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EXPERIMENTAL

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Improved short-term survival with polyethylene glycol modified hemoglobin liposomes in critical normovolemic anemia

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Abstract *Objective:* To investigate the efficacy of a polyethylene glycol (PEG) modified formulation of liposome-encapsulated hemoglobin (LEH) as an oxygen-carrying blood substitute in the treatment of critical normovolemic anemia. *Design and setting:* Prospective, controlled, randomized experimental study in a university research facility. *Subjects:* 14 anesthetized and mechanically ventilated beagle dogs. *Interventions:* Animals were splenectomized and hemodiluted by exchange of whole blood for iso-oncotic hetastarch (HES). Target parameter of the hemodilution protocol was the individual critical hemoglobin concentration (Hb_{crit}) corresponding with the onset of O_2 supply dependency of total body O_2 consumption. At Hb_{crit} animals were randomized to receive a bolus infusion (20 ml/kg) of either LEH ($n = 7$) or normal saline (NS; $n = 7$). Subsequently animals were observed without further intervention. *Measurements and results:* The primary endpoint was survival time after the completion of treatment; secondary endpoints were parameters of central hemodynamics, O_2 transport and tissue oxygenation. Animals in the LEH group survived significantly longer after completion of treatment (149 ± 109 vs. 43 ± 56 min). Immediately after treatment LEH-treated animals presented with a more stable cardiovascular condition. After 30 min tissue O_2 tension on the surface of a skeletal muscle was significantly higher in the LEH group (23 ± 8 vs. 9 ± 2 mmHg). Nevertheless, treatment with LEH did not decrease mortality within the observation period. *Conclusions:* In this present experimental study the infusion of a PEG-modified LEH provided adequate tissue oxygenation, hemodynamic stability, and a prolongation of survival time after critical anemia. However, these effects were sustained for only a short period of time.

Keywords Artificial O_2 carriers · Liposome-encapsulated hemoglobin · Hemoglobin · Critical normovolemic anemia · Transfusion · Blood

Introduction

Safe and effective alternatives to the transfusion of allogeneic blood are continuously gaining importance in view of the immanent risks and increasing costs of banked blood products. Typical risks still associated with allogeneic blood transfusions include accidental mistransfusion ("clerical error"), transmission of infectious

diseases, immunomodulation, and transfusion-related lung injury [1]. Moreover, public health systems are facing intensive costs resulting from increasing costs of blood products [2] as well as from transfusion-related morbidity [3]. Therefore the initial treatment of an acute blood loss usually consists in the infusion of acellular (i.e., crystalloid and/or colloidal) fluids. The goal is the maintenance of normovolemia, and the consequence is a dilution of the cell mass remaining in the vasculature with a corresponding dilutional anemia. Once the patient's individual transfusion trigger is met, the transfusion of allogeneic red blood cells (RBC) is required [4–6].

Artificial O_2 carriers on the basis of human or bovine hemoglobin can substitute the O_2 transport function of RBCs and therefore have the potential to reduce allogeneic blood transfusions [7]. Depending on their chemical modification and molecular size, preparations of stroma-free hemoglobin exert vasoconstrictive effects of variable intensity. Vasoconstriction has in part been confirmed as harmful for microcirculatory blood flow and tissue oxygenation [8–11] and is predominantly explained by binding of the endogenous vasodilator nitric oxide (NO scavenging) [12]. Since NO is located in the interstitial space of the vascular wall, a prerequisite for NO scavenging is that Hb molecules permeate the endothelial layer via gap junctions [13].

Encapsulating Hb molecules into liposomes (liposome-encapsulated hemoglobin, LEH) is postulated to reduce NO scavenging because the diameter of Hb liposomes exceeds the size of endothelial gaps, thereby preventing the permeation of the endothelial barrier [14]. The present study used a formulation of polyethylene glycol (PEG) modified LEH (Terumo, Japan) in its original indication, i.e., as an alternative to allogeneic RBC transfusions when tissue oxygenation becomes impaired by acute anemia. The O_2-carrying blood substitute is made of human Hb originating from outdated banked blood. Moreover, this LEH features low O_2 affinity, which should facilitate O_2 offloading to the tissues. Therefore we hypothesized that LEH should provide adequate tissue oxygenation and hence increase survival time after treatment of critical normovolemic anemia. Preliminary results of this were presented at the Euroanesthesia Congress in Munich in 2007 [15].

Materials and methods

After approval by the local governmental review board, experiments were performed in 14 healthy beagle dogs of either sex (body weight 12.7 ± 1.8 kg). All animals received good care in compliance with the Guide for the Care and Use of Laboratory Animals. Prior to the current investigation the study design and target parameters were checked for feasibility and validity by means of preliminary pilot experiments (see Chap. 4, Electronic Supplementary Material, ESM).

