Title
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Journal
Journal of Trauma and Acute Care Surgery, 21(11)

ISSN
2163-0755

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Publication Date
1981-11-01

DOI
10.1097/00005373-198111000-00005

0022-5282/81/2111-0938$02.00/0
THE JOURNAL OF TRAUMA
Vol. 21, No. 11
Printed in U.S.A.

Resuscitation with Pyridoxalated Stroma Free Hemoglobin: Tolerance to Sepsis

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The contribution of the reticuloendothelial system (RES) in pyridoxalated stroma-free hemoglobin (SFH-P) clearance may be insignificant. The magnitude of this is not at present clear. Any compromise of RES function would militate against its potential benefit as an oxygen-carrying resuscitation fluid. The relationship between lethal hemorrhagic shock resuscitation with SFH-P and subsequent host depression was examined in a rat model. Host tolerance to a standard intra-abdominal polymicrobial septic challenge was assessed 5 days after hemorrhagic shock. Shock resuscitation with pyridoxalated stroma-free hemoglobin was equal to or better than all other resuscitation groups evaluated. Tolerance to a standard septic challenge 5 days after resuscitation was no different between resuscitation groups. There appears to be no compromise to host defense in general in tolerating intra-abdominal sepsis 5 days following shock resuscitation with pyridoxalated stroma-free hemoglobin.

There is currently an expanded awareness that post-traumatic patients have increased susceptibility to infection due to host defense depression (1, 2, 5, 11, 21). The significance of this relationship is that infection and sepsis with associated multiple organ failure continues to be the major antecedent of post-traumatic mortality (4, 20).

As survival is ultimately dependent on the severity of shock and injuries, early *adequate* fluid resuscitation of trauma patients continues to be the mainstay of early treatment (15, 25). Whether the type of resuscitation fluid has any appreciable effect on host defense during the post-traumatic period is a concern recently raised (3, 9, 16, 22).

If stroma-free hemoglobins, especially chemically modified ones, are to have any advantage over more standard resuscitation fluids, it must not be at the expense of compromising host defense systems. In this study we evaluated the potential for significant host depression following administration of SFH for severe hemorrhagic shock. Animals successfully resuscitated were subsequently challenged with a standard intraperitoneal polymicrobial fecal solution 5 days after the shock insult. The effect on survival was determined.

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Presented at the 40th Annual Session of the American Association for the Surgery of Trauma, Phoenix, Arizona, September 18-20, 1980.
Supported in part by VA Research Service.

MATERIALS AND METHODS

Animals. All experiments reported were performed within a 3-month period using Sprague-Dawley rats (275–350 gm) obtained from a single vendor. They were housed together with continuous access to food and water.

Experimental Design and Groups. Animals were randomly assigned to resuscitation groups the morning of each experiment. They were subjected to lethal hemorrhagic shock and then resuscitated as discussed below. Following infusion, or in the case of controls the end of the hypotensive period, arterial cannulae were removed and the animals returned to their cages for observation. Survivors were subsequently challenged with a standard intraperitoneal injection of human fecal mix and 48-hour survival determined.

All resuscitation fluids were prepared in advance. Resuscitation groups were 1) controls: no resuscitation—NRC ($N = 30$); 2) shed blood—SB ($N = 30$); 3) Ringer's lactate 2½ × shed blood volume—RL.2.5 × SB ($N = 18$); 4) pyridoxalated SFH 1 × shed blood volume—SFH-P 1 × SB ($N = 12$); 5) pyridoxalated SFH 2 × shed blood volume—SFH-P 2 × SB ($N = 13$); 6) pyridoxalated SFH and Ringer's lactate mixed 1:1 × shed blood volume—SFH-P:RL, 1:1 × SB ($N = 12$). Pyridoxalated SFH solution was from a single batch for all experiments. It was prepared in our laboratory by previously described methods (13, 19). Composition data: hemoglobin concentration, 6.6 gm/dL; % methemoglobin, 2.7%; P_{50}, 19.9 torr; total osmolarity, 282 mOsm/L; pH, 7.4; Na+, 129 mEq/L; K+, 4.7 mEq/L. Solutions were stored at 4° C until use.

