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Resuscitation with Recombinant Hemoglobin rHb2.0 in a Rodent Model of Hemorrhagic Shock

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Background: **Hemoglobin solutions combine volume effect, oxygen-carrying capacity, and vasoactive properties, the latter facilitating restoration of global hemodynamics but endangering microvascular resuscitation. Hemoglobin-evoked vasoconstriction probably is due to nitric oxide scavenging, which can be reduced by genetic modifications of the heme pocket. This study compares resuscitation with a nonhemoglobin colloid and two recombinant hemoglobin solutions with wild-type and reduced nitric oxide–scavenging capacity.**

Methods: **Twenty-seven awake Syrian golden hamsters fitted with dorsal skinfold chambers underwent a 30 min-hemorrhagic shock (mean arterial pressure [MAP] 30–35 mmHg) and resuscitation with shed blood volume of either 6% dextran 60 (Biophausia, Uppsala, Sweden), recombinant hemoglobin 1.1 (rHb1.1; wild-type nitric oxide–scavenging capacity; 10 g/dl), or recombinant hemoglobin 2.0 (rHb2.0; reduced nitric oxide–scavenging capacity; 10 g/dl; both Baxter Healthcare, Boulder, CO). Macrohemodynamic and laboratory parameters were assessed; microvascular parameters in the skinfold chamber were analyzed by intravital microscopy.**

Results: **Hemorrhagic shock reduced functional capillary density (FCD) by 70% and caused significant metabolic acidosis. Colloid resuscitation led to incomplete recovery of MAP and FCD. Infusion of rHb1.1 completely restored MAP but not FCD, with the smallest arteriolar diameters found in this group. FCD was restored best by resuscitation with rHb2.0, although MAP was lower than in rHb1.1-treated animals. Metabolic acidosis was resolved by both hemoglobin solutions, but not by dextran.**

Conclusion: **After resuscitation with rHb1.1, arteriolar vasoconstriction quickly restored MAP, but this was achieved at the expense of FCD. In contrast, after resuscitation with rHb2.0, the recovery of MAP could be translated into a significantly improved FCD.**

THE primary objective of resuscitation from hemorrhagic shock is the reconstitution of blood volume, cardiac output, and nutritional organ perfusion.[1] Reestablishment of microvascular perfusion is particularly important because it is crucial for long-term survival.[2] Resuscitation from severe hemorrhagic shock using crystalloid or colloidal solutions means transforming hypovolemia into a normovolemic dilutional anemia. This allows for enhanced oxygen delivery by increasing cardiac output. But as the second determinant of oxygen delivery, *i.e.*, the erythrocyte mass, remains unchanged, the potential of volume resuscitation *per se* necessarily is limited. Beyond a certain threshold, blood transfusion would be the therapy of choice, but is usually not available for primary resuscitation.

Actually, two different concepts of artificial oxygen carriers are investigated for avoiding anemia from becoming the limiting factor: Synthetic perflubron emulsions with their linear oxygen binding kinetics are designed to increase arterial oxygen content and have been shown to improve oxygen availability in the tissue after hemorrhagic shock.[3] Hemoglobin-based oxygen carriers (HBOCs) are designed to provide volume effect and supplemental oxygen-carrying capacity at the same time. Experiences gathered so far with first-generation HBOCs were prosperous, but they still reveal important side effects such as arteriolar vasoconstriction, which has the potential to offset the benefit of additional oxygen-carrying capacity. On the one hand, increased systemic vascular resistance may help to effectively restore mean arterial pressure (MAP) after shock and resuscitation, but on the other hand, this can limit cardiac output and global oxygen delivery.[4] Arteriolar vasoconstriction is considered to be one of the key factors for hypertension and the remaining deficits of capillary perfusion after resuscitation with first-generation HBOCs.[5] Several studies have shown that permissive hypotension in the situation of uncontrolled bleeding yields better outcome and survival,[6] whereas hypertension elicited by hemoglobin solutions may increase blood losses in trauma patients.

Among the potential mechanisms responsible for vasoactivity, nitric oxide scavenging by the infused hemoglobin molecules seems to be the most important factor.[7] Nitric oxide scavenging by free hemoglobin is thought to be even more powerful if extravasated across the endothelial barrier.[8] Neither intramolecular chemical modifications nor the polymerization of the molecules could completely eliminate the vasoactive side effects of first-generation HBOCs.[9] In contrast, recombinant technology allows for a site-directed genetic modification of the heme pocket and was used for developing hemoglobin molecules with a drastically reduced rate of nitric oxide scavenging. Using these second-generation hemoglobin solutions, a direct relation between the rate of nitric oxide scavenging and the pressure response could be shown in a rat model of 50% blood exchange.[7] In a pig model of uncontrolled hemorrhage, Malhotra *et al.*[10] observed improved cardiac output as well as increased oxygen delivery after resuscitation with rHb2.0 as compared with the first-generation hemoglobin solution Di-

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Table 1. Characteristics of Dextran 60, rHb1.1, and rHb2.0

	Dex	rHb1.1	rHb2.0
Hb, g/dl	—	10	10
MW, Da	60.000	64.000	Polymerized
COP, mmHg	39	42	63–68
Viscosity, cP	2.8	1.9	2.1–2.4
P_{50}, mmHg	—	29–32	31–33
Hill coefficient	—	2.2–2.5	1.1
$k'_{NO,OX}$, $\mu M^{-1} \cdot s^{-1}$	—	60	2.5

COP = colloid osmotic pressure; Dex = 6% dextran 60; Hb = hemoglobin; $k'_{NO,OX}$ = rate of reaction between oxyhemoglobin and nitric oxide; MW = molecular weight; P_{50} = oxygen tension at 50% hemoglobin saturation; rHb = recombinant hemoglobin.

aspirin Crosslinked Hemoglobin (DCLHb; Baxter Healthcare, Boulder, CO).

The aim of this study was to compare the effects of the second-generation hemoglobin solution rHb2.0 (reduced nitric oxide–scavenging capacity) with those of the first-generation hemoglobin solution rHb1.1 (wild-type nitric oxide–scavenging capacity) in dorsal skinfold chamber fitted Syrian golden hamsters after resuscitation from severe hemorrhagic shock, with particular regard to the microcirculatory situation.

