cells, electrolytes and metabolic products.

**Hemoglobin solutions.** All Hb solutions were prepared in Ringer's acetate (Sodium, 148 mEq; Potassium, 3 mEq; Calcium 4mEq; Chloride 127 mEq, Acetate 28 mM). Solutions of stroma-free hemolysate (SFH), high-pressure liquid chromatographically purified adult human Hb (HbA<sub>0</sub>), and  $\alpha\alpha$ Hb were prepared in a sterile Hb production facility according to previously published methods (33). Characteristics of the Hb solutions are given in Table 1. Hb concentration and methemoglobin fraction were measured by modifications of the Drabkin (26) and Evelyn and Malloy (12) techniques respectively. P<sub>50</sub> (P<sub>O<sub>2</sub></sub> at which hemoglobin is half saturated) and the Hill coefficient (n) were calculated from the oxygen dissociation curves measured as previously described (25). Endotoxin was measured using a kinetic turbidimetric modification of the Limulus amoebocyte lysate assay (9). The rabbit pyrogen test was performed according to published standards (24). Inorganic phosphate was measured on a blood chemistry analyzer (Cobas-Fara; Roche Diagnostic Systems, Nutley, NJ) by the method of Daly and Ertingshausen (5).

**Control solutions.** Sterile bottles of 7% (w/v) HSA in Ringer's acetate were prepared in an adjoining hospital pharmacy from commercial salt-poor albumin, concentrated salts, and water for injection. The albumin solutions were used within four hours of preparation.

RL for injection was purchased from approved medical supply houses and opened fresh for each experiment.

**Animals.** Thirty-five 8- to 12-week-old female Yorkshire swine, weighing 8 to 12 kg, were acquired from a commercial specific pathogen-free breeder in groups of 4 to 8 animals. The animals were observed in quarantine for 1 week to document weight gain and then used during the following 6 weeks.

**Randomization.** A random number table was generated to determine the order of allocation of animals into each repetition of the four arms of the study. After each animal had reached 15 kg and had been prepared surgically, it was allocated against the next available assignment. Animals that were removed from the study, either because of instrumentation failure or death, were replaced by the next available animal.

Thirty swine survived the surgical procedures, had functioning catheters, and were hemorrhaged. Of these, one animal died during hemorrhage and never received a resuscitation fluid. A second animal died two hours after resuscitation with  $\alpha\alpha$ Hb and was found at necropsy to have a catheter-related left coronary sinus thrombosis and a large apical transmural myocardial infarction that was several days old, predating the hemorrhage and resuscitation. The remaining twenty-eight swine, seven in each group, are described.

At the time the study began, no HbA<sub>0</sub> was available, and the first animal resuscitated with Hb received SFH. The next four animals assigned to receive Hb received HbA<sub>0</sub> until the limited supply of this material was consumed. The final two animals that were assigned to receive Hb received SFH. In the charts, all the SFH and HbA<sub>0</sub> animals are referred to as the Hb group to be distinct from the  $\alpha\alpha$ Hb group. Clinically significant differences within the Hb group are specifically designated by SFH and HbA<sub>0</sub>.

**Surgical preparation.** The animal preparation is shown in Fig. 1. After an overnight fast, swine were sedated with intramuscular atropine (0.08 mg/kg), ketamine (2.2 mg/kg), and xylazine (2.2 mg/kg).



An endotracheal tube was inserted, and anesthesia was administered as a mixture of isoflurane, nitrous oxide, and oxygen. Sterile surgical technique was used throughout. Through a midline neck incision, a central venous catheter (1 mm ID) was introduced into one internal jugular vein, and a flow-directed Swan-Ganz catheter (Pentacath; Viggo-Spectramed, Oxnard, CA) was inserted under waveform guidance into the pulmonary artery through the other internal jugular vein. A carotid artery catheter (1 mm ID) was placed in one carotid artery. The catheters were brought through the skin on the dorsal surface of the neck, and the incision was closed. Following this procedure, a midline laparotomy was performed, and the spleen was removed. The abdomen was irrigated, and the incision was closed.

Finally, an aortic catheter (2 mm ID) was inserted, and a left renal artery flow probe (6R, Transonic Systems, Inc., Ithaca, NY) was attached through a left retroperitoneal incision and brought through the skin on the dorsal midline. After closure of the retroperitoneal incision, nylon pouches to hold the external ends of the catheters were sewn to the skin, and the catheters were irrigated with heparin saline and capped. Anesthesia was discontinued, the animal was moved to a transfer cage and observed until awake and then returned to the animal care facility.

Dehydration. Four to six days after surgery, when daily body weight gain was established again, swine were transferred to metabolic cages where they lived for the two days before and seven days after hemorrhage and resuscitation. Water was withheld for the two days before hemorrhage, and food was withdrawn 12 hours before hemorrhage. Animals were observed and weighed each day. Daily blood samples were drawn for blood cell counts and plasma chemistries, and daily urine collections were obtained from the floor of the metabolic cage for volume and creatinine concentration.

Hemorrhage. On the day of hemorrhage and resuscitation, the swine was delivered to the laboratory in its metabolic cage. The awake swine was allowed to assume a recumbent position in a restricted portion of the cage, and the catheters and flow probe wires

were removed from their pouches and connected to transducers and recording devices as shown in Fig. 1. When the swine was resting and the recordings of aortic, pulmonary artery, and central venous blood pressure were stable, two sets of baseline hemodynamic measures, blood gases, blood cell counts, and plasma chemistries were obtained fifteen minutes apart. Aortic blood (25 ml/kg) was then withdrawn over the next hour by a syringe pump in four aliquots of 10, 7, 5 and 3 ml/kg in successive 15-minute intervals. Withdrawn blood was stored in CPDA-1 at room temperature for subsequent reinfusion. At the end of each 15-minute interval of logarithmic hemorrhage, hemodynamic measurements and blood samples for blood gases, blood cell counts, and plasma chemistries were obtained.