Hemorrhage Model. Following assignment to a resuscitation group animals were anesthetized with intraperitoneal pentobarbital (4 mg/100 mg body weight). A small caliber (PE-50) polyethylene catheter was inserted into the aorta through the left femoral artery to a point below the renal arteries and above the aortic bifurcation. The amount of catheter needed for accurate placement was previously determined. This cannula was used for withdrawal and infusion of blood and fluids as well as arterial pressure measurements.

Arterial pressure was continuously monitored using a pressure transducer connected to a strip chart recorder. An initial mean arterial pressure of 90 torr was chosen and animals with less than this were excluded from further study.

Each animal was injected with heparin (100 U/kg) and the mean arterial pressure was lowered to and maintained at 30 torr by continuous withdrawal of blood for a 60-minute period. Constant observation of the blood pressure was utilized to guide subsequent withdrawal of blood to maintain this pressure. Following 60 minutes of sustained shock rapid resuscitation was achieved by administering the preselected fluid regimen. Subsequently, animals were monitored for an additional 10 minutes until stabilized, catheters removed, wounds closed, and the animals returned to their cages. Survival at 24 hours was determined and all animals were followed for 5 days. Body weight and total shed blood volume were recorded for each animal. Severity of hemorrhage was calculated for each animal as follows (6)

$$\% \text{ hemorrhage} = \frac{\text{volume shed blood}}{7\% \times \text{total body weight (gm)}}.$$

Septic Challenge Model. Animals surviving 5 days were then challenged by an intraperitoneal injection of human fecal mix known to produce 50% mortality in matched control animals. Some animals surviving hemorrhagic shock for 24 hours post-resuscitation did not undergo subsequent fecal challenge. The majority of these animals suffered catheter-related extremity necrosis requiring sacrifice before inclusion in the septic challenge model. This was no more than two animals per group.

Human fecal mix was prepared with peptone/yeast/glucose broth and 10% barium sulfate as reported by Nichols et al. (18). Individual batches were stored at −60° C for 2 weeks without significant deterioration of viable bacterial counts. Quantitative cultures revealed: *E. coli.*, 10^7; *Strep. fecalis*, $10^{5.2}$; *Bacteroides fragilis*, 10^9; and *Clostridia perfringens*, $10^{8.2}$. Cultures and dose response curves for each batch were checked regularly to monitor and select a dose which was consistently lethal to 50% of control animals.

Survival following injection of septic challenge was determined after 48 hours. Animals injected manifested the equivalent of a clinical sepsis picture. Initially hyperactive, they became progressively quiescent and hypercapnic. After about 24 hours they maintained a fixed stance with extremities widely based and had diffuse piloerection—as if guarding. They neither ate nor drank. At 48 hours they were either dead or had resumed activity. (The numbers of animals successfully resuscitated and subsequently challenged are shown in Table IV.)

Statistical Analysis and Control Maintenance. The two-part model maintained controls differently for each component. For the hemorrhage model control was based on duplicating the experimental situation for each animal as measured by per cent of total blood hemorrhaged as an index of severity of shock (Table I).

In the sepsis model, matched controls were challenged and 48-hour survival was determined concurrently for each group of post-resuscitation animals. Further, fecal dose response was determined to adjust or abandon a fecal batch and assess lethality by volume. Our 'ideal' dose was 0.5 cc associated with a 50% mortality (Table II).

Shock severity index or per cent total blood volume hemorrhage was evaluated by analysis of variance. Severity of hemorrhage within each group was compared for 24-hour survivors versus nonsurvivors as shown in Table I. Resuscitation groups were evaluated against controls and each other for shock survival and sepsis survival using Chi-square analysis.

RESULTS

Controls. Table I shows the severity of hemorrhage for each group. There were no statistical differences between groups, all having approximately 50% of the total blood volume removed. Also intragroup nonsurvivors were no different from survivors when evaluated at 24 hours.

Table II shows the mean dose response to five batches of fecal mix. The mean dose to achieve a 50% survival after 48 hours was 0.6 ± 0.1 ml. Thus there was very little difference in the intraperitoneal volume of the septic challenge even though several batches were used during the course of the experiments.

