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Kinetics of liposome-encapsulated hemoglobin after 25% hypovolemic exchange transfusion

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Abstract

Liposome-encapsulated hemoglobin (LEH) is being developed as an oxygen therapeutic. In this work, we evaluated a neutral formulation of PEGylated LEH for its circulation and distribution properties in rodent models of 25% hypovolemic exchange transfusion. About 25% of blood in rats and rabbits was exchanged with LEH that had been previously labeled with ^{99m}Tc radionuclide. The distribution of ^{99m}Tc-LEH was followed by gamma camera imaging and intermittent blood sampling during 48 h, and counting the tissue-associated radioactivity after necropsy at 48 h. On the basis of circulation kinetics, the half-life of ^{99m}Tc-LEH in blood was 30 and 39.8 h in rats and rabbits, respectively. Apart from blood, major organs of accumulation of LEH after 48 h included liver (rats, 10.3% and rabbits, 5.4% of injected dose) and spleen (rats, 2.4% and rabbits, 0.8% of injected dose). The results demonstrate that LEH circulates for a prolonged time after administration and that the animals tolerate at least 25% of blood exchange without any distress. Subsequent to the enhanced uptake in the RES, the rats clear LEH from the circulation faster than the rabbits.

Keywords: Liposome-encapsulated hemoglobin; Blood substitute; Exchange transfusion; Liposomes

1. Introduction

Hemoglobin-based substitutes of red blood cells are gradually emerging as oxygen therapeutics for use in accidental blood loss, major surgery or organ transport (Winslow, 2000, 2003; Stowell, 2002). Besides being free of blood-borne pathogens, these products have the advantages of being easily purified, stored for a relatively long period of time and used regardless of the recipient's blood type. Administration of free hemoglobin is not recommended because of its instability in circulation and toxicity secondary to the strong binding of nitric oxide. Polymerization, cross-linking and polymer conjugation are a few approaches that are being examined to eliminate these drawbacks of free hemoglobin (Reiss, 2001; Squires, 2002). Another

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approach that has commanded particular attention is the encapsulation of hemoglobin inside lipid vesicles. Termed liposome-encapsulated hemoglobin (LEH), it is an oxygen carrier that mimics the membrane-enclosed cellular structure of red cells in relation to the oxygen transport and delivery, and eventual metabolic disposition (Rudolph, 1995; Sakai et al., 1996; Phillips et al., 1999). The spatial isolation of hemoglobin by a lipid bilayer potentially minimizes the cardiovascular/hemodynamic effects associated with the other modified forms of hemoglobin. Moreover, it is possible to co-encapsulate reductants, antioxidative enzyme system and oxygen-affinity modifiers with hemoglobin to artificially resurrect the red blood cell environment.

An optimum formulation of LEH will circulate in blood for a prolonged period while maintaining its oxygen-carrying property intact. It is also important that LEH is well tolerated when infused in large quantities intravenously. Previously, we reported a neutral lipid-based formulation of LEH with high encapsulation efficiency, and compared its circulation and distribution with negatively charged LEH at a relatively small lipid dose of about 16 mg. Role of post-insertion of poly (ethylene glycol)-linked phosphatidylethanolamines (PEG-PE) was also elucidated (Awasthi et al., 2004). In this report, we present results from a study where 25% of circulating blood in rats and rabbits was exchanged with LEH. The LEH consisting of distearoylphosphatidylcholine (DSPC), cholesterol and alpha-tocopherol was post-inserted with PEG-DSPE. The PEG-neutral LEH was radiolabeled with ^{99m}Tc, and its circulation kinetics in rat and rabbit models of 25% hypovolemic exchange transfusion was monitored up to 48 h after administration.