Materials and Methods

Test Solutions

Both hemoglobin solutions used in this study were provided by Baxter Healthcare; 6% dextran 60 was purchased from Biophausia (Uppsala, Sweden). rHb1.1 and rHb2.0 are hemoglobin solutions expressed in recombinant *Escherichia coli*.[11] For higher stability after administration, both molecules feature genetically fused α-chains that increase half-life *in vivo* by preventing dissociation into dimers. rHb1.1 is an unpolymerized pseudotetramer with wild-type nitric oxide–scavenging capacity, which is bearing the naturally occurring Presbyterian mutation for reducing the high oxygen affinity of extraerythrocytic hemoglobin. rHb2.0 is polyethylene glycol polymerized and, due to its genetically modified distal heme pocket, has a 20- to 30-fold reduced nitric oxide–scavenging capacity as compared with rHb1.1 (table 1; according to the product sheets and Burhop and Doyle[12]).

Animal Model

The experimental protocol conforms to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* and was approved by the Ethical Governmental Committee of Bavaria. Experiments were performed in Syrian golden hamsters of 50–70 g body weight (Charles River, Sulzfeld, Germany). Animals were kept in acclimated rooms with free access to water and pellet food. For investigation of microcirculation in the striated skin muscle, dorsal skinfold chambers were implanted 48–72 h before experiments.[13] At least 24 h before experimentation, polyethylene catheters were inserted into the carotid artery and jugular vein for induction and maintenance of shock, for resuscitation, and for monitoring and measurements. Both procedures were performed during ketamine–xylazine anesthesia (130 and 20 mg/kg body weight intraperitoneal, respectively).

Experimental Protocol

For optimal accessibility of the dorsal skinfold chamber preparation, the animals were placed in perforated acrylic tubes, which allowed for undisturbed breathing of room air during experiment. Hemorrhagic shock was induced by withdrawal of blood from the arterial catheter at a rate of 0.4 ml/min, until a MAP of 30–35 mmHg was reached. During the following 30 min, this blood pressure was maintained by further withdrawal or reinjection of blood, as necessary. At the end of shock, animals were randomly assigned to one of the experimental groups (n = 9), and shed blood volume was replaced by the same volume of dextran, rHb1.1, or rHb2.0 at a rate of 0.2 ml/min using the venous catheter. Measurements were performed under baseline conditions; at the end of shock; and at 15, 30, and 60 min after volume replacement.

Macrohemodynamic Measurements and Blood Sample Analysis

Mean arterial pressure was continuously monitored *via* the fluid-filled carotid catheter connected to a pressure transducer (DTX Plus; Becton Dickinson GmbH, Heidelberg, Germany). The signal was amplified (Hugo Sachs, March, Germany) and processed on-line (Datalog GmbH, Mönchenglattbach, Germany); heart rate was derived from the pressure tracings. Hematocrit and hemoglobin content, arterial blood gases, lactate, and base excess were analyzed in 90-μl blood samples (Chiron 860; Chiron Diagnostics GmbH, Fernwald, Germany); the withdrawn sample volume was replaced by the same volume of 0.9% saline solution.

Microcirculatory Analysis

The dorsal skinfold chamber comprises striated muscle as well as soft tissue and thus may be representative for the musculoskeletal system as a whole. The model was successfully used to elucidate numerous physiologic and pathologic microcirculatory phenomena including the vascular response to hemorrhage and resuscitation. Currently, the model is one of the most frequently used for the investigation of HBOC effects on the microcirculation.

Before baseline measurements, in each animal's skinfold chamber, approximately four arteriolar (8–60 μm) and six venular vessel segments (15–60 μm) and six capillary fields were selected for observation. This was

realized by means of a modified Archoplan epi-illumination microscope equipped with a 20×/0.5-W water immersion objective (Zeiss, Oberkochen, Germany) and a computer-controlled, stepping motor–driven x–y table for precisely repeatable measurements in the preselected areas. Plasma was stained with 150.000-kd fluorescein isothiocyanate–dextran 0.5% (15 mg/kg body weight; Sigma Chemicals, Taufkirchen, Germany) for (1) determination of arteriolar and venular vessel diameters (μm); (2) venular erythrocyte velocity (mm/s); (3) endothelial integrity assessed as the quotient of fluorescence in (f_i) and directly next to (f_e) the observed vessel segment; and (4) functional capillary density (FCD; cm/cm^2), defined as the total length of erythrocyte-perfused capillaries per area.

Functional capillary density has previously been described as a reliable indicator for tissue perfusion.[14] Being mainly dependent on the perfusion pressure over the capillary bed,[15] a number of other factors, such as viscosity[16] and ischemia–reperfusion injury[17] (*e.g.*, leukocyte plugging, endothelial cell swelling), have been described as determinants of FCD.

Rhodamine 6G 0.05% (15 mg/kg body weight; Sigma Chemicals) was used as fluorescent dye for *in vivo* staining of leukocytes. Leukocyte–endothelium interaction was analyzed for 30 s and classified as (1) rolling leukocytes identified as cells moving significantly slower than centerline velocity and (2) adherent leukocytes that were not moving for 30 s. By means of adequate light sources, sets of optical filters, and an integrated charge-coupled device camera (FK6990 IQ-S; Pieper, Schwerte, Germany), images were displayed on a monitor and recorded on videotape.

Blinded off-line analysis was performed later on using a computer-assisted microcirculatory analysis system (CapImage 6.01; Zeintl, Heidelberg, Germany).[18]

Statistical Analysis

A nonnormal distribution of the data was assumed. A Kruskal-Wallis H statistic followed by the Student-Newman-Keuls test was used to check for significant intergroup differences (Jandel SigmaStat for Windows 2.0; Erkath, Germany). All values are given as median (25th/75th quartiles). $P < 0.05$ (two-tailed) was considered significant.

Results

Macrohemodynamic Measurements and Blood Sample Analysis

Under baseline conditions, all parameters were in the normal range physiologically encountered in hamsters. Before and during hemorrhagic shock, none of the investigated parameters revealed significant intergroup differences (table 2).

Mean arterial pressure before hemorrhage was 90 (85/92) mmHg. For induction and maintenance of hemorrhagic shock at a target MAP between 30 and 35 mmHg, in the average a blood volume of 42 ml/kg body weight was withdrawn, corresponding to an estimated 60% of the hamster's total blood volume.