Table 1 Composition and physical properties of the LEH formulation

Liposome diameter (mean)	236 nm
pH	7.3
Hb concentration	6.2 g/dl
Viscosity (at 25°C)	1.94 cp
LEHcrit	25.5%
Met-Hb rate	3.7%
P_{50}	51.4 mmHg
Hill coefficient	1.88
PEG 5000 DSPE	0.14 g/dl
Inositol hexaphosphate	0.08 g/dl
Cholesterol	1.08 g/dl
Stearic acid	0.80 g/dl
Soybean hydrogenated phosphatidylcholine	2.20 g/dl
Plasma half-life	36 h

Characterization of the LEH

LEH was obtained from Terumo (Kanagawa, Japan). Its physicochemical properties are listed in Table 1. The preparation of LEH has been described in detail elsewhere [16].

Anesthesia and ventilation

Animals were fasted with free access to water 12 h before the experiments. After intramuscular premedication with 10 mg/kg ketamine (Ketavet, Parke-Davis, Berlin, Germany) and 1 mg/kg midazolam (Ratiopharm, Ulm, Germany), anesthesia was induced by intravenous injection of 3 mg/kg propofol (Braun, Melsungen, Germany), 10 μg/kg fentanyl (Janssen, Neuss, Germany), and 0.2 mg/kg pancuronium (Curamed, Karlsruhe, Germany) and maintained by continuous infusion of propofol ($0.16\,mg\,kg^{-1}\,min^{-1}$), midazolam ($0.01\,mg\,kg^{-1}\,min^{-1}$), and fentanyl ($0.8\,\mu g\,kg^{-1}\,min^{-1}$). For assessment of oxygen tension (tpO_2) on a skeletal muscle, muscular paralysis was achieved by continuous infusion of pancuronium ($0.1\,mg\,kg^{-1}\,min^{-1}$). Estimated fluid losses were replaced by 5 ml/kg per hour of a balanced electrolyte solution (Tutofusin, Baxter, Unterschleissheim, Germany). Animals were orotracheally intubated and ventilated with ambient air at a rate of 14 cycles per minute and a positive endexpiratory pressure of $5\,cmH_2O$ (Servo 900B, SiemensElema, Solna, Sweden). Tidal volume was individually adjusted to provide arterial normocapnia and was then maintained throughout the entire protocol.

Instrumentation and monitoring

Animals were placed in supine position and a five-lead electrocardiogram (II, V5) was installed for detection of

Table 2 Parameters of hemodynamics and myocardial function (*BVI*, blood volume index; *HR*, heart rate; *MAP*, mean arterial pressure; *MPAP*, mean pulmonary arterial pressure; *CI*, cardiac index; *SVI*, stroke volume index; *SVRI*, systemic vascular resistance index; *PVRI*, pulmonary vascular resistance index; *CVP*, central venous pressure; *LVP*, left ventricular pressure; *LVEDP*, left ventricular enddiastolic pressure; *CPP*, coronary perfusion pressure; dp/dt_{max}, maximum left ventricular pressure increase; dp/dt_{min}, maximum left ventricular pressure decrease; *LVSWI*, left ventricular stroke work index; *RVSWI*, right ventricular stroke work index)