2. Materials and methods

The phospholipids, distearoylphosphatidylcholine and poly(ethylene glycol)$_{5000}$-distearoylphosphatidylethanolamine (PEG$_{5000}$-DSPE) were obtained from Avanti Polar Lipids (Pelham, AL). Cholesterol (Chol) was purchased from Calbiochem (La Jolla, CA) and α-tocopherol was purchased from Aldrich (Waukegan, IL). Glutathione (GSH), octyl-β-glucoside (OBG) and pyridoxal-5′ phosphate (PLP) were from Sigma (St. Louis, MO). The radiopharmaceutical ^{99m}Tc-sodium pertechnetate was obtained commercially (Amersham Health Nuclear Pharmacy, San Antonio, TX). For animal experiments, anesthetics xylazine and ketamine were from Phoenix Scientific, Inc. (St. Joseph, MO) and Fort Dodge Animal Health (Fort Dodge, IA), respectively. Frozen human stroma-free oxy-hemoglobin (O_2-Hb) was a kind gift from US Army, Walter Reed Army Institute of Research – Biological Resources Division (Washington, DC). All other chemicals were from Sigma (St. Louis, MO).

2.1. Preparation of LEH

Neutral LEH (DSPC/Chol/α-tocopherol, 51.4:46.4:2.2) post-inserted with PEG-DSPE was prepared by the technique reported previously (Awasthi et al., 2004). The manufacturing process was a combination of microfluidization and ultrafiltration as shown in the schematic (Fig. 1). During LEH manufacturing and hemoglobin recycling, hemoglobin was in carbonyl hemoglobin (CO-Hb) form that stabilizes hemoglobin against temperature-sensitive degradation. For PEGylation, PEG$_{5000}$-DSPE solution was added to a dilute suspension of LEH, such that the concentration of PEG$_{5000}$-DSPE was below its critical micelle concentration (Sou et al., 2000). In order to convert CO-Hb back to oxy-hemoglobin (Oxy-Hb), the PEGylated LEH was exposed to bright visible light from a 500-W halogen lamp under saturating oxygen atmosphere at 4–8 °C (Sakai et al., 1996). The final LEH was suspended in 5% hydroxyethyl starch (Baxter, Deerfield, IL). The unencapsulated hemoglobin was collected as filtrate during 500 kDa (MWCO) ultrafiltration step (Fig. 1) and concentrated by another ultrafiltration step (10 KDa MWCO) for use in subsequent batches of LEH. This recycling was performed at least three times, and the final LEH preparation used in this study was a combined mixture of all three LEH batches made out of recycled hemoglobin.

2.2. Characterization of LEH

The phospholipid concentration of the LEH was determined by the method of Stewart (1980). The oxygen affinity (p50) was measured on a Hemox-analyzer (TCS Scientific Corp., New Hope, PA). Amount of encapsulated hemoglobin was determined by monitoring absorbance of the OBG lysate of LEH at 540 nm