After resuscitation with 100% of shed blood volume, MAP reached the highest values in the rHb1.1 group with 87 (83/90) mmHg, whereas rHb2.0-treated animals stabilized at 74 (72/81) mmHg and dextran-resuscitated animals recovered to 61 (59/72) mmHg with no further tendency to increase (table 2). Both hemoglobin groups had significantly higher MAP than the dextran group at all time points after resuscitation. At the same time, difference of MAP between the rHb1.1 and rHb2.0 groups was found to be significant (fig. 1).

As compared with baseline, heart rate was reduced in all animals during the entire shock period. It was restored to baseline values in either of the recombinant hemoglobin groups, whereas significantly lower values were found in the dextran group for the first 30 min after resuscitation, indicating a delayed recovery of heart rate.

Because of the fluid shift evoked by hemorrhage, all animals' mean hematocrit and hemoglobin concentrations decreased from 46 (45/48)% and 15 (15/16) g/dl under baseline conditions to 26 (25/29)% and 9.1 (8.6/9.7) g/dl at the end of shock. Volume replacement decreased hematocrit further on to approximately 14% in all groups, whereas hemoglobin concentration stabilized at 9.1 (8.8/9.6) g/dl in the rHb1.1 group and at 8.0 (7.8/8.4) g/dl in the rHb2.0 group. Only in the dextran group did it decrease further on to approximately 4.6 (4.3/5.4) g/dl.

Arterial blood gas analysis revealed a pronounced compensatory hyperventilation during shock in all animals, which significantly increased arterial carbon dioxide tension ($Paco_2$) from 73 (71/83) to 135 (127/138) mmHg and decreased $Paco_2$ from 39 (38/41) to 30 (28/37) mmHg. Despite this, significant metabolic acidosis developed as reflected by a marked increase of lactate to 12.6 (10.4/14.8) mM and a base excess of −13.1 (−16.6/−11.1) mM just before resuscitation. Resuscitation with both hemoglobin solutions allowed for fast recovery of lactate level and base excess, reaching baseline values at the end of the experiments. In contrast, after resuscitation with dextran, metabolic parameters showed a tendency toward normalization, but remained clearly elevated as compared with baseline even 60 min after resuscitation, with a lactate of 5.8 (4.2/6.3) mM and a base excess of −3.4 (−5.0/−0.1) mM (fig. 2). Both recombinant hemoglobin solutions reversed shock induced hyperventilation, although $Paco_2$ remained slightly elevated at 89 (80/91) mmHg in the rHb1.1 group and 98 (89/113)

Table 2. Hemodynamic, Respiratory, and Metabolic Parameters (n = 9)

	Baseline	Shock	15 min	30 min	60 min
Mean arterial pressure, mmHg					
Dex	85 (85/92)	34 (33/35)	61 (59/73)	64 (59/69)	64 (62/70)
rHb1.1	94 (92/95)	33 (32/35)	87 (83/90)*	89 (87/94)*	94 (85/97)*
rHb2.0	90 (85/91)	34 (31/35)	74 (72/81)*†	76 (73/77)*†	77 (73/79)*†
Heart rate, beats/min					
Dex	390 (368/423)	240 (213/303)	340 (315/353)	340 (310/363)	340 (330/398)
rHb1.1	410 (378/423)	260 (228/333)	390 (368/403)*	390 (348/415)*	390 (360/433)
rHb2.0	400 (390/425)	240 (238/305)	400 (353/410)*	410 (368/433)*	400 (375/435)
Hematocrit, %					
Dex	46 (45/49)	26 (25/29)	12 (12/15)	13 (13/15)	15 (14/16)
rHb1.1	46 (45/48)	26 (24/28)	13 (12/14)	13 (12/14)	14 (13/16)
rHb2.0	46 (45/47)	28 (26/29)	13 (13/14)	14 (12/15)	14 (12/15)
Hemoglobin concentration, g/dl					
Dex	15.6 (15.2/16.4)	8.9 (8.6/10.0)	4.6 (4.3/5.4)	4.9 (4.5/5.4)	5.2 (5.0/5.8)
rHb1.1	15.7 (15.4/15.9)	9.1 (8.4/9.6)	9.1 (8.8/9.6)*	9.2 (8.9/9.5)*	9.4 (9.1/10.0)*
rHb2.0	15.4 (15.1/15.8)	9.1 (8.9/9.8)	8.0 (7.8/8.4)*†	8.2 (7.8/8.3)*†	8.1 (7.7/8.4)*†
Pa_{O_2}, mmHg					
Dex	75 (72/81)	129 (124/137)	124 (115/128)	113 (109/128)	121 (116/128)
rHb1.1	71 (70/75)	134 (129/140)	87 (83/115)*	88 (80/94)*	89 (80/91)*
rHb2.0	80 (72/87)	136 (127/139)	103 (97/112)*	98 (91/109)*†	98 (89/113)*†
Pa_{CO_2}, mmHg					
Dex	41 (38/44)	31 (28/37)	37 (35/44)	39 (32/47)	37 (30/44)
rHb1.1	40 (34/41)	29 (29/37)	44 (40/47)	44 (41/45)	41 (38/42)
rHb2.0	39 (38/41)	28 (23/34)	43 (41/45)	42 (41/45)	42 (39/44)
pH					
Dex	7.44 (7.43/7.46)	7.23 (7.19/7.28)	7.28 (7.24/7.29)	7.33 (7.30/7.35)	7.39 (7.37/7.41)
rHb1.1	7.47 (7.44/7.50)	7.25 (7.20/7.28)	7.33 (7.29/7.39)*	7.39 (7.36/7.42)*	7.46 (7.42/7.47)*
rHb2.0	7.45 (7.43/7.47)	7.29 (7.23/7.39)	7.36 (7.34/7.38)*	7.40 (7.38/7.42)*	7.42 (7.41/7.46)*
Base excess, mM					
Dex	3.5 (1.5/5.0)	−14.6 (−17.0/−10.5)	−9.2 (−11.7/−4.7)	−7.2 (−10.1/−2.6)	−3.4 (−5.0/−0.1)
rHb1.1	3.5 (2.1/4.1)	−14.2 (−17.1/−11.1)	−3.0 (−7.2/2.0)*	1.1 (−3.0/3.4)*	−2.6 (1.0/6.8)*
rHb2.0	2.7 (1.8/4.3)	−12.2 (−15.4/−10.7)	−2.1 (−3.7/−0.8)*	0.6 (−0.5/1.5)*	2.5 (1.7/3.8)*
Lactate, mM					
Dex	1.4 (1.2/1.8)	12.8 (10.9/15.1)	8.3 (5.8/11.3)	7.1 (3.6/9.3)	5.8 (4.2/6.3)
rHb1.1	1.7 (1.4/1.9)	12.7 (11.8/16.8)	3.4 (2.1/6.7)*	1.2 (0.9/3.7)*	0.9 (0.7/2.0)*
rHb2.0	1.4 (1.3/1.5)	11.4 (9.1/13.5)	4.4 (2.8/5.6)*	2.3 (1.6/3.4)*	1.2 (1.0/2.2)*

Data are presented as median (1st/3rd quartiles).