	Baseline	Hb_{crit}	0 min	30 min
BVI (ml/kg)				
LEH	85 ± 14	n.d.	83 ± 7	n.d.
NS	82 ± 8	n.d.	87 ± 3	n.d.
HR (1/min)				
LEH	128 ± 23	213 ± 21*	193 ± 21*	205 ± 33
NS	131 ± 28	211 ± 28*	190 ± 37	208 ± 49
MAP (mmHg)				
LEH	97 ± 14	80 ± 18*	97 ± 17*,**	86 ± 19
NS	95 ± 19	73 ± 17*	70 ± 22	59 ± 16
MPAP (mmHg)				
LEH	18 ± 5	30 ± 10*	41 ± 15*	30 ± 8*
NS	18 ± 5	31 ± 14*	30 ± 14	27 ± 7
CI ($l\,min^{-1}\,m^{-2}$)				
LEH	3.1 ± 0.5	7.8 ± 2.3*	5.6 ± 1.1*	6.1 ± 1.9
NS	3.0 ± 0.4	6.3 ± 1.3*	5.6 ± 1.1	6.2 ± 2.0
SVI ($ml\,m^{-2}$)				
LEH	24 ± 5	36 ± 10*	29 ± 6	31 ± 12
NS	23 ± 3	30 ± 7*	30 ± 6	32 ± 16
SVRI ($dyne\,s^{-1}\,cm^{-5}\,m^{-2}$)				
LEH	2492 ± 493	827 ± 263*	1327 ± 350*,**	1167 ± 519
NS	2484 ± 624	870 ± 362*	837 ± 349	712 ± 389
PVRI ($dyne\,s^{-1}\,cm^{-5}\,m^{-2}$)				
LEH	293 ± 141	232 ± 151	449 ± 213*,**	312 ± 152
NS	240 ± 254	201 ± 234	154 ± 269	142 ± 119
CVP (mmHg)				
LEH	3.2 ± 3.4	5.4 ± 2.9	6.4 ± 3.9	6.5 ± 4.7
NS	3.7 ± 1.7	8.6 ± 2.4*	9.4 ± 6.0	10.2 ± 3.2
LVP (mmHg)				
LEH	120 ± 13	114 ± 14	123 ± 17	126 ± 28**
NS	133 ± 30	118 ± 10	106 ± 17*	93 ± 18
LVEDP (mmHg)				
LEH	6.8 ± 3.0	9.1 ± 7.2	9.4 ± 4.8	6.8 ± 5.5
NS	9.6 ± 4.1	11.6 ± 6.0	20.1 ± 9.0*,**	17.9 ± 5.9**
CPP (mmHg)				
LEH	68 ± 16	37 ± 16*	57 ± 17**	43 ± 14
NS	66 ± 23	28 ± 21*	27 ± 27	23 ± 16
dp/dt_{max} ($mmHg\,s^{-1}$)				
LEH	3444 ± 932	5767 ± 3045	5291 ± 1222*,**	6984 ± 3832
NS	3865 ± 1691	3661 ± 1514	2803 ± 1231	2508 ± 829
dp/dt_{min} ($mmHg\,s^{-1}$)				
LEH	−5048 ± 1503	−5624 ± 3161	−5449 ± 1991**	−5414 ± 2755
NS	−6122 ± 2767	−4044 ± 1289	−2534 ± 1734	−1919± 786
LVSWI ($Nm\,10^{-3}\,m^{-2}$)				
LEH	316 ± 78	383 ± 117	373 ± 84	340 ± 121
NS	290 ± 47	284 ± 64	281 ± 109	230 ± 47
RVSWI ($Nm\,10^{-3}\,m^{-2}$)				
LEH	60 ± 23	144 ± 55*	164 ± 85	121 ± 52
NS	55 ± 12	120 ± 50*	116 ± 55	105 ± 25

* $p < 0.05$ vs. previous, ** $p < 0.05$ LEH vs. NS

arrhythmias and ST-segment changes. An electronic tip-manometer catheter (PC 370, Millar Instruments, Houston, Tex., USA) was inserted into the left ventricle via the left carotid artery. A double-lumen catheter (Arrow, Reading, Pa., USA) was inserted into the cranial vena cava and a Swan-Ganz-Catheter (Baxter, Irvine, Calif., USA) was floated into a branch of the pulmonary artery. A large bore hemodialysis catheter and a 6-F introducer sheath (both from Arrow) were inserted into the right femoral vein and artery, respectively. For continuous measurement of cardiac output a thermodilution-catheter (Pulsion, Munich, Germany) was placed into the left femoral artery. A Foley catheter (Rüsch, Kernen, Germany) was inserted into the urinary bladder via an inferior minilaparotomy. An area of 3 × 5 cm of the adductor muscle of the lower limb was dissected free from surrounding tissue for measurement of tpO_2. To avoid autotransfusion dogs were splenctomized via midline laparotomy. Body core temperature was kept constant using a warming pad. Measurements are described in detail in the ESM (Chap. 2).

Table 3 Parameters of O_2 transport and tissue oxygenation (DO_2I, index of O_2 delivery; VO_2I, index of O_2 consumption; $paCO_2$, arterial carbon dioxide tension; paO_2, arterial O_2 tension; pvO_2, mixed venous O_2 tension; CaO_2, arterial O_2 content; *BE*, base excess; $tpO_{2muscle}$, tissue O_2 tension on the surface of a skeletal muscle; *HV*, rate of hypoxic tpO_2 values)