**Resuscitation.** Five minutes after the end of the hour-long hemorrhage, the resuscitation fluid was administered over 5 to 10 minutes through the central venous line. Hemoglobin and albumin solutions were administered in a volume of 25 ml/kg, Ringer's lactate was given in a volume of 75 ml/kg. Hemodynamic measurements and blood samples for blood gases, blood cell counts, and plasma chemistries were obtained at five time points during the three hours directly following hemorrhage. At the end of that period, the blood which had been withdrawn in the course of the hemorrhage was filtered and returned to the animal, a final set of hemodynamic measurements was made, and blood samples were drawn. Finally, the catheters were flushed with heparin and, along with the flow-probe wires, were placed back in the dorsal pouches, and the animals were given free access to food and water.

**Metabolic follow-up.** The swine spent the next seven days in their metabolic cages, during which time they were weighed, aortic blood was drawn for blood cell counts and plasma chemistries, and urine was collected for volume measurement and urine creatinine determination daily. The swine had free access to food and water at all times during this period.

**Euthanasia.** After seven days, the animals were transferred to the necropsy suite where they were anesthetized with sodium pentobarbital, intubated endotracheally, and maintained on isoflurane

while their right ureter was dissected, cannulated, and two 20-minute urine specimens were collected. The animals were euthanized with an intravascular overdose of sodium pentobarbital.

**Necropsy.** Body tissues and cavities were examined grossly, and tissue abnormalities were recorded. Gross lesions and representative sections of cerebrum, cerebellum, pituitary, eye, heart, lung, liver, stomach, small intestine, pancreas, large intestine, mesenteric lymph node, kidney, adrenal gland, urinary bladder, and uterus were removed and immersed in 10% neutral buffered formalin.

**Histopathology.** After fixation, tissues were prepared for light microscopic examination. Tissue sections (5  $\mu$ m) were cut from paraffin embedded blocks, mounted, and stained with hematoxylin and eosin.

All histopathologic abnormalities observed were recorded, and then all tissues were reviewed and graded for all lesions on a 6-point scale, ranging from absent (0) to severe (5).

**Measurements and calculations.** Systemic, pulmonary artery, and central venous blood pressures were measured using pressure transducers (P23XL, Gould, Cleveland, OH) connected to the aortic, pulmonary artery, and central venous catheters. Transducer outputs were recorded continuously on a strip-chart recorder (2400S; Gould, Cleveland, OH). Heart rate was measured from the pressure recordings. Cardiac output was measured in triplicate by thermodilution with the Swan-Ganz catheter and a cardiac output computer (COM-1; American Edwards Laboratory, Irving, CA). Left-renal artery blood flow was measured from the time-averaged output of the ultrasonic flow probe connected through a flow meter (Model 201, Transonic Systems, Inc., Ithaca, NY). Daily urine output was determined by measuring the filtered 24-hour urine samples collected from the bottom of the metabolic cage with a graduated cylinder. The pre-necropsy, 20-minute right-kidney urine volumes were measured from the output of the right ureteral catheter collected in a graduated cylinder.

Ten milliliters of aortic blood were removed during each of the daily samplings while the swine was in the metabolic cage. One milliliter of blood was placed in a citrated tube and used for cell counting and total Hb concentration determination (System 9000; Baker Instruments, Allentown, PA). One milliliter of blood was placed in 2 ml of ice-cold perchloric acid (70% wt/vol), which was used to determine whole blood lactate in the blood chemistry analyzer. Four milliliters of blood were placed in a heparinized tube and were centrifuged to remove the blood cells. The plasma sodium, potassium, creatinine, BUN, Hb and methemoglobin concentrations were then measured along with determination of the plasma activities for creatine kinase (CK), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) on the blood chemistry analyzer. The final 4 ml of blood was heparinized, centrifuged, and the plasma was frozen for repeated studies as necessary.

On the day of hemorrhage and resuscitation, 12 ml of blood were drawn at each sampling time. Ten milliliters were drawn for the above measurements, and 1 ml of aortic and 1 ml of pulmonary artery blood were drawn into heparinized syringes, which were used to measure pH,  $PCO_2$ , and  $PO_2$  in a blood gas analyzer (Model 170; Corning Medical, Medfield, MA).

Data from the chart recorder and chemistry laboratory reports were entered into a computer spreadsheet (RS1; BBN Software, Boston, MA), derived values were calculated, and manipulation of data for statistical analysis also was performed in programmed routines and defined procedures (using RPL in RS1).

Mean blood pressure ( $BP_{Mean}$ ) was calculated as shown in equation #1 from aortic systolic and diastolic pressures (Torr). A similar calculation was used to determine mean pulmonary artery pressure.

$$BP_{Mean} = BP_{Diastolic} + (BP_{Systolic} - BP_{Diastolic})/3 \quad (1)$$

Systemic and pulmonary vascular resistance (SVR) was calculated as shown in equation #2, in which CO is the cardiac output (l/kg). Pulmonary vascular resistance was calculated by a similar formula.

$$SVR = BP_{\text{Mean}} / CO \quad (2)$$

Stroke volume (SV) was calculated as shown in equation #3, in which HR is the heart rate (beats/min).

$$SV = CO / HR \quad (3)$$

Stroke work (SW) was calculated as shown in equation #4.