* $P < 0.05$ *vs.* Dex. † $P < 0.05$, rHb2.0 *vs.* rHb1.1.

Dex = 6% dextran 60; Pa_{CO_2} = arterial carbon dioxide tension; Pa_{O_2} = arterial oxygen tension; rHb = recombinant hemoglobin.

mmHg in the rHb2.0 group 60 min after resuscitation. With dextran resuscitation, hyperventilation was not resolved, as reflected by decreased Pa_{CO_2} and significantly increased Pa_{CO_2} around 120 mmHg, persisting during the whole postshock observation time.

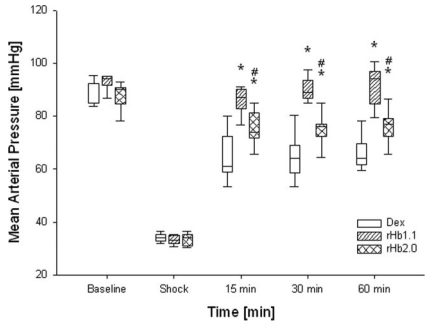

Fig. 1. Mean arterial blood pressure (n = 9). Data are presented as median (1st/3rd quartiles) and 5th/95th percentiles. Dex = dextran 60; rHb = recombinant hemoglobin. * $P < 0.05$ *versus* Dex. # $P < 0.05$, rHb2.0 *versus* rHb1.1.

Microcirculatory Analysis

In hemorrhagic shock, the diameter changes of the observed arteriolar and venular vessel segments remained in the baseline range (table 3). Mean venular erythrocyte velocity was decreased from 0.45 (0.38/0.68) mm/s to 0.04 (0.02/0.08) mm/s by hemorrhage. At

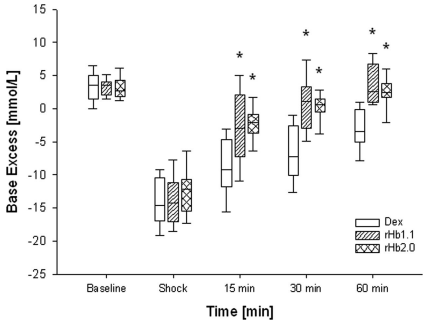

Fig. 2. Base excess (n = 9). Data are presented as median (1st/3rd quartiles) and 5th/95th percentiles. Dex = dextran 60; rHb = recombinant hemoglobin. * $P < 0.05$ *versus* Dex.

Table 3. Microcirculatory Parameters (n = 9)

	Baseline	Shock	15 min	30 min	60 min
Venular diameter, μm					
Dex	24 (22/30)	21 (18/26)	25 (19/27)	26 (20/27)	25 (20/25)
rHb1.1	24 (22/29)	21 (18/22)	23 (21/29)	26 (22/31)	24 (23/33)
rHb2.0	25 (22/26)	20 (16/26)	25 (23/29)	26 (23/29)	25 (23/28)
Venular blood flow, nl/s					
Dex	0.16 (0.12/0.24)	0.01 (0.00/0.04)	0.07 (0.03/0.22)	0.10 (0.03/0.23)	0.08 (0.03/0.18)
rHb1.1	0.16 (0.15/0.27)	0.01 (0.00/0.02)	0.26 (0.14/0.35)	0.30 (0.17/0.43)*	0.26 (0.17/0.36)
rHb2.0	0.24 (0.09/0.26)	0.02 (0.01/0.03)	0.19 (0.13/0.25)	0.21 (0.15/0.24)	0.17 (0.16/0.23)
Venular rolling leukocytes, n/min					
Dex	8.3 (4.7/9.4)	NA	3.3 (1.7/17.1)	4.7 (1.3/16.3)	5.3 (2.1/11.5)
rHb1.1	6.0 (3.9/8.8)	NA	3.0 (2.5/8.1)	5.0 (4.0/9.7)	5.7 (2.7/10.8)
rHb2.0	6.3 (5.1/8.1)	NA	5.0 (3.6/9.4)	7.3 (4.9/10.3)	9.7 (4.8/12.5)
Venular adherent leukocytes, n/mm^2					
Dex	35.2 (14.1/59.7)	NA	24.6 (11.8/83.1)	16.2 (8.4/28.8)	13.9 (0.0/18.1)
rHb1.1	22.8 (11.7/49.7)	NA	22.9 (0.0/63.0)	7.9 (0.0/89.5)	46.0 (13.7/92.9)*
rHb2.0	24.9 (13.4/38.9)	NA	61.7 (31.9/91.4)	71.2 (18.9/84.8)	64.8 (20.2/111.8)*
Arteriolar diameter, μm					
Dex	34 (29/36)	34 (32/35)	38 (35/40)	41 (37/42)	40 (37/42)
rHb1.1	33 (26/36)	32 (27/42)	34 (30/40)	34 (30/38)	32 (29/34)*
rHb2.0	30 (23/31)	33 (27/35)	40 (34/43)	36 (34/43)	38 (34/45)†
Arteriolar leakage, f_i/f_e					
Dex	0.35 (0.30/0.43)	0.36 (0.32/0.44)	0.33 (0.28/0.38)	0.29 (0.27/0.35)	0.28 (0.25/0.33)
rHb1.1	0.38 (0.35/0.43)	0.41 (0.36/0.46)	0.38 (0.33/0.42)	0.36 (0.34/0.42)*	0.38 (0.34/0.40)*
rHb2.0	0.33 (0.30/0.34)	0.36 (0.31/0.39)	0.30 (0.28/0.32)	0.29 (0.28/0.34)†	0.31 (0.31/0.33)†
Functional capillary density, cm/cm^2					
Dex	187 (176/201)	28 (12/83)	111 (53/140)	126 (69/157)	108 (64/167)
rHb1.1	187 (166/200)	19 (11/46)	78 (66/113)	84 (72/105)	113 (77/125)
rHb2.0	184 (180/206)	28 (12/58)	145 (123/163)*†	160 (127/174)*†	154 (130/168)*†

Data are presented as median (1st/3rd quartiles).