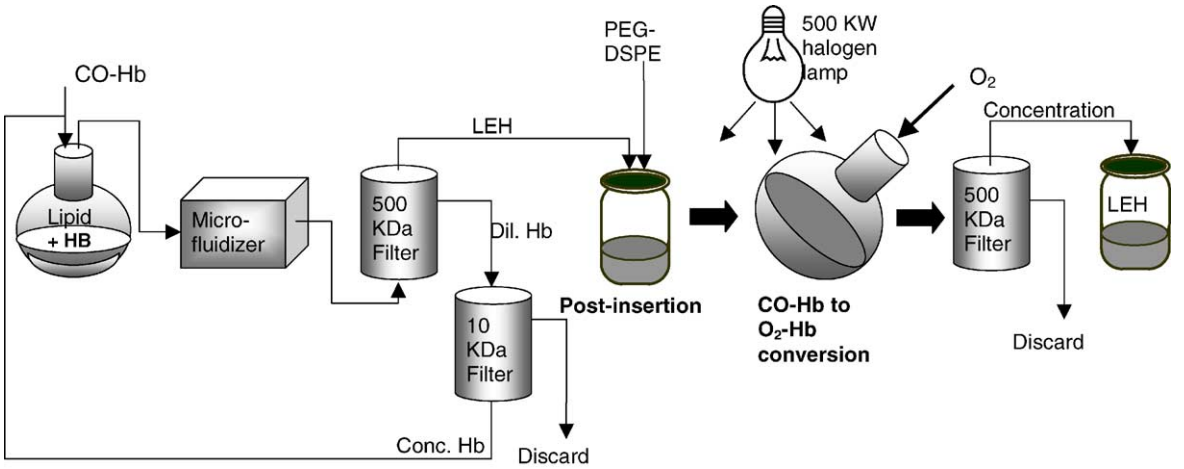

Fig. 1. Schematics of a continuous LEH manufacturing process for producing PEG-neutral LEH. In the first step, lipid component is mixed with aqueous phase containing concentrated CO-Hb solution to generate a homogenous suspension. The mixture is introduced into a Microfluidizer to reduce the particle size of LEH before filtering (500 kDa MWCO) off unencapsulated CO-Hb. Unencapsulated CO-Hb is concentrated again by ultrafiltration (10 kDa MWCO) and re-introduced in the first step of the next cycle. The LEH preparation is taken for post-inserting PEG-DSPE at 55 °C. CO-Hb in the PEG-LEH is converted from carbonyl form to oxy form by exposure to light and oxygen saturation. This is followed by concentration of dilute LEH by ultrafiltration (500 kDa MWCO).

(Tomita et al., 1968). Methemoglobin content was measured in hemoglobin as well as LEH (Matsuoka, 1997). The particle size was determined by photon correlation spectroscopy using a Brookhaven particle size analyzer equipped with argon laser, BI-9000AT digital correlator and BI-200SM goniometer (Holtsville, NY). Each sample was sized for 2 min with detector at 90° angle, and sample housed in a 25 °C bath. The data were analyzed by non-negatively constrained least squares (CONTIN) using dynamic light scattering software – 9KDLSW, beta version 1.24 supplied with the instrument. Conversion of carbonyl hemoglobin to oxyhemoglobin required exposure to intense light and oxygen saturation. The extent of lipid oxidation was estimated by monitoring thiobarbituric acid reactive substrates (TBARs) (Szebeni et al., 1984). The absorption values at 532 nm were transformed into concentrations of malonodialdehyde (MDA) using $\varepsilon = 1.5 \times 10^5\ M^{-1}cm^{-1}$ (Gutierrez et al., 2003).

2.3. Radiolabeling of LEH

The LEH was labeled essentially by the method developed by Phillips et al. (1992). Only a portion of LEH (2–3 ml) was radiolabeled, and the radiolabeled portion was mixed with rest of the unlabeled LEH to prepare infusible preparation.

2.4. Animal biodistribution and imaging studies

The animal experiments were performed according to the NIH Animal Use and Care Guidelines, and were approved by the Institutional Animal Care Committee of the University of Texas Health Science Center at San Antonio.

2.4.1. Rat exchange model

The rat exchange transfusion model has been described earlier (Goins et al., 1995). Left femoral artery of male Sprague Dawley rats (350–450 g, $n = 7$) was cannulated with a polyethylene tube catheter, filled with heparin (1000 U/ml), and subcutaneously tunneled and secured at the nape. After closing the surgical area, the rats were given 2 days to recover from the procedure. On the day of the exchange, the rats were anesthetized with isoflurane gas (2% in oxygen at 2 l/min) and 25% of blood was withdrawn through the tubing at the rate of 0.5 ml/min. Total volume of blood was estimated as 5.7% of body weight. The tubing was filled with heparin again and after giving 10 min time for

equilibration, ^{99m}Tc-LEH (volume equal to the amount of blood withdrawn) was infused through the tail vein (0.5 ml/min). Blood samples (50 μl) were withdrawn at various times through the arterial catheter for counting of blood borne radioactivity. Dynamic gamma camera images were acquired for 30 min after the start of transfusion and static images were acquired at 4, 24 and 48 h of infusion. Images were acquired with a matrix size of 256 × 256 for time sufficient to obtain significant radioactive counts. Further details of the imaging are discussed below under rabbit exchange model.