$$SW = SV \cdot BP_{\text{Mean}} \quad (4)$$

The arterial and mixed venous oxygen contents ( $Co_2$ ) were calculated as shown in equation #5 as the sum of the oxygen content of the swine RBCs, the plasma Hb or  $\alpha\text{Hb}$  if present, and the plasma, in which ( $So_2$ ) is the fractional saturation of Hb in the compartment, (MCH) is the mean porcine RBC Hb (g/dl), [RBC] is the RBC concentration ( $\cdot 10^9$ /l), [Hb]<sub>p</sub> is the plasma concentration of cell free Hb (g/dl), [Hb<sup>3+</sup>]<sub>p</sub> is the plasma methemoglobin concentration (g/dl), ( $\alpha$ ) is the Bunsen solubility coefficient for oxygen (ml/dl) assumed to be the same for swine plasma and human plasma (.003 ml/Torr) (4),  $PO_2$  is the measured oxygen partial pressure (Torr), and (Hct) is the hematocrit (v/v).

$$Co_2 = 1.36So_2MCH[RBC] + 1.36So_2([Hb]_p - [Hb^{3+}]_p) + \alpha PO_2(1 - Hct) \quad (5)$$

The  $So_2$  was calculated by the Hill equation, equation #6, using oxygen half-saturation pressures ( $P_{50}$ ) and Hill coefficient values (n) for porcine RBCs from the data of Willford and Hill (31) and for administered Hbs from Table 1.

$$So_2 = PO_2^n / (P_{50}^n + PO_2^n) \quad (6)$$

Oxygen delivery was calculated from the Fick equation, equation #7, in which ( $MO_2$ ) is the oxygen delivery to tissue (ml/kg/min), and arterial (A) and mixed venous (V)  $CO_2$  are designated by subscripts.

$$MO_2 = CO(C_{A}O_2 - C_{V}O_2) \quad (7)$$

Creatinine clearance (CCr) was calculated as in equation #8, in which  $[Cr]_{ur}$  is the urine creatinine concentration (mg/dl),  $V_{ur}$  is the urine volume (ml), (t) is the collection period (min), and  $[Cr]_p$  is the plasma creatinine concentration (mg/dl).

$$CCr = [Cr]_{ur} V_{ur} / [Cr]_p t \quad (8)$$

**Statistics.** Differences between treatment groups and changes in group means of measurements over time were analyzed during clinically relevant time intervals (i.e., the periods of water restriction, hemorrhage, the three hours after hemorrhage, and the seven days of metabolic observation). Significant differences were determined by a two-way analysis of variance (procedure ANOVA TWOWAY in RS1; BBN Software Products Corporation, Cambridge, MA) with time and treatment groups as the categorical variables. A value of  $P < 0.05$  was defined as a significant difference throughout the study. The use of the words "increase" or "rise" and "decrease," "decline," or "fall" indicate statistically significant changes.

The correlation of maximum plasma creatinine concentration and urine volume was performed with the standard Pearson technique.

## RESULTS

**Dehydration.** Forty-eight hours of water deprivation induced a 10% loss in swine body mass (Table 2). During this same period, hematocrit increased by 9.5%, plasma sodium concentration rose 10%, and the plasma protein concentration increased by 13.5%. The dehydration induced a slight increase in plasma creatinine but did not alter plasma creatinine clearance.

**Hemorrhage.** With the removal of 25 ml/Kg of blood over one hour, there was an average 35% decrease in systemic blood pressure, pulmonary artery pressure, and cardiac output, accompanied by a 28% increase in heart rate (Table 3). Cardiac stroke volume decreased by half, as did renal artery blood flow. Over the one hour of hemorrhage, Hct and plasma protein concentrations decreased significantly, while plasma creatinine levels rose, along with a six-fold increase in whole blood lactate. Arterial  $PO_2$  increased by 36%, and venous  $PO_2$  decreased by 28%. This was accompanied by a 33% drop in  $PCO_2$ , while arterial pH remained unchanged. There was little variability among animals in the physiologic and biochemical changes induced by timed water deprivation and fixed-volume hemorrhage, and no important differences were observed between treatment groups.

**Resuscitation.** Blood pressures rose to prehemorrhage levels upon resuscitation with RL and HSA, but increased with resuscitation with Hb and  $\alpha$ Hb to levels that were 40 and 20 Torr higher in the systemic and pulmonary circulations and were sustained during the subsequent 3 hours (Fig. 2A and 2B). Cardiac output rose immediately with RL and HSA, but remained depressed with HB and  $\alpha$ Hb (Fig. 2C). Systemic and pulmonary vascular resistances were markedly elevated after resuscitation with Hb and  $\alpha$ Hb compared with the slight decreases observed with RL and HSA (Fig. 2D and 2E), despite the fact that all treatments resulted in the same level of hemodilution as indicated by a uniform Hct decline to  $14.2 \pm 0.5\%$  (Fig. 4A). Heart rate decreased with resuscitation in all groups (Fig. 3A). Stroke volume was initially elevated in the RL and

HSA groups and rose only minimally in the Hb treated groups (Fig. 3B). Stroke work was markedly increased in all groups (Fig. 3C).

Upon resuscitation with all treatment solutions, the animals' hyperventilation ceased. As expected, arterial oxygen content was higher in animals resuscitated with Hb and  $\alpha$ Hb compared with RL and HSA (Fig. 4B), but mixed venous oxygen content was also higher in the hemoglobin groups (Fig. 4C). Calculated oxygen uptake by tissue was the same in all groups (Fig. 4D), and elevated blood lactate concentrations all returned to baseline levels after 3 hours (Fig. 4E). Arterial pH increased from a low of  $7.36 \pm 0.02$  immediately after resuscitation to  $7.50 \pm 0.01$  after 3 hours.

Two deaths occurred among the 28 animals during the three hours of hemodynamic monitoring. One animal, which had received RL, achieved restoration of blood pressure and high cardiac output but died 25 minutes post-resuscitation with an uncorrected lactic acidosis. The second animal, which had received  $\alpha$ Hb, died 25 minutes post-resuscitation with marked systemic and pulmonary hypertension, low cardiac output, pulmonary edema, and arterial hypoxia. All other animals recovered.