* $P < 0.05$ *vs.* Dex. † $P < 0.05$, rHb2.0 *vs.* rHb1.1.

Dex = 6% dextran 60; NA = not applicable; rHb = recombinant hemoglobin.

30 and 60 min after resuscitation, venular blood cell velocity in both recombinant hemoglobin groups was significantly higher than in the dextran group. At the same time, arteriolar vasoconstriction was found in the rHb1.1 group, whereas it was absent in both of the other groups.

No significant differences were found in the number of rolling leukocytes measured after resuscitation among the three groups. Leukocyte sticking after shock was found to be significantly reduced in the dextran group 30 and 60 min after resuscitation (fig. 3).

Endothelial integrity as determined by 150.000-kd fluorescein isothiocyanate–dextran extravasation remained unchanged during shock, but as compared with both other groups, it was significantly higher for the rHb1.1-treated animals at 30 and 60 min after shock.

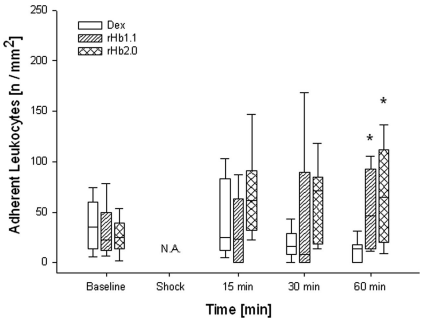

Fig. 3. Adherent leukocytes (n = 9). Data are presented as median (1st/3rd quartiles) and 5th/95th percentiles. Dex = dextran 60; NA = not applicable; rHb = recombinant hemoglobin. * $P < 0.05$ between groups.

Baseline FCD was 187 (178/201) cm/cm^2. During shock, it was measured as 27 (12/64) cm/cm^2, corresponding to a reduction of more than 85%. Compared with baseline, resuscitation with dextran resulted in a reperfusion value of 108 (64/167) cm/cm^2, which amounted to 58% of the observed capillaries. In the rHb1.1 group, capillary perfusion recovered to only 113 (77/125) cm/cm^2, which corresponds to 60% of the baseline value. In contrast, in the rHb2.0 group, reperfusion reached 154 (130/168) cm/cm^2 or more than 84% of baseline at 60 min after resuscitation, thereby being significantly superior to both other groups (fig. 4).

Discussion

The major finding of this study is that the hemoglobin solution with reduced nitric oxide–scavenging capacity allows for a more complete restoration of microvascular perfusion as compared with a colloid or a first-generation hemoglobin solution. Even though vasoactivity of rHb1.1 resulted in a better restoration of macrohemody-

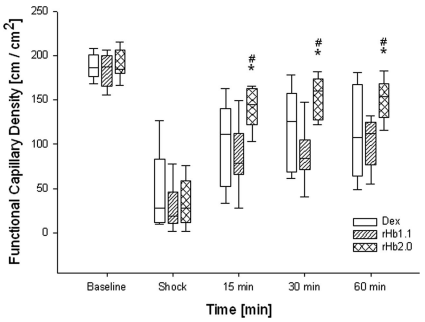

Fig. 4. Functional capillary density (n = 9). Data are presented as median (1st/3rd quartiles) and 5th/95th percentiles. Dex = dextran 60; rHb = recombinant hemoglobin. *$P < 0.05$ between groups. $P < 0.05$, rHb2.0 *versus* rHb1.1.

namic parameters, this was not paralleled by a congruent improvement of the microcirculatory situation.

After trauma and hemorrhagic shock, three peaks of mortality can be identified.[19] Whereas the first reflects immediate death due to injury of the heart, aorta, or central nervous system, a few hours later a second peak of mortality can be ascribed to the underlying patterns of injuries such as hematothorax, pelvic fracture, or abdominal trauma. In the following days, a third rise in mortality is related to the occurrence of acute respiratory distress syndrome, multiple organ dysfunction syndrome, and sepsis.

Primary resuscitation is targeted on the control of bleeding, normalization of blood volume, and the restoration of global hemodynamic parameters. To prevent secondary complications such as delayed organ failure, resuscitation has to reestablish a sufficient microcirculation providing adequate oxygen supply and allowing for a washout of hypoxic metabolites. Clinically, successful resuscitation is commonly judged by global metabolic parameters, as are base excess and lactate. On the level of microcirculation, in a model similar to ours, FCD was found to be predictive for successful resuscitation from hemorrhagic shock.[20]

In preclinical testing, most first-generation HBOCs like DCLHb and rHb1.1 effectively restored global hemodynamic and metabolic parameters that are clinically accessible.[5,21–25] Thereupon, clinical trials were initiated, among them multicenter trials in patients with severe traumatic hemorrhagic shock in the United States[26] and Europe.[27] However, because one of these trials[26] revealed an unexpected high mortality in DCLHb-treated trauma patients, further testing had to be terminated prematurely. Besides methodologic problems that arose from this study,[28,29] hemodynamic side effects were considered to be harmful to the compromised patient potentially due to detrimental effects on organ perfusion.[28,30] Furthermore, in the clinical setting of uncontrolled bleeding, it has been described that the rapid normalization of blood pressure, *e.g.*, by HBOC vasoactivity, may enlarge blood loss.[31] For those reasons, second-generation HBOCs were designed to be void of vasoactivity.