	Baseline	Hb_{crit}	0 min	30 min
DO_2I (ml min^{-1} m^{-2})				
LEH	509 ± 115	288 ± 104*	254 ± 147	167 ± 110*
NS	480 ± 105	212 ± 45*	174 ± 49	230 ± 48*
VO_2I (ml min^{-1} m^{-2})				
LEH	148 ± 28	150 ± 33	n.d.	145 ± 49
NS	142 ± 29	147 ± 30	n.d.	138 ± 38
$paCO_2$ (mmHg)				
LEH	35 ± 4	39 ± 6	44 ± 8	44 ± 7
NS	35 ± 3	40 ± 6	35 ± 8	34 ± 2
paO_2 (mmHg)				
LEH	93 ± 11	107 ± 35	98 ± 38	60 ± 20*
NS	96 ± 19	94 ± 26	126 ± 34**	111 ± 21**
pvO_2 (mmHg)				
LEH	47 ± 8	32 ± 6*	29 ± 4	22 ± 5
NS	43 ± 5	37 ± 10*	34 ± 12	31 ± 6
CaO_2 (ml dl^{-1})				
LEH	17.1 ± 2.4	3.8 ± 0.1*	4.2 ± 1.6*	3.0 ± 1.0
NS	16.0 ± 1.9	3.6 ± 0.6*	3.2 ± 0.7	3.8 ± 0.4
O_2 extraction rate (%)				
LEH	21 ± 10	40 ± 17*	62 ± 9*	58 ± 5**
NS	16 ± 17	32 ± 20*	36 ± 24	30 ± 23
pH				
LEH	7.36 ± 0.07	7.31 ± 0.08	7.27 ± 0.10	7.22 ± 0.11
NS	7.35 ± 0.05	7.31 ± 0.03	7.30 ± 0.05	7.27 ± 0.07
BE (mmol/l)				
LEH	−5.1 ± 4.0	−5.7 ± 2.8	−7.7 ± 3.2	−10.6 ± 5.5
NS	−5.9 ± 1.9	−6.1 ± 1.6	−8.6 ± 3.5	−9.0 ± 3.4
Lactate (mmol/l)				
LEH	1.5 ± 0.7	2.7 ± 1.1*	2.2 ± 1.0**	4.0 ± 2.5**
NS	1.6 ± 0.7	3.5 ± 1.4*	4.7 ± 2.1	5.9 ± 4.1
$tpO_{2muscle}$ (mmHg)				
LEH	42 ± 5	15 ± 7*	19 ± 5	24 ± 7**
NS	43 ± 5	13 ± 5*	14 ± 6	9 ± 2
HV (%)				
LEH	0 ± 0	21 ± 20*	15 ± 6	11 ± 14**
NS	0 ± 0	41 ± 22*	45 ± 56	53 ± 7

*$p < 0.05$ vs. previous, **$p < 0.05$ LEH vs. NS

Experimental protocol

Upon completion of surgical preparation and installation of the different measuring devices a 60-min stabilization period was allowed to elapse to achieve stable baseline conditions. After recording the first data set (baseline) a hemodilution protocol was initiated by simultaneous exchange of circulating blood for an iso-oncotic 6% hetastarch (HES) solution using a bidirectional precision pump (Havard Apparatus, Holliston, Mass., USA; exchange rate 1 ml kg^{-1} min^{-1}). Target parameter of the hemodilution protocol was the animal's individual critical Hb concentration (Hb_{crit}; for determination of Hb_{crit} see ESM, Chap. 1). When Hb_{crit} was met, the second data set was collected (Hb_{crit}) and animals were randomized to receive a bolus infusion (20 ml/kg) of either LEH ($n = 7$) or normal saline (NS; $n = 7$); At baseline, the groups did not differ significantly in any of the parameters investigated (see Tables 2, 3). During infusion of the respective fluid FIO_2 was elevated to 0.6 and was reduced to 0.21 as soon as infusion was completed. The rationale was to achieve initially a complete O_2 loading of the LEH (low O_2 affinity; see Table 1) but reduce the impact of beneficial effects provided by hyperoxic ventilation on the target parameters [17, 18]. After completion of the bolus infusion the third data set (0 min) was recorded, and measurement of survival time was initiated. Animals were left without any further intervention, and data were collected every 30 min.

Statistics

Statistical analysis was performed with the SAS 9.1 software package (SAS Institute, Cary, N.C., USA). All parameters are presented as mean ± SD. Distribution of data was assessed by the Shapiro–Wilk test. In the case of normal distribution the time effect on the parameters investigated and the differences between groups were tested by repeated measures analysis of variance. Posthoc analysis of within-group differences detected by analysis of variance was performed by the Student–Newman–Keul test and posthoc analysis of between-group differences

by Student's t test. In the case of nonnormal distribution the time effect on the parameters and differences between the groups were tested by analysis of variance on ranks. Post-hoc analysis of differences within and between groups was performed by Wilcoxon's signed rank test. Statistical analysis of tpO_2 was performed by analysis of medians of the 240 single tpO_2 values obtained at each observation. For all parameters investigated statistical significance was accepted at $p < 0.05$.

Results

Hemodilution to Hb_{crit}

Hemodynamics and myocardial function

Induction of critical normovolemic anemia required the exchange of 85 ± 16 ml/kg blood for iso-oncotic HES in the LEH group and 100 ± 18 ml/kg in the NS group (n.s.). The investigated cardiovascular parameters are listed in Table 2. No significant differences between the groups were detected at Hb_{crit} (Table 2). In both groups heart rate, cardiac index, mean pulmonary arterial pressure, and right ventricular stroke work index increased significantly, while systemic vascular resistance index (SVRI), mean arterial pressure, and coronary perfusion pressure decreased ($p < 0.05$). The increase in central venous pressure by 5 mmHg was statistically significant only in the NS group.