At the end of three hours of hemodynamic observation, the blood which had been removed was returned with approximately 63 ml of CPDA-1 anticoagulant that was present in the storage bag. This volume load averaged 435 ml and was associated with an increase in systemic and pulmonary arterial blood pressures in the RL and HSA groups. However, cardiac output remained unchanged in all groups.

Metabolic observation. Plasma hemoglobin concentration decreased exponentially in the Hb group with a half-time of  $4.4 \pm 0.3$  hours and biexponentially in the  $\alpha$ Hb group with an initial half-time of  $4.7 \pm 0.7$  hours and a final half-time of  $13.8 \pm 0.2$  hours (Fig. 5A). Hb was not detected after 24 hours, and  $\alpha$ Hb was not detected after 48 hours.



With the restoration of free access to food and water, body mass and plasma sodium concentrations were restored to baseline levels within 24 hours (data not shown). Animals in all four groups gained an average of 1.9 Kg during the following six days.

Plasma CK and LDH activity were increased in both Hb and  $\alpha$ Hb treated animals in the days immediately after resuscitation (Fig. 5B and 5C). CK activities were increased more than five times above upper limits of normal for one day in the Hb group and two days in the  $\alpha$ Hb group, returning to normal during the following two days in each group. LDH activities were elevated to twice the upper limits of normal for one day in the Hb group and two days in the  $\alpha$ Hb group and thereafter decreased slowly toward normal during the remainder of the week. The RL and HSA groups did not manifest elevations in plasma activities of either enzyme. Plasma ALT activity was normal in all groups throughout the study (Fig. 5D).

Five of the seven animals receiving Hb, including two of the four animals that received HbA<sub>1</sub> and all three that received SFH, developed elevations of plasma creatinine during the three days following resuscitation (Fig. 6A). No elevations of creatinine concentration were seen in any of the other three treatment groups. The same five animals had elevated urine nitrogen concentrations, but the group mean was significantly elevated only on the first day (Fig. 6B). The Hb group exhibited marked diuresis over the three days after resuscitation with collected urine volumes more than twice that in any other group (Fig. 6C). These elevations in plasma creatinine correlated with increased urine volume. The diuresis did not alter the steady gain in body weight (Fig. 6D). Creatinine clearances, calculated either from the daily 24-hour urine collections or from the pre-necropsy right-ureter collections did not vary among the groups (data not shown).

The first animal that had been resuscitated with RL died on the fifth day of the metabolic observation period. Necropsy revealed multiple septic emboli in this animal, and *Staphylococcus aureus* was cultured from its blood. All data from this animal are included in the

study, and thereafter animals in the study received cephalosporin (0.33 g i.v., twice daily).

**Histopathology.** All 28 swine underwent necropsy and histologic evaluation. Several tissues, including kidney and brain, were not examined in the one swine treated with  $\alpha$ Hb that died acutely with pulmonary edema. No histopathologic change was seen more frequently in Hb- or  $\alpha$ Hb-treated animals than in controls.

## DISCUSSION

We set out to measure the toxicity of hemoglobin-based oxygen-carrying resuscitation fluids under the anticipated conditions of their field use. To this end we attempted to create a model of resuscitation from hemorrhagic shock in water-deprived swine which would produce the renal injury associated with administration of unmodified hemoglobin. Furthermore, we attempted to determine if  $\alpha\alpha\text{Hb}$ , a chemically defined example of a modified Hb that is cross-linked to prevent tetramer dissociation into dimers, produced detectable renal injury under these extreme conditions.

Water deprivation led to reproducible dehydration. Deprivation for 48 hours led to a 10% loss of weight, a 10% increase in Hct and plasma sodium, and a 12% increase in plasma protein concentration. The 10% weight loss represented a loss of approximately 15% of the whole body water. The changes in Hct, sodium, and protein indicate significant dehydration of intravascular, interstitial, and cellular water spaces. This dehydration was achieved without overt behavioral changes or clinical neurologic signs. Histopathologic evidence of sodium toxicity was not found in the tissues examined at necropsy (21). Hemorrhage of 25 ml/kg over one hour led to shock. This hemorrhagic shock was marked by a decrease in blood pressure and cardiac output. Despite compensatory tachycardia, hyperventilation, and an increased fraction of transported oxygen utilized, whole blood lactate increased to levels associated with fatality if untreated (28). This shock was associated with decreased renal blood flow and an immediate increase in plasma creatinine. Despite the severity of the hemorrhagic shock, 90% of the animals survived for assessments of toxicity, and there were no persistent metabolic consequences of shock *per se*.

Resuscitation restored intravascular volume in all four groups. The increment in intravascular volume achieved with the resuscitation was initially equivalent in all groups as judged by the dilution of the remaining red cell mass. Judging from the Hct changes, the restored blood volume was slightly in excess of the blood volume before hemorrhage as expected, because the protein solutions

have colloid osmotic activity that is slightly greater than that of swine plasma. The intravascular volume expansion was not maintained fully in the RL group, but this did not cause any untoward effects.

Three toxicities were seen in the hemoglobin-treated groups. The first was a marked hypertensive response to all of the Hb solutions. This was manifest as both systemic and pulmonary hypertension and reduced cardiac output. The second toxicity was marked by the elevations of CK and LDH activity in both groups receiving Hb solutions. The tissue or tissues involved in this injury have not been determined. The third toxicity was an acute renal failure with elevation of plasma creatinine that was seen only in the group which received unmodified Hb. All three toxicities raise concerns over clinical applications of these products.