Hemorrhagic Shock. Twenty-four hour survival data for each hemorrhagic shock resuscitation group are shown in Table III. As previously reported (15) there was

TABLE I
Control of hemorrhage severity

Resuscitation Group	$\bar{X}$ % Hemorrhage		Significance
	Total Group	Non-Survivors	
I Non-resus. control	46 ± 7	46 ± 4	N.S.
II Shed blood	48 ± 5	46 ± 3	N.S.
III R.L. 2.5 × SB	49 ± 6	47 ± 3	N.S.
IV SFH-P 1 × SB	48 ± 5	46 ± 4	N.S.
V SFH-P 2 × SB	50 ± 5	51 ± 3	N.S.
VI SFH-P:RL; 1:1 × SB	47 ± 6	47 ± 2	N.S.

$\bar{X}$ ± SE % total blood volume.

TABLE II
$\bar{X}$ fecal mix—dose response

Dose Range (cc)	$\bar{X}$ ± SE Survival: Lab Controls Overall ($N = 200$)
1) 0.1–0.2	80% ± 8
2) 0.5–0.7	50% ± 10
3) 0.8–1.0	10% ± 10

$N = 5$.

TABLE III
Shock resuscitation: 24-hr % survival*

	No. Deaths/No. Resuscitated	% Survival
I Non-resus. control	25/30	17%
II Shed blood—SB	2/30	94%
III R.L. 2.5 × SB	5/18	73%
IV SFH-P, 1 × SB	4/12	67%
V SFH-P, 2 × SB	3/13	77%
VI SFH-P:RL; 1:1 × SB	1/12	93%

* Portion of larger experiment data (15).

a significant difference between all resuscitating fluids and nonresuscitated controls ($p < 0.01$). Ringer's lactate and SFH at one and two times shed blood volume were equally effective as shed blood. Ringer's lactate was not statistically different from SFH-P at one and two times shed blood volume. SFH-P and Ringer's lactate in equal volumes to shed blood gave better protection than Ringer's lactate alone at 2½ times the shed blood volume ($p < 0.05$). SFH-P and lactated Ringer's 1:1 was not significantly better than shed blood, but statistically equivalent.

Sepsis Tolerance. There were no significant differences between groups regarding loss of animals from subsequent septic challenge. There were insufficient nonresuscitated controls ($N = 5$) who underwent subsequent sepsis to allow statistical analysis. In each of the other groups at least ten animals were available for intraperitoneal challenge 5 days post shock resuscitation.

As seen in Table IV all groups had a survival pattern which was not statistically different from control (52% survival). There is a suggestion that SFH-P when given either as twice the volume of shed blood or in tandem with equivalent volumes of Ringer's lactate enhances survival. The small number of animals in each group does not hold this as a statistically valid conclusion, however. There appears to be no significant depression of host defense mechanisms detectable 5 days after hemorrhagic shock resuscitation for any resuscitation regimen evaluated herein. In this regard SFH does not appear to produce any major problems.

DISCUSSION

Successful resuscitation of the traumatized patient and ultimately survival are at least dependent on the severity

TABLE IV
5-day post-shock resuscitation: Sepsis survival

	No. Deaths—48 hrs Post-sepsis / No. Alive 5 Days Post-resuscitation	% Survival
I Nonshocked controls	29/56	52%
II Shed blood	5/10	50%
III R.L. 2.5 × SB	7/12	42%
IV SFH-P, 1 × SB	4/8	50%
V SFH-P 2 × SB	3/10	70%
VI SFH-P:RL; 1:1 × SB	3/10	70%

of injury, time course between injury and resuscitation, severity of shock, number of associated injuries, and concomitant bacterial contamination. The need to provide adequate volume resuscitation of all fluid compartments is well demonstrated for experimental and clinical fluid resuscitation regimens (7, 15, 25).

The common denominator in massive injury is maintenance of host integrity and defense mechanisms of various forms: barrier integrity, critical serum opsonins or mediators (among them fibronectin), cellular immunity, and ongoing cellular metabolism. Many specific aspects of host defense 'well being' have been evaluated and manipulated in an attempt to affect a change in ultimate outcome.