After resuscitation with rHb2.0, in our experiments MAP was significantly lower and arteriolar diameters were significantly larger than in the rHb1.1 group. This is in agreement with other studies, in which no hypertensive response was observed[7,32,33] and vascular resistance was found to be lowered upon resuscitation with rHb2.0.[33] As demonstrated by Malhotra *et al.*,[10] the absence of vasoconstriction after resuscitation with rHb2.0 allows for an improved restoration of cardiac output as compared with resuscitation with DCLHb. After colloid resuscitation, vascular resistance is not increased, but dilutional anemia may endanger the recovery of cardiac performance after shock.[21] This was reflected by the insufficient restoration of heart rate and MAP in the dextran group of the current study. Therefore, in both hemoglobin groups, the additional oxygen transport capacity may have supported cardiac performance, but only the absence of vasoconstriction in the rHb2.0 group allowed for the improved recovery of cardiac output.

Both lactate and negative base excess reflect oxygen debt during hemorrhagic shock and are reliable predictors for outcome after resuscitation.[34] Values of base excess of −11.8 mM in patients during shock correspond to a 50% mortality[35]; we attained base excess values even lower than that. In a similar model, it has previously been shown that mortality would reach 100% if shock were extended to 60 min.[5]

Both rHb2.0 and rHb1.1 have been found to be superior in restoring base excess and lactate to baseline values as compared with the colloid control. Similar results were obtained previously with other first-generation hemoglobin solutions.[22,23,36] Therefore, global oxygen delivery even in the rHb1.1 group despite reduced capillary perfusion presumably was sufficient, probably because facilitated oxygen diffusion by plasma dissolved hemoglobin has contributed to tissue oxygenation.

Functional capillary density in the rHb2.0 group was significantly higher than in the rHb1.1 group, but values of base excess were identical at any time. Performing resuscitation from hemorrhagic shock with rHb1.1 in a rat model, Loeb *et al.* [24] demonstrated that some organs are particularly endangered by increased resistance, namely the skeletal muscle, liver, gastrointestinal organs, and brain, whereas blood flow in other organs was found to be increased. In this context, the decrease of FCD observed in the rHb1.1 group may represent so-called occult hypoperfusion resulting in a "hidden" tissue acidosis which would not be paralleled by acidemia.[37]

The incomplete recovery of FCD in the dextran and rHb1.1 groups probably had different reasons. With the larger arteriolar diameters found after colloid resuscitation, FCD was curtailed by the low MAP, whereas in the rHb1.1-resuscitated animals, arteriolar diameters were significantly lower and thus precapillary resistance was

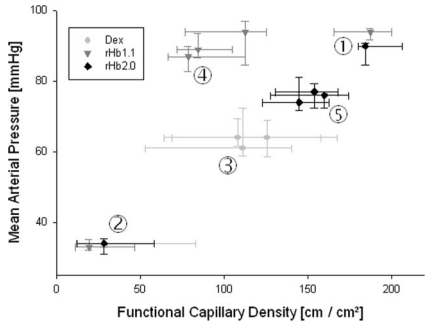

Fig. 5. Functional capillary density as a function of the mean arterial pressure. For *1* and *2*, each mean value represents one group (n = 9); for *3*, *4*, and *5*, each mean value represents one postshock time point (15, 30, and 60 min). *1* = Baseline, all groups; *2* = shock, all groups; *3* = dextran 60 (Dex) resuscitation; *4* = recombinant hemoglobin (rHb) 1.1 resuscitation; *5* = rHb2.0 resuscitation. Note that *1*, *2*, *3*, and *5* seem to follow a linear trend, whereas in data set *4* (rHb1.1 resuscitation), the increased mean arterial pressure is not paralleled by an increased functional capillary density. Data are presented as median (1st/3rd quartiles).

increased. The effects observed after resuscitation with dextran and rHb1.1 are in accord with the findings of previous studies. In a similar shock model, Botzlar *et al.*[25] and Nolte *et al.*[5] reported an inadequate restoration of MAP accompanied by compromised capillary perfusion after resuscitation with dextran. In these studies, the investigated first-generation hemoglobin solutions UPBHb[25] and DCLHb[5] allowed for a more effective restoration of MAP, but no superiority could be shown for the restitution of capillary perfusion.

In the current study, both hemoglobin solutions restored MAP significantly better than dextran. But whereas rHb1.1 resuscitation evoked vasoconstriction, arteriolar diameters in the rHb2.0 group were the same as in the dextran group. Apparently, this is why in the rHb2.0 group the higher MAP could be translated into an increased FCD, whereas in the rHb1.1 group vasoconstriction may have curtailed capillary perfusion (fig. 5).

Furthermore, endothelial cell dysfunction and perivascular edema as observed after application of first-generation hemoglobin solutions may evoke increased capillary resistance, inhomogeneous blood flow distribution, and deficits of capillary perfusion. This may be another explanation why we found no correlation between reversal of base excess/lactate and FCD.

Severe hemorrhagic shock induces endothelial cell dysfunction, which increases microvascular permeability and may aggravate hypovolemia by additional fluid losses into the interstitial space. Moreover, the inhibition of nitric oxide synthesis by N^G-nitro-L-arginine methylester as well as $\alpha\alpha$-crosslinked hemoglobin have been shown to induce endothelial cell contraction, thereby promoting endothelial leakiness.[38] Accordingly, resuscitation with nitric oxide scavenging HBOC causes hemoglobin-induced hemoglobin extravasation.[39] This may result in blood volume contraction as demonstrated for $\alpha\alpha$-crosslinked hemoglobin after hemodilution and resuscitation from hemorrhagic shock.[40,41] In the current study, vascular leakage was increased only in the rHb1.1 group; extravasation of hemoglobin into the vessel wall may aggravate nitric oxide scavenging–induced vasoconstriction, which has been demonstrated for rHb1.1 by model analysis.[42] This mechanism can be mitigated by polymerization of the hemoglobin molecule.[9] rHb2.0 is polymerized and has a low nitric oxide–scavenging capacity. Therefore, this compound should not induce endothelial cell contraction and vascular leakiness, and Kavdia *et al.*[42] have demonstrated by model analysis that, even in case of extravasation of unpolymerized rHb2.0, this should not lower the nitric oxide concentration in the vessel wall below the activation range of guanylate cyclase.

The higher erythrocyte velocities in both recombinant hemoglobin groups presumably are related to the higher MAP and FCD of these groups. The slightly (not significantly) increased erythrocyte velocity in the rHb1.1 group as compared with the rHb2.0 group may be explained by (1) increased arteriovenous shunting and (2) shunting of the blood into the adjacent soft tissue, which also may be drained by the venules observed in the striated muscle.