Oxygen transport and tissue oxygenation

Starting from baseline Hb concentrations of 13.4 ± 1.6 g/dl in the LEH group and 13.0 ± 1.6 g/dl in the NS group, Hb_{crit} was met at 2.7 ± 0.6 and 2.7 ± 0.7 g/dl, respectively. No significant differences were detected between the groups at Hb_{crit} regarding the investigated parameters of O_2 transport and tissue oxygenation (Table 3). In both groups hemodilution to Hb_{crit} was associated with a significant decrease in CaO_2 and DO_2. The increase in O_2 extraction ratio was accompanied by a significant decrease in mixed venous pO_2. Critical impairment of tissue oxygenation was reflected by increased lactate concentrations ($p < 0.05$) and by the typical left shift in tpO_2 histograms (Fig. 1), with decreased tpO_2 medians and an increased rate of hypoxic tpO_2 values ($p < 0.05$).

Treatment of critical anemia

According to the protocol, animals received 253 ± 33 ml LEH or 251 ± 35 ml NS (n.s.). The maintenance of normovolemia was confirmed by an additional blood volume measurement directly after completion of treatment (Table 2).

Hemodynamics and myocardial function

In the LEH group heart rate and cardiac index decreased significantly, while mean arterial pressure, coronary perfusion pressure, mean pulmonary arterial pressure, systemic and and pulmonary vascular resistance indices, and dp/dt_{max} increased ($p < 0.05$). In the NS group left ventricular pressure decreased by 9% and left ventricular enddiastolic pressure increased by 41% ($p < 0.05$). Mean arterial pressure, systemic and pulmonary vascular resistance indices, and dp/dt_{max} were significantly higher and left ventricular enddiastolic pressure and dp/dt_{min} significantly lower in the LEH than the NS group ($p < 0.05$), indicating superior cardiovascular performance.

Oxygen transport and tissue oxygenation

While CaO_2 did not change significantly in the NS group, the infusion of LEH increased CaO_2 by 19% ($p < 0.05$). The O_2 extraction rate increased only in the LEH group ($p < 0.05$); tpO_2 medians increased slightly (n.s.) and the left shift in the tpO_2 histogram began to return (Fig. 1). Lactate concentration was significantly lower in the LEH than in the NS group but remained elevated compared with baseline. After termination of treatment paO_2 was significantly higher in the NS group.

Observation period

Survival time ranged from 47 to 345 min in the LEH group and from 5 to 164 min in the NS group (149 ± 109 min vs. 43 ± 56 min, $p < 0.05$), indicating a significant prolongation in survival time attributable to treatment with the LEH (Fig. 2).

Hemodynamics and myocardial function

Within the first 30 min after the bolus infusion mean pulmonary arterial pressure decreased significantly overall, but this decrease was statistically significant only within the LEH group. Cardiac index increased by 9% in the LEH group and by 11% in the NS group (n.s.). At 30 min after termination of treatment superior left-ventricular performance in LEH-treated animals was reflected by higher left ventricular pressure and lower left ventricular enddiastolic pressure ($p < 0.05$).

Oxygen transport and tissue oxygenation

Thirty minutes after completion of bolus infusion the following differences between the groups were observed: The left shift in tpO_2 histograms was reversible only in the LEH group (Fig. 1). Accordingly, the median of tpO_2 values were significantly higher, while the rate of hypoxic