Of recent interest is the awareness that initial resuscitation efforts can potentially affect host defense. Newer information has raised the issue of risk/benefit ratio for initial resuscitation with albumin-containing fluids (8, 14), dextran (16), and SFH solutions (22). Although it is generally appreciated that replacement and maintenance of intravascular fluid are critical, there may be risks as yet undefined. With an increased appreciation of the role of opsonins and serum or cell mediators in the host defense mechanism process each blood component has been examined, implicated, or absolved of immediate detrimental effects (3, 7). Transfusion practice has changed based on these observations.

This setting forces a reconsideration of the potential toxicity of hemoglobin solutions or other oxygen-carrying blood substitutes in a more clinically oriented model (10).

The early toxicities of SFH, coagulopathy and nephrotoxicity, have been completely eliminated or resolved through the use of improved manufacturing techniques. Any potential benefit of increased oxygen delivery associated with hemoglobin solutions could possibly be at the expense of the 'RES' or host defense depression. This would detract from the benefits attributable to SFH. In the model presented here, this mechanism does not appear to be operative. A closer look at individual RES defense components is necessary to precisely define the situation.

Not knowing the specifics of interaction between SFH and the various components of the RES, we implemented a more general model using survival as an end point. Our

animal model of hemorrhagic shock with subsequent septic challenge more closely mirrors the clinical situation. Should a resuscitation fluid impair the host defense mechanism, we would expect to see an increased mortality with the septic challenge. It is possible that the RES is significantly overwhelmed or blocked by the resuscitation fluid in the immediate post-resuscitation period. Our model did not detect this because we challenged the animals 5 days following shock and resuscitation. A septic challenge in an earlier time frame may have produced different results.

Focusing on an earlier time frame of potential host depression, Schneidkraut and Koegering showed that hemoglobin solutions did not affect subsequent tolerance to an endotoxin when compared to hemolyzed blood and sheep blood (22). Their study speaks against a generalized blockade induced by SFH as measured by standard RES function tests. They, in fact, explain their observations by offering the hepatocyte as the normal metabolic site for SFH solution. Thus one would not expect depression of the RES. By labeling the globin moeity rather than the heme, Seki has recently shown that hepatocytes preferentially metabolize hemoglobin, but are functionally dependent on haptoglobin (23, 24). He could cause RES appearance of label by exceeding the binding capacity of haptoglobin. This suggests that some RES metabolism of SFH is real, but these studies did not measure RES depression.

De Venuto (9) in an extensive review addressed the question of pharmacokinetics and created a three-compartment excretion model based on data from the literature. This model fails to address the potential influence of injury in altering clearance kinetics as a function of RES capacity. They have recently demonstrated, experimentally, reversal of hepatic necrosis associated with shock and SFH resuscitation by increasing the vascular volume and repeated dosing with the SFH solutions (10). This is in agreement with our shock resuscitation data from the standpoint of volume alone. They have not explored the RES system.

The renal contribution to the metabolism of hemoglobin solutions normally or in shock is not well understood. In severe gradations of shock, however, clearance appears to be a function of renal blood flow and subsequent filtration fraction, both of which are decreased (12). If indeed the renal clearance mechanism is impaired, the RES may be overly burdened and the potential for blockade increased. However, the data of Schneidkraut and the present study do not support this concept. This must remain an unanswered question until the role of the RES and its component subsystems in hemoglobin clearance and metabolism are defined.

The problem addressed here is not a new one for synthetic bloods. The National Research Council in 1963 established criteria which in retrospect were prophetic (17). These compounds were to neither alter hemostasis or agglutination of any type, nor be metabolized in such a way as to cause delayed function or impaired metabolism, sepsis tolerance, or general cardiopulmonary renal physiology changes. These are still valid criteria today, and indeed SFH in its current form meets or exceeds these 'ideal' criteria.

From our data we can draw only one reasonable conclusion. There is no difference in survival to a standard septic challenge intraperitoneally at 5 days following resuscitation from significant hemorrhagic shock with pyridoxalated SFH. From the standpoint that this parallels the normal time frame in which infection appears clinically, this is a reasonable argument.