2.4.2. Rabbit exchange model

Male New Zealand white rabbits (n = 3), weighing 2.0–2.5 kg, were anesthetized by intramuscular injection of ketamine/xylazine mixture (50 and 10 mg/kg body weight, respectively). Patency of arterial and venous lines was established by an angiocath and a butterfly catheter, respectively. Total 25% of circulating blood was withdrawn through arterial line (0.5 ml/min), and animals were given 10 min to equilibrate before infusing equal volume of ^{99m}Tc-LEH through a venous line. After intravenous administration of ^{99m}Tc-LEH, anterior whole body scintigrams (64 × 64 matrix) of the rabbits were acquired using a Picker Model Dyna 4 Gamma Camera (Cleveland, OH) interfaced to a Pinnacle computer (Medasys, Miami, FL). A low-energy, high-resolution collimator was used and the camera was peaked at 140 keV with ±20% window. Arterial blood samples (100 μl) were obtained at various times after LEH injection.

After imaging at 48 h, the animals were euthanized by an overdose of an euthanasia solution (Beuthenesia, Veterinary Labs, Inc., Lenexa, KS). Various organs were excised, washed with saline, weighed and appropriate tissue samples were counted in a gamma counter (Perkin-Elmer, Boston, MA). Total blood volume and muscle mass were estimated as 5.7% and 40% of body weight, respectively (Frank, 1976; Petty, 1982). A diluted sample of injected LEHs served as a standard for comparison.

2.5. Data analysis

The circulation kinetics data (Fig. 4) represent radioactivity at various times as percent of circulating radioactivity of the first sample taken after finishing the infusion in the animals. On the other hand, accumulation of LEH in various organs at 48 h of biodistribution is calculated as percent of injected radioactivity. All data were corrected for decay of ^{99m}Tc-radioactivity ($T_{1/2}$ = 6 h).

3. Results

The purpose of this study was to evaluate the kinetics of PEG-neutral LEH after 25% hypovolemic exchange transfusion in rodents. The physical characteristics of the preparation are shown in Table 1. MDA concentration in oxygenated LEH was 0.33 ± 0.02 μM, while that in control LEH was 0.25 ± 0.01 μM. One major advance in the manufacturing of LEH used in this study was the recycling of unencapsulated hemoglobin. The recycling of unencapsulated hemoglobin was performed without any effect on the ultimate oxygen carrying property of the final LEH comprising of a combination of first, second and third batches of LEH. The oxygen dissociation curve of the LEH is shown in Fig. 2. To monitor the distribution of LEH in vivo, it was labeled with ^{99m}Tc radionuclide. The radioactivity was imaged by gamma camera and, on necropsy, accumulation of LEH in various organs was estimated by counting the tissue-associated ^{99m}Tc-radioactivity. About 25% of estimated circulating blood was exchanged with LEH without any apparent respiratory

Table 1
Properties of PEG-neutral LEH used in 25% hypovolemic exchange transfusion studies

Parameter	Value
p50 (mm Hg)	19.9
Size (nm)	133.1 ± 31.7
Hemoglobin (g/dl)	3.4
Phospholipid (mg/ml)	59
Osmolality (mOsmol/kg)	320
Colloidal oncotic pressure (mm Hg)	20.2
Percent PEG-DSPE insertion	52.14%
Tc-99m radiolabeling efficiency	41%
Methemoglobin (%)	<10%
Average volume of LEH injected animal (ml)	5.7 (rat) and 32 (rabbit)
Average radioactivity injected per animal (Mbq)	28 (rat) and 74 (rabbit)
Average radioactivity injected per animal (Mbq)	28 (rat) and 74 (rabbit)
Approximate phospholipid dose per animal (g)	0.34 (rat) and 1.9 (rabbit)