Unmodified Hb was associated with an acute, high-output renal failure. This injury occurred in half of the animals that were resuscitated with HPLC purified, pyrogen-negative, low-residual-phosphate HbA<sub>0</sub>. Tetramericly stabilized  $\alpha\alpha$ Hb solutions, containing more endotoxin and phosphate contamination, did not cause this injury. These findings are consistent with the higher threshold for renal injury that has been reported with other hemoglobins that have been modified to prevent dissociation (19). The safety of any hemoglobin in the face of a protein-losing glomerulopathy is unknown.

The elevations of plasma CK and LDH activity suggest tissue damage. The histologic analysis of the animals in our study did not reveal a site of active injury, but most of the tissues were collected seven days after the administration of Hb and at least five days after the height of the enzyme activity. Analysis of isoenzymes in the plasma samples from these studies is underway. Several causes of tissue injury have been considered as reasons for the enzyme elevation. White and his colleagues reported that Hb administration in rabbits was associated with brain and myocardial necrosis and a vascular inflammatory lesion marked by heterophil cell paving of the endothelium progressing to arteritis with heterophil invasion of

the muscular layer (29). Hypertension from sustained pressor administration can cause vascular injury (15). Interference with endothelium-derived nitric oxide (NO) can lead to white blood cell adhesion to endothelium and invasion of vessel walls (16). Smith and her colleagues have reported kidney and centrilobular liver injury after  $\alpha$ Hb (23). DeVenuto and his colleagues reported that Hb-associated centrilobular liver injury appears to be related to reduced intravascular volume (6).

From their experience administering Hb/saline solutions to humans, Amberson and his colleagues concluded that Hb is a vasoconstrictor (1). They described a woman who was given 2.3 L of Hb/saline and developed blood pressures 40 Torr above normal before she died with acute renal failure. In all reported human trials of a variety of Hb products, 26 of 55 patients in whom blood pressure was measured developed hypertension (32). In trials of fixed-volume resuscitation in animals, increases in blood pressure greater than those achieved with equal volumes of whole blood have been reported in rats (14), cats (2), and dogs (20).

The hypertension seen in this study was severe, and, despite the 40-Torr rise in mean blood pressure in the Hb-treated animals, cardiac output decreased. Systemic and pulmonary vascular resistances in the Hb-treated animals were more than twice those of the animals which were resuscitated with non-Hb solutions. The increased vascular resistance was probably the immediate cause of death of one of the 14 Hb-treated animals in the study and also of the animal dropped from the study with the myocardial infarction. Moss and his colleagues (18), Hauser and Shoemaker (10), and Vlahakes and his colleagues (27) all reported data showing that exchange transfusions with Hb solutions were associated with decreased cardiac output, while blood pressure remained unchanged or increased.

Increased vascular resistance can be caused by increased viscosity, vascular plugging, or vasoconstriction. Pig blood diluted with Hb or  $\alpha$ Hb has reduced viscosity, and the histology showed no evidence of vascular plugging (11). The increased vascular resistance

is assumed to be due to vasoconstriction. This vasoconstriction may have been related to the well-documented interaction of cell-free Hb and NO (17). Blockade of NO production causes hypertension of this magnitude (8). Hb binds NO as a heme ligand and may be involved in oxidative reactions that remove NO as a biological transducer.

Improved oxygen transport, the anticipated benefit of a Hb-based resuscitation fluid, was not realized in this study. The decrease in cardiac output that was associated with the vasoconstriction in the Hb-treated animals was equal to the increase in oxygen-carrying capacity provided by the Hb. Thus, there was no net gain in oxygen transport, and conventional crystalloid or colloid solutions provided equally rapid correction of the elevated whole blood lactate.

Hb-based RBC substitutes have been under development for more than 50 years, but much of that time has been spent awaiting developments in parallel fields such as protein processing, sterile manufacturing, and vascular biology. Now, with pure and sterile modified Hb solutions available for study and with plausible mechanisms for some of the observed toxicity, a new era of research into mechanisms and therapy of hemoglobinemic disease and blood substitute development is arriving.

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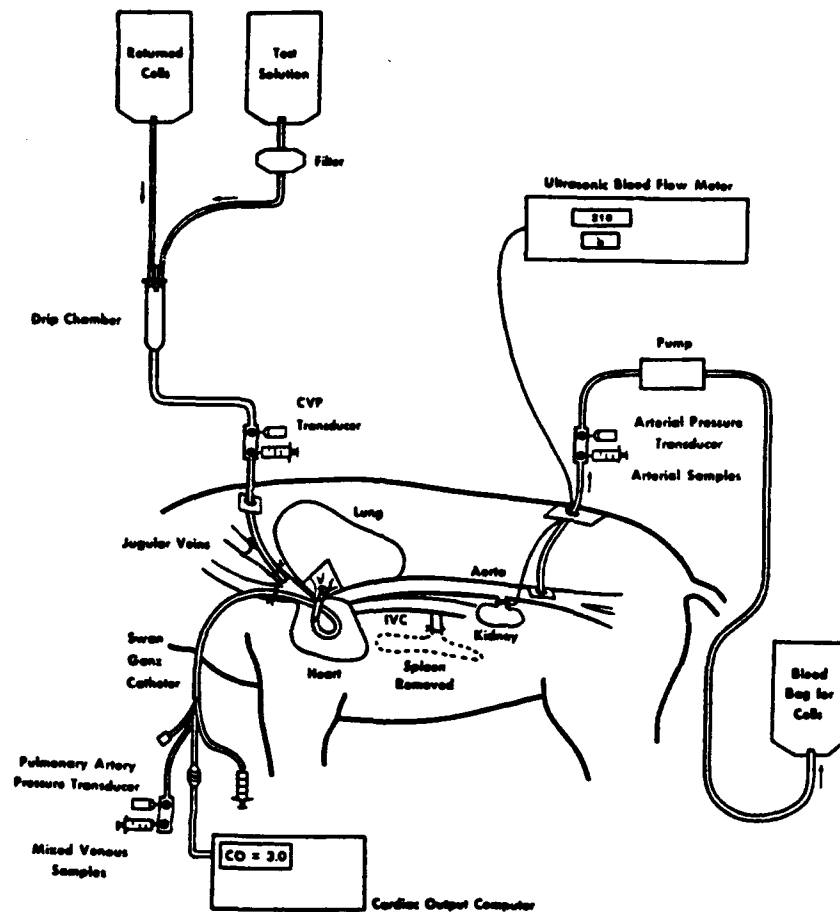