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DISCUSSION

DR. CHARLES L. RICE (Michael Reese Hospital and Medical Center, Chicago, IL 60616): Thank you, Mr. Chairman. As with any product, two areas need to be addressed before a product can be advocated for use in the clinical situation. One is the efficacy and the other is the safety of the product.

Doctor Greenburg and his coworkers in San Diego have shown, as have we in Chicago, that stroma-free hemoglobin, especially in the pyridoxalated form, does function as a respiratory protein. It is as yet unclear how well it functions in a mixed population of red cells and hemoglobin, since the amount of off-loading from the stroma-free hemoglobin in the presence of red cells remains uncertain.

The other issue is that of safety and the authors have addressed themselves in this study to the possibility of a depression of host response.

The question of renal toxicity remains open. The question of whether or not stroma-free hemoglobin is itself immunogenic has been answered in the negative, so the reticuloendothelial system and the host defense in general issue has been raised.

It was first raised by Doctor Rabner in the 1950's when he showed that hemoglobin solution was cleared by the reticuloendothelial system.

As Doctor Hoyt mentioned, this model was chosen to represent the clinical situation. I have several questions for the authors.

First of all, survival from peritonitis is not purely a function of the reticuloendothelial system. This study, it seems to me, needs a little additional information. First, some substance known to impair the reticuloendothelial system, such as either carbon or latex, to test the sensitivity of their intraperitoneal injection of feces for reticuloendothelial dysfunction.

Second, an infection with some alternative method must be to infect the animal with a more reticuloendothelial dependent model, such as the infusion of intravenous *Bartonella* or *Salmonella.* Patients with sickle cell disease are known to have impaired reticuloendothelial systems because of their prolonged hemolysis, and they are definitely more susceptible to *Salmonella.*

My last question is: based on the numbers given in the abstract, it seems to me, Doctor Hoyt, that your probability of having detected a difference, if one was present, was only about 45%. I wonder if you have any plans to repeat this experiment with a larger number of animals?

Thank you for the privilege of discussing the paper.

DR. DAVID B. HOYT (Closing): I will address the last comment first and simply say that, yes, we have expanded the numbers in each resuscitation group. I agree that the numbers presented are small, but I don't think the trend will change. Even at this point each group is in excess of ten animals, with the exception of two, and all have matched controls of another ten animals each.

Doctor Rice raises two other issues. First, what is the contribution of the kidneys and RES to the metabolic management of the hemoglobin solutions. It is very confusing to interpret the literature. One doesn't know how each investigator's quality control, during manufacture, differs. Therefore many studies are not comparable regarding toxicity. It is clear that if free of stromal elements renal toxicity is insignificant.

As for the RES involvement, there is a very nice study by Seki from the Japanese literature in the last year, where, using a radiolabel technique, they labeled the globin moiety rather than the heme group with radiolabeled iron. Using this, they were then able to sequentially dose rats and evaluate the relative participation of the RES or lack thereof. As they increased hemoglobin concentration, they could show that after haptoglobin was saturated, the RES became involved, presumably through phagocytosis of hemoglobin. It is not too surprising if you're talking about the RES, that you may have to invoke a humoral mediator, at least that seems to be the pattern with many other interactions. For hemoglobin, the mediator is haptoglobin. Whether adjuvant haptoglobin should be used with these preparations is something that would be just speculating.

Finally, Doctor Rice questions whether this study says anything specifically about RES blockade. Perhaps this raises the validity of the abdominal septic challenge as a test of RES function.

We've shown in another study, using a nonspecific intraperitoneal stimulator not too different from the Simmons group at Minnesota, that intraperitoneal containment can correlate with mortality and containment is defined simply as an inflammatory response which traps bacteria. This can be correlated with peripheral appearance of viable organisms and ultimately mortality. Perhaps RES is too specific for what we are attempting to assess. What we really are implying is that host defense in general is assessed with this model. First, local containment and later, handling of disseminated infection. Certainly the RES plays an active role in both of these.

I don't know if this answers the questions, but I hope it suggests some of the rationale behind our experimental design.