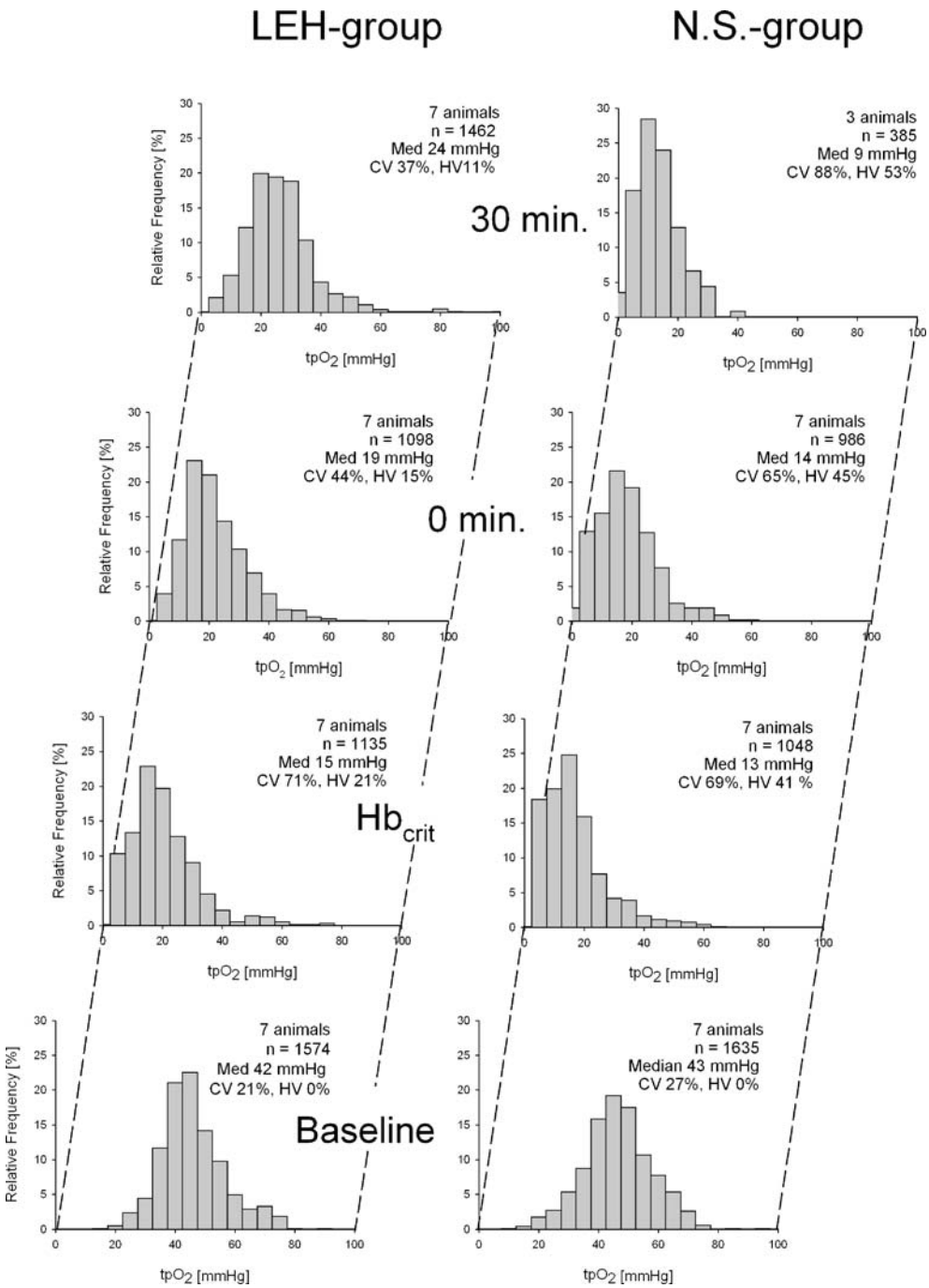

Fig. 1 Sum histograms of all tpO_2 values obtained on the surface of a skeletal muscle. Time points of measurement (*from bottom to top*): baseline, Hb_{crit}, 0 min and 30 min after termination of treatment. *x-Axis*, tpO_2 values are displayed in classes of 5 mmHg; *y-axis*, relative frequency of the tpO_2 values. At Hb_{crit} the histograms are left-shifted in both groups, indicating that the relative frequency of low tpO_2 values had increased. This effect was reversible in the LEH group

tpO_2 values was lower in the LEH group ($p < 0.05$). Lactate concentration was still lower than in the LEH group ($p < 0.05$) but remained elevated compared with baseline. O_2 extraction was significantly higher in the LEH group. Although DO_2I decreased by 34% in the LEH group ($p < 0.05$) and increased by 32% in the NS group ($p < 0.05$), DO_2I did not differ significantly between the groups. While cardiac index increased slightly in both groups (see above), CaO_2 decreased by 29% in the LEH group ($p < 0.05$) and increased by 19% in the NS group ($p < 0.05$). In the LEH group paO_2 and pvO_2 decreased within the first 30 min after completion of treatment and remained decreased within the observation period. Thirty minutes after infusion of LEH, LEH-transported O_2 contributed 35.6% of total body DO_2 (vs. 57.3% RBC-transported O_2). The higher O_2 extraction rate in the LEH group was most likely related to the utilization of LEH-transported O_2: after 30 min 41.6% of O_2 consumed was calculated to come from LEH (vs. 50.8% RBC-transported O_2).