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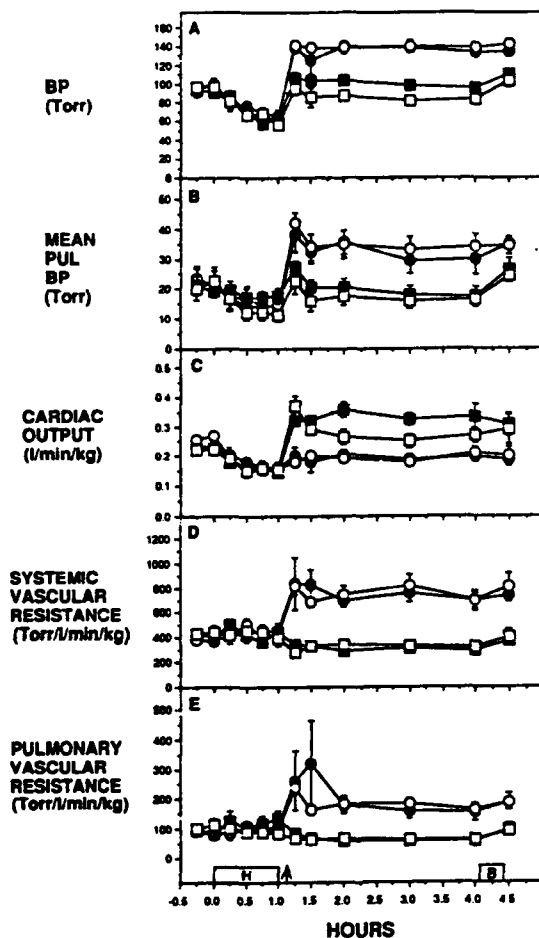
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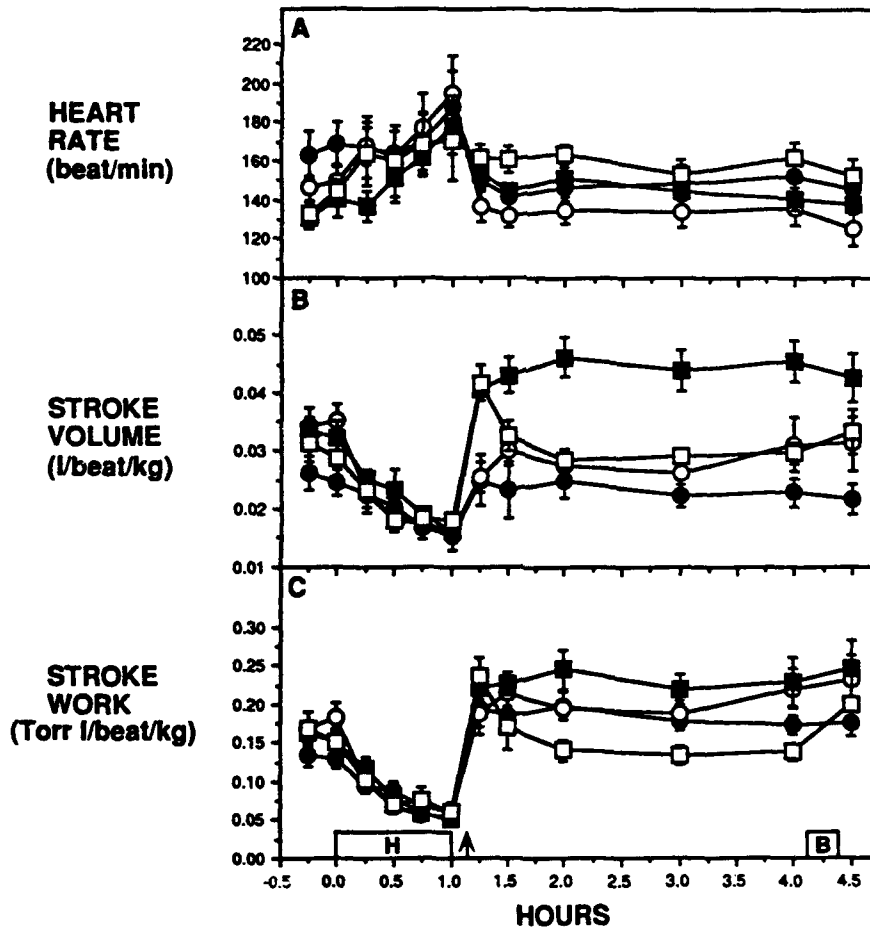
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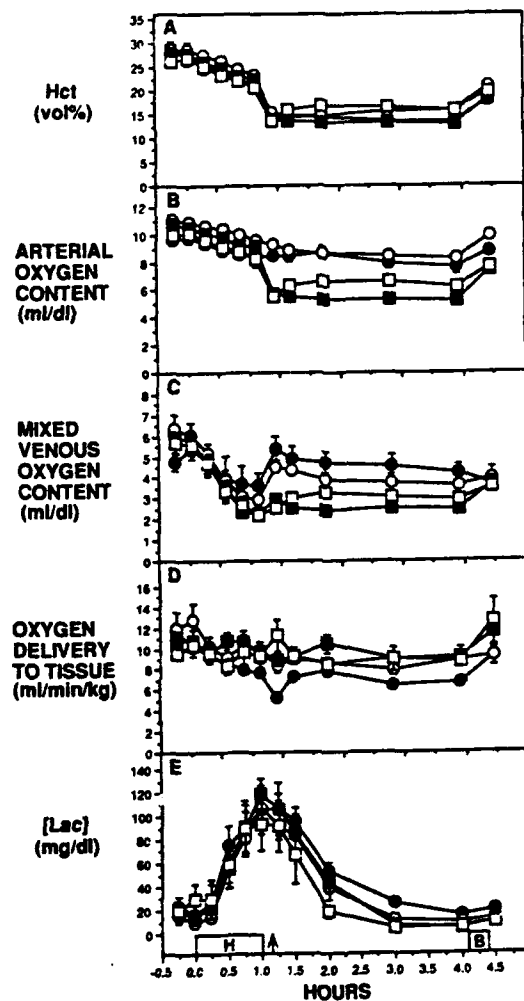
**FIG. 1.** Diagram of the surgical preparation and of catheter and transducer attachment. Swine were splenectomized and instrumented six to eight days prior to hemorrhage and resuscitation. Blood was removed from the aorta with a syringe pump, while resuscitation fluids and shed blood were pumped by hand into the jugular vein. Hemoglobin and blood were infused through appropriate filters.