Previously, in the case of extreme hemodilution, a positive correlation has been described for plasma viscosity and capillary perfusion.[43] In our experiments, postshock capillary perfusion was found to be the lowest in the rHb1.1 group (1.9 cP) and the dextran group (2.8 cP), whereas it was significantly higher in the rHb2.0 group (2.1–2.4 cP), *i.e.*, no correlation between viscosity and FCD could be observed. The differences in viscosity were small and were diminished by adding the solutions to the circulating blood volume. Furthermore, because of the differences in macrohemodynamic parameters as well as oxygen transport and nitric oxide–scavenging capacities, no final conclusion on the effect of viscosity can be drawn from our study.

The amount of recombinant hemoglobin administered for resuscitation from hemorrhagic shock was the same in both groups. Nevertheless, lower hemoglobin concentrations were measured in the rHb2.0 group after resuscitation, possibly indicating an increased fluid shift due to the higher colloid osmotic pressure of rHb2.0. However, it is uncertain whether the difference in hematocrit is responsible for the increased MAP in the rHb1.1 group because a dose-dependent increase of the vascular resistance seems to exist only for rHb1.1 and not for rHb2.0.[44]

The model of the dorsal skinfold chamber in the Syrian golden hamster is frequently used for studies in intact microcirculation and in unanesthetized animals, especially in the context of hemodilution, ischemia–reperfusion injury, and hemorrhagic shock. Because the investigated tissue is striated musculature, the microcirculatory situation

observed in the skinfold chamber is likely to be representative for a large tissue mass in the organism. However, as in many other rodent models, access to some major cardiovascular parameters (*e.g.*, left ventricular function, cardiac output or systemic vascular resistance) is difficult, and blood sampling (*e.g.*, for additional viscosity measurements) is limited because of the small size of the animals.

In conclusion, the current study shows that resuscitation from severe hemorrhagic shock with rHb2.0 was more effective than resuscitation with the first-generation rHb1.1 or the colloidal control dextran. Although MAP did not reach the level of the rHb1.1 group and recovery of metabolic parameters was comparable, rHb2.0 resuscitation yielded the best microcirculatory recovery. This may mainly be ascribed to the reduced nitric oxide–scavenging capacity because the lack of vasoactivity seems to allow for a better translation from macrohemodynamic into microcirculatory recovery.