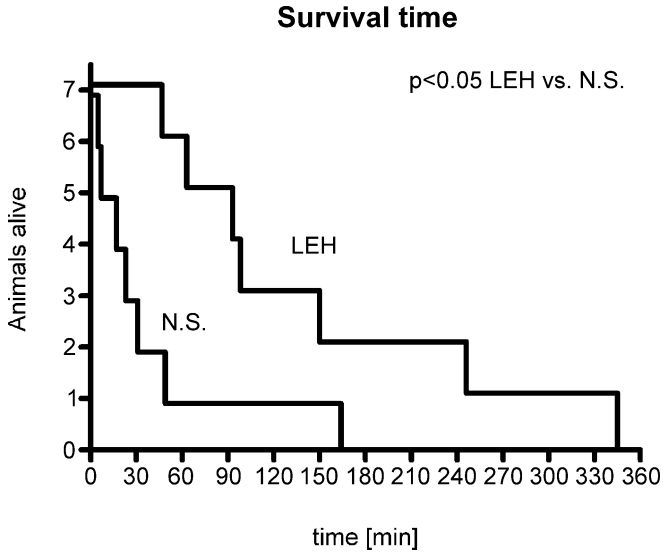

Fig. 2 Survival time after completion of treatment

Discussion

The main finding of the present study is that LEH effectively restores tissue oxygenation in critical normovolemic anemia, as reflected by directly measured parameters of tissue oxygenation, by a more stable cardiovascular condition, and by prolonged survival time compared with placebo treatment. However, these positive effects were sustained for only a short period of time. Whether safety problems or limited efficacy of the LEH terminated its beneficial effects in our experimental model remains to be elucidated.

Placebo control was performed with NS, which is void of any volume expansion effect, and therefore the infusion of 20 ml/kg did not exert an effect on the target parameters investigated (Tables 2, 3). Since LEH is a suspension of oncotically inert Hb liposomes in saline, NS appears to be the best matched control solution to LEH, with the only difference that NS does not provide additional O_2 carriers. Safe and effective O_2-carrying blood substitutes are expected (a) to be at least as efficacious as banked RBCs regarding O_2 transport and tissue oxygenation and (b) to exert fewer side effects than allogeneic blood. The efficacy of all prevalent hemoglobin-based blood substitutes in improving O_2 transport and tissue oxygenation is well documented in the literature (Table 4). However, the spectrum of potential side effects (in particular vasoconstriction) has not yet been fully documented [7, 19–22].

In the treatment of critical normovolemic anemia the efficacy of LEH should become apparent by means of restoring tissue oxygenation, stabilization of macro- and microhemodynamic parameters and, finally, by prolongation of survival time. These therapeutic goals were initially realized by infusion of the blood substitute: O_2 transported by the LEH contributed significantly to DO_2, covered a relevant part ($> 45\%$) of total body O_2 demand, and provided sufficient myocardial oxygenation as reflected by superior cardiac performance (dp/dt_{max}, dp/dt_{min}). Compared with placebo, superior peripheral tissue oxygenation was indicated by significantly higher tpO_2 values and lower lactate concentration in animals treated with LEH.

Table 4 Physicochemical characteristics and current state of clinical research on hemoglobin based oxygen carriers [*PHP*, pyridoxilated, polyethylene-glycol conjugated Hb (Curacyte Health Sciences, Munich, Germany); *HemAssist*, diaspirin-crosslinked Hb (DCLHb, Baxter Healthcare, Round Lake, USA); *r-Hb 1.1*, recombinant Hb version 1.1 (Somatogen, Boulder, USA, later Baxter Healthcare); *r-Hb 2.0*, recombinant Hb version 2.0 (Baxter Healthcare); *Hemopure*, polymerized bovine Hb (HBOC 201, Biopure, Cambridge, USA); *polyheme*, pyridoxylated, glutaraldehyde-polymerized Hb (Northfiled Lab., Evanston, USA); *Hemolink*, Hb raffimer (Hemosol, Toronto, Canada); *Hemospan*, mMaleimide-activated polyethylene glycol-modified Hb (MP4, Sangart, San Diego, USA)]

	Source of Hb	Conc. (g/dl)	MW (kDa)	P50 (mmHg)	Indication	Phase of clinical testing
PHP	Human	8	123	23.6	Hemodynamic instability in septic shock	II/III
HemAssist	Human	10	65	32	Reduction in perioperative transfusion rate	Up to III, stopped
r-Hb 1.1	Recombinant	5–10	64	31–32	Reduction in perioperative transfusion rate	I/II, stopped
r-Hb 2.0	Recombinant	10	320	31–32	Reduction in perioperative transfusion rate	I/II, stopped
Hemopure	Bovine	13	250	38	Reduction in perioperative transfusion rate	III, approved since 2001 in South Africa, FDA licence application filed in 2002
Polyheme	Human	10	150	26–32	Reduction in perioperative transfusion rate	III, pivotal prehospital study completed in autumn 2006
Hemolink	Human	10	120–180	39	Reduction in perioperative transfusion rate	III, stopped
Hemospan	Human	4	95	6	Reduction in perioperative transfusion rate	III

Obviously these factors substantially lengthened survival in animals treated with LEH. The rapid improvement in tissue oxygenation was most likely achieved by low O_2 affinity and facilitated O_2 offloading from the LEH. By means of sufficient tissue oxygenation these O_2-transport characteristics have previously been demonstrated to be effective in models of cerebral ischemia and exchange transfusion in rats [23, 24], in isolated perfused beating hearts [25], in moderate hemorrhagic shock in rabbits [26] and dogs [18, 27], and in priming cardiopulmonary bypass in mongrel dogs [28].