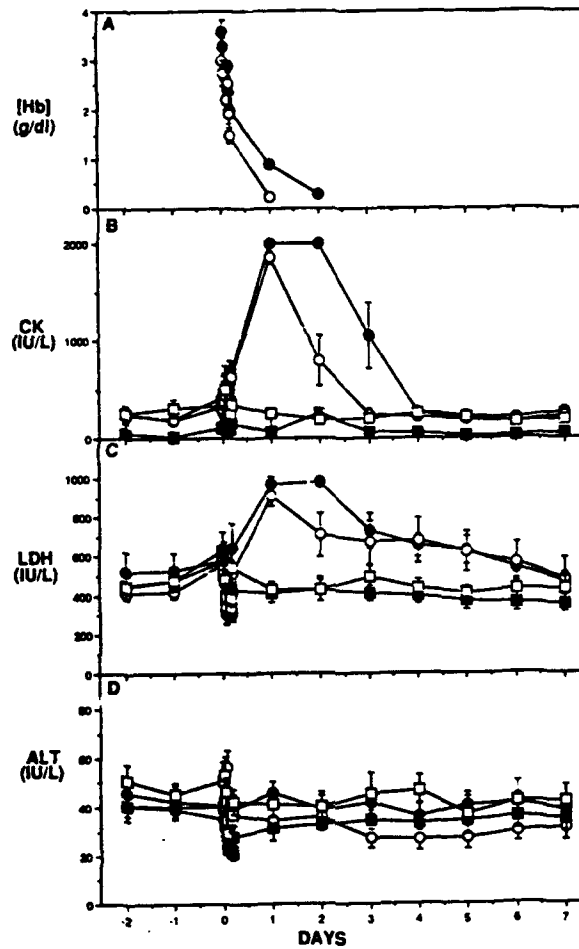
**FIG. 2.** Hemodynamic effects of hemorrhage and resuscitation with Ringer's lactate ( $\square$ ), Human Serum Albumin ( $\blacksquare$ ), unmodified hemoglobin ( $\circ$ ), and  $\alpha\text{Hb}$  ( $\bullet$ ). A: mean arterial blood pressure. B: mean pulmonary blood pressure. C: cardiac output. D: systemic vascular resistance. E: pulmonary vascular resistance. Period of hemorrhage is indicated by the enclosed "H" along the abscissa, resuscitation by the arrow, and reinfusion of the animal's blood by the enclosed "B". Values are means  $\pm$  SEM.



**FIG. 3.** Heart rate effects of hemorrhage and resuscitation with Ringer's lactate ( $\square$ ), Human Serum Albumin ( $\blacksquare$ ), unmodified hemoglobin ( $\circ$ ), and  $\alpha$ Hb ( $\bullet$ ). **A:** heart rate. **B:** stroke volume. **C:** stroke work. Period of hemorrhage is indicated by the enclosed "H" along the abscissa, resuscitation by the arrow, and reinfusion of the animal's blood by the enclosed "B". Values are means  $\pm$  SEM.