References

1. Messmer K, Kreimeier U: Microcirculatory therapy in shock. Resuscitation 1989; 18 (suppl):51–61
2. Heckbert SR, Vedder NB, Hoffman W, Winn RK, Hudson LD, Jurkovich GJ, Copass MK, Harlan JM, Rice CL, Maier RV: Outcome after hemorrhagic shock in trauma patients. J Trauma 1998; 45:545–9
3. Paxian M, Rensing H, Geckeis K, Bauer I, Kubulus D, Spahn DR, Bauer M: Perflubron emulsion in prolonged hemorrhagic shock: Influence on hepatocellular energy metabolism and oxygen-dependent gene expression. ANESTHESIOLOGY 2003; 98:1391–9
4. Hess JR, MacDonald VW, Brinkley WW: Systemic and pulmonary hypertension after resuscitation with cell-free hemoglobin. J Appl Physiol 1993; 74: 1769–78
5. Nolte D, Botzlar A, Pickelmann S, Bouskela E, Messmer K: Effects of diaspirin-cross-linked hemoglobin (DCLHb) on the microcirculation of striated skin muscle in the hamster: A study on safety and toxicity. J Lab Clin Med 1997; 130:314–27
6. Revell M, Greaves I, Porter K: Endpoints for fluid resuscitation in hemorrhagic shock. J Trauma 2003; 54:63–7
7. Doherty DH, Doyle MP, Curry SR, Vali RJ, Fattor TJ, Olson JS, Lemon DD: Rate of reaction with nitric oxide determines the hypertensive effect of cell-free hemoglobin. Nat Biotechnol 1998; 16:672–6
8. Alayash AI: Hemoglobin-based blood substitutes: Oxygen carriers, pressor agents, or oxidants? Nat Biotechnol 1999; 17:545–9
9. Doyle MP, Apostol I, Kerwin BA: Glutaraldehyde modification of recombinant human hemoglobin alters its hemodynamic properties. J Biol Chem 1999; 274:2583–91
10. Malhotra AK, Kelly ME, Miller PR, Hartman JC, Fabian TC, Proctor KG: Resuscitation with a novel hemoglobin-based oxygen carrier in a swine model of uncontrolled perioperative hemorrhage. J Trauma 2003; 54:915–24
11. Looker D, Abbott-Brown D, Cozart P, Durfee S, Hoffman S, Mathews AJ, Miller-Roehrich J, Shoemaker S, Trimble S, Fermi G: A human recombinant haemoglobin designed for use as a blood substitute. Nature 1992; 19:356:258–60
12. Burhop KE, Doyle MP: The development and preclinical testing of a second-generation recombinant hemoglobin solution, rHb2.0 for injection, Microcirculatory Effects of Hemoglobin Solutions. Progress in Applied Microcirculation. Vol 25. Edited by Messmer K, Burhop KE, Hutter J. Basel, Karger, 2004, pp 48–64
13. Endrich B, Asaishi K, Götz A, Messmer K: Technical report- a new chamber technique for microvascular studies in unanesthetized hamsters. Res Exp Med (Berl) 1980; 177:125–34
14. Nolte D, Zeintl H, Steinbauer M, Pickelmann S, Messmer K: Functional capillary density: An indicator of tissue perfusion? Int J Microcirc Clin Exp 1995; 15:244–9
15. Lindbom L, Arfors KE: Mechanisms and site of control for variation in the number of perfused capillaries in skeletal muscle. Int J Microcirc Clin Exp 1985; 4:19–30
16. Messmer K, Lewis DH, Sunder-Plassmann L, Klövekorn WP, Mendler N, Holper K: Acute normovolemic hemodilution: Changes of central hemodynamics and microcirculatory flow in skeletal muscle. Eur Surg Res 1972; 4:55–70
17. Menger MD, Steiner D, Messmer K: Microvascular ischemia-reperfusion injury in striated muscle: Significance of "no reflow." Am J Physiol 1992; 263: H1892–900
18. Klyscz T, Jünger M, Jung F, Zeintl H: Cap Image-ein neuartiges computerunterstütztes Videobildanalysesystem für die dynamische Kapillarmikroskopie. Biomed Tech (Berl) 1997; 42:168–75
19. Baker CC, Oppenheimer L, Stephens B, Lewis FR, Trunkey DD: Epidemiology of trauma deaths. Am J Surg 1980; 140:144–50
20. Kerger H, Waschke KF, Ackern KV, Tsai AG, Intaglietta M: Systemic and microcirculatory effects of autologous whole blood resuscitation in severe hemorrhagic shock. Am J Physiol 1999; 276:H2035–43
21. Meisner FG, Kemming GI, Habler OP, Kleen MS, Tillmanns JH, Hutter JW, Bottino DA, Thein E, Meier JM, Wojtczyk CJ, Pape A, Messmer K: Diaspirin crosslinked hemoglobin enables extreme hemodilution beyond the critical hematocrit. Crit Care Med 2001; 29:829–38
22. Siegel JH, Fabian M, Smith JA, Costantino D: Use of recombinant hemoglobin solution in reversing lethal hemorrhagic hypovolemic oxygen debt shock. J Trauma 1997; 42:199–212
23. Schultz SC, Hamilton IN Jr, Malcolm DS: Use of base deficit to compare resuscitation with lactated Ringer's solution, Haemaccel, whole blood, and diaspirin cross-linked hemoglobin following hemorrhage in rats. J Trauma 1993; 35:619–25
24. Loeb AL, McIntosh LJ, Raj NR, Longnecker DE: Resuscitation after hemorrhage using recombinant human hemoglobin (rHb1.1) in rats: Effects on nitric oxide and prostanoid systems. Crit Care Med 1998; 26:1071–80
25. Botzlar A, Nolte D, Messmer K: Effects of ultra-purified polymerized bovine hemoglobin on the microcirculation of striated skin muscle in the hamster. Eur J Med Res 1996; 1:471–8
26. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G: Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: A randomized controlled efficacy trial. JAMA 1999; 282:1857–64
27. Kerner T, Ahlers O, Veit S, Riou B, Saunders M, Pison U: DCL-Hb for trauma patients with severe hemorrhagic shock: The European "On-Scene" Multicenter Study. Intensive Care Med 2003; 29:378–85
28. Sloan EP, Koenigsberg M, Brunett PH, Bynoe RP, Morris JA, Tinkoff G, Dalsey WC, Ochsner MG: *Post hoc* mortality analysis of the efficacy trial of diaspirin cross-linked hemoglobin in the treatment of severe traumatic hemorrhagic shock. J Trauma 2002; 52:887–95
29. Riou B, Landais P, Vivien B, Stell P, Labbene I, Carli P: Distribution of the probability of survival is a strategic issue for randomized trials in critically ill patients. ANESTHESIOLOGY 2001; 95:56–63
30. Winslow RM: $\alpha\alpha$-Crosslinked hemoglobin: Was failure predicted by preclinical testing? Vox Sang 2000; 79:1–20
31. Capone AC, Safar P, Stezoski W, Tisherman S, Peitzman AB: Improved outcome with fluid restriction in treatment of uncontrolled hemorrhagic shock. J Am Coll Surg 1995; 180:49–56
32. von Dobschütz E, Hutter J, Hoffmann T, Messmer K: Recombinant human hemoglobin with reduced nitric oxide–scavenging capacity restores effectively pancreatic microcirculatory disorders in hemorrhagic shock. ANESTHESIOLOGY 2004; 100:1484–90
33. Raat NJ, Liu JF, Doyle MP, Burhop KE, Klein J, Ince C: Effects of recombinant-hemoglobin solutions rHb2.0 and rHb1.1 on blood pressure, intestinal blood flow, and gut oxygenation in a rat model of hemorrhagic shock. J Lab Clin Med 2005; 145:21–32
34. Dunham CM, Siegel JH, Weireter L, Fabian M, Goodarzi S, Guadalupi P, Gettings L, Linberg SE, Vary TC: Oxygen debt and metabolic acidemia as quantitative predictors of mortality and the severity of the ischemic insult in hemorrhagic shock. Crit Care Med 1991; 19:231–43
35. Rutherford EJ, Morris JA Jr, Reed GW, Hall KS: Base deficit stratifies mortality and determines therapy. J Trauma 1992; 33:417–23
36. Kerger H, Tsai AG, Saltzman DJ, Winslow RM, Intaglietta M: Fluid resuscitation with O2 *versus* non-O2 carriers after 2 h of hemorrhagic shock in conscious hamsters. Am J Physiol 1997; 272:H525–37
37. Bergentz SE, Carlsten A, Gelin LE, Krebs J: Hidden acidosis in experimental shock. Ann Surg 1969; 169:227–32
38. Baldwin AL: Modified hemoglobins produce venular interendothelial gaps and albumin leakage in the rat mesentery. Am J Physiol 1999; 277:H650–9
39. Dull RO, DeWitt BJ, Dinavahi R, Schwartz L, Hubert C, Pace N, Fronticelli C: Quantitative assessment of hemoglobin-induced endothelial barrier dysfunction. J Appl Physiol 2004; 97:1930–7
40. Caron A, Mayer JC, Menu P, Alayash A, Marie PY, Vigneron C: Measurement of blood volume after haemodilution with haemoglobin-based oxygen carriers by a radiolabelled-albumin method. Transfus Med 2001; 11:433–42
41. Migita R, Gonzales A, Gonzales ML, Vandegriff KD, Winslow RM: Blood volume and cardiac index in rats after exchange transfusion with hemoglobin-based oxygen carriers. J Appl Physiol 1997; 82:1995–2002
42. Kavdia M, Tsoukias NM, Popel AS: Model of nitric oxide diffusion in an arteriole: Impact of hemoglobin-based blood substitutes. Am J Physiol Heart Circ Physiol 2002; 282:H2245–53
43. Tsai AG, Friesenecker B, McCarthy M, Sakai H, Intaglietta M: Plasma viscosity regulates capillary perfusion during extreme hemodilution in hamster skinfold model. Am J Physiol 1998; 275:H2170–80
44. Resta TC, Walker BR, Eichinger MR, Doyle MP: Rate of NO scavenging alters effects of recombinant hemoglobin solutions on pulmonary vasoreactivity. J Appl Physiol 2002; 93:1327–36