In the present study, however, no animal in the LEH group survived longer than 6 h. In contrast to this, the transfusion of RBC concentrates enabled 6-h survival in three pilot experiments (see Chap. 4, ESM). In our own previous experimental studies the reduction in 6-h mortality was the primary endpoint of efficacious treatment of situations with critically impaired tissue oxygenation. In particular, ventilation with pure oxygen (FIO_2 1.0) decreased 6-h mortality in critical normovolemic anemia [29], severe hemorrhagic shock [30], and critical methemoglobinemia [31]. Moreover, 6-h mortality in critical normovolemic anemia was also reduced by vasopressor support with norepinephrine [32].

This raises the question as to why the initially positive effects of the LEH were not sustained for 6 h. Compared with other hemoglobin based oxygen carriers (Table 4), the present blood substitute features a rather low Hb content (6 g/dl). Although the infusion of LEH resulted in a significant increase in CaO_2, it may be speculated that this effect was insufficient to provide a sustained improvement in tissue oxygenation. Indeed, CaO_2 and DO_2I decreased during the first 30 min after completion of LEH infusion. Unexpectedly, paO_2 decreased beyond the baseline level within the first 30 min after LEH infusion, which progressively compromised the saturation of the LEH. Both the moderate Hb content and the limited O_2 saturation of LEH may have compromised its long-term efficacy. Although LEH infusion resulted in a significantly lower lactate concentration than did placebo treatment, lactate remained still elevated compared with baseline.

Simultaneously with the decrease in paO_2 we observed a significant increase in systemic and pulmonary vascular resistance indices. Although LEH is not thought to scavenge NO, these findings do indicate a certain vasoactivity of the LEH. Several authors have discussed high p50 and facilitated O_2 offloading as a possible trigger of vasoconstriction. According to the so-called autoregulation theory, vasoconstriction is elicited when arteriolar vessel walls are exposed to excess O_2 delivery. The consequence of increased diffusive O_2 delivery is a paradoxical decrease in O_2 uptake by the tissues [33, 34]. If this applies to systemic as well as to pulmonary circulation, the above decrease in paO_2 may have resulted from impaired pulmonary perfusion and O_2 uptake.

Moreover, LEH preparations are reported to activate platelets and phagocytic cells of the reticuloendothelial system [11]. Additionally, Szebeni and Alving [35] and Szebeni and coworkers [36] suggest a complement-mediated "pseudoallergic reaction" acutely following the infusion of LEH. This evokes classical and alternative pathways of complement activation, and variable degrees of inflammatory effects have been documented in rats, pigs, and men.

The severity of the response to LEH infusion seems to be species dependent. In monkeys hemodiluted to 3 g/dl Hb no adverse effects on cardiovascular performance or pulmonary gas exchange were observed after infusion of the LEH (personal communication from the manufacturer). In own pilot experiments in pigs the top-load infusion of LEH resulted in fatal pulmonary hypertension and right-ventricular failure (unpublished data). These unfavorable effects have been attributed to the presence of pulmonary intravascular macrophages, which are specifically found in the pulmonary endothelium of pigs [37]. The exposure of these macrophages to particulate stimuli results in the release of several proinflammatory mediators and acute pulmonary hypertension [38].

We therefore decided to perform the experiments in dogs that are void of pulmonary intravascular macrophages. The hemodynamic response of dogs to particulate stimuli is moderate, but pronounced blood cell alterations have been reported in response to infusion of various liposome solutions [39]. However, the impact of these alterations on proinflammatory diathesis and pulmonary gas exchange is presently not fully understood. In two dogs treated with LEH lungs were removed at the end of the experiment for histopathological evaluation. The major finding of this analysis was an accumulation of neutrophils in pulmonary vessels without any signs of pulmonary edema, congestion, or cellular infiltration. All in all, our data indicate that LEH improves tissue oxygenation in critical normovolemic anemia. In the early phase after infusion O_2 transport properties were sufficient to outweigh side effects of the LEH. The nature of intrinsic side effects, their species-dependency, and potential safety-related issues still remain to be elucidated. Overall, the blood substitute provided a short-term positive effect in our experimental model.

Conclusion

In the present experimental study the infusion of PEG-modified LEH provided sufficient tissue oxygenation in the early phase after treatment of critical normovolemic anemia. However, these effects were sustained for only a short time. The underlying causality leading to the termination of the effectiveness remains to be elucidated.

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