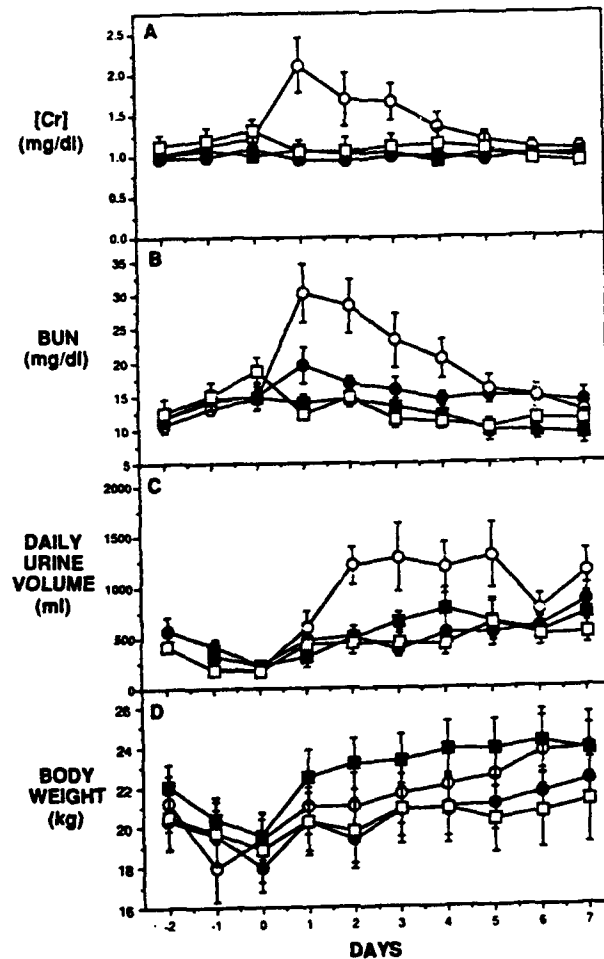


**FIG. 4.** Whole blood oxygen content and delivery in animals hemorrhaged and resuscitated with Ringer's lactate (□), Human Serum Albumin (■), unmodified hemoglobin (○), and  $\alpha$ Hb (●). A: hematocrit. B: arterial oxygen content. C: mixed venous oxygen content. D: oxygen uptake. E: whole blood lactate concentration. Period of hemorrhage is indicated by the enclosed "H" along the abscissa, resuscitation by the arrow, and reinfusion of the animal's blood by the enclosed "B". Values are means  $\pm$  SEM.



**FIG. 5.** Relation of free hemoglobin to plasma activities of cellular enzymes in animals hemorrhaged and resuscitated with Ringer's lactate (□), Human Serum Albumin (■), unmodified hemoglobin (○), and  $\alpha\alpha$ Hb (●). A: plasma hemoglobin concentration. B: creatine kinase activity. C: lactate dehydrogenase activity. D: alanine aminotransferase activity. Treatment was given on day 0. Values are means  $\pm$  SEM.





**FIG. 6. Renal function in animals hemorrhaged and resuscitated with Ringer's lactate (□), Human Serum Albumin (■), unmodified hemoglobin (○), and ααHb (●). A: plasma creatinine concentration. B: plasma urea nitrogen concentration. C: daily urine output. D: body mass. Treatment was given on day 0. Values are means ± SEM.**

Table 1. Characteristics of the Hemoglobin Solutions

Product	$\alpha\alpha\text{Hb}$	$\text{HbA}_0$	SFH
Hemoglobin conc (g/dl)	9.9	8.4	11.4
Methemoglobin (%)	7	1	2
$P_{50}$ (Torr)	29	13	13
Hill Coeff (n)	2.3	2.8	2.8
COP (Torr)	34.5	27.9	41.9
Endotoxin (EU/ml)	1.2	0.1	0.02
Rabbit Pyrogen Test	+	-	-
Inorganic Phosphorus (g/ml)	2.2	1.9	8.4

$P_{50}$ , oxygen pressure at half saturation of hemoglobin at 37 C and pH 7.4

COP, colloid osmotic pressure calculated as  $\text{COP} = 2.8[\text{Hb}] + .02[\text{Hb}]^2 + .005[\text{Hb}]^3$

TABLE 2. Effect of water deprivation of swine

Treatment	n	Body Mass (Kg)	Hct (%)	[Na] <sub>p</sub> (meq/l)	[Protein] <sub>p</sub> (g/dl)	[Cr] <sub>p</sub> (g/dl)	CCr (ml·min <sup>-1</sup> ·Kg <sup>-1</sup> )
Water Replete	28	21.0±0.7	25.3±0.5	141.5±0.9	5.2±0.1	0.97±0.05	1.37±0.11
Water Deprived	28	18.9±0.6	27.7±0.6	155.8±1.3	5.9±0.1	1.14±0.05	1.16±0.09

Values are means±SEM of n animals subjected to 48 hours of water deprivation. Animals were without food for the last 12 hours of the procedure. Hct, hematocrit; [Na]<sub>p</sub>, plasma sodium concentration; [Protein]<sub>p</sub>, plasma protein concentration; [Cr]<sub>p</sub>, plasma creatinine concentration; CCr, creatinine clearance. \*P<0.05.

TABLE 3. Hemodynamic and metabolic changes in swine with hemorrhage

Measure	n	Baseline	n	Hemorrhage
BP <sub>Mean</sub> (Torr)	28	95.4±1.8	28	62.3±3.3*
PAP <sub>Mean</sub> (Torr)	27	21.6±1.8	26	14.3±1.4*
CO (l·min <sup>-1</sup> ·Kg <sup>-1</sup> )	27	0.24±0.01	27	0.15±0.01*
RBF (ml·min <sup>-1</sup> ·Kg <sup>-1</sup> )	25	8.7±6.0	25	4.2±0.6*
HR (beats·min <sup>-1</sup> )	28	143±5	28	183±9*
Hct (%)	28	27.7±0.6	28	21.9±0.5*
[Protein] <sub>p</sub> (g/dl)	28	5.9±0.1	28	5.2±0.1*
[Cr] <sub>p</sub> (g/dl)	28	1.14±0.05	28	1.47±0.07*
[Lac] <sub>wb</sub> (g/dl)	28	18±4	28	106±9*
PaO <sub>2</sub> (Torr)	27	77±2	27	105±3*
PaCO <sub>2</sub> (Torr)	27	42±1	27	28±1*
PvO <sub>2</sub> (Torr)	27	36±1	24	26±1*

Values are means±SEM of n animals before and immediately after hemorrhage (25 ml/Kg over 1 hour). BP, mean aortic pressure; PAP, pulmonary artery pressure; CO, cardiac output; RBF, left renal artery blood flow; HR, heart rate; [Lac]<sub>wb</sub>, whole blood lactate concentration; PaO<sub>2</sub> and PvO<sub>2</sub>, arterial and mixed venous PO<sub>2</sub>, respectively; PaCO<sub>2</sub>, arterial PCO<sub>2</sub>. \*P<0.05.2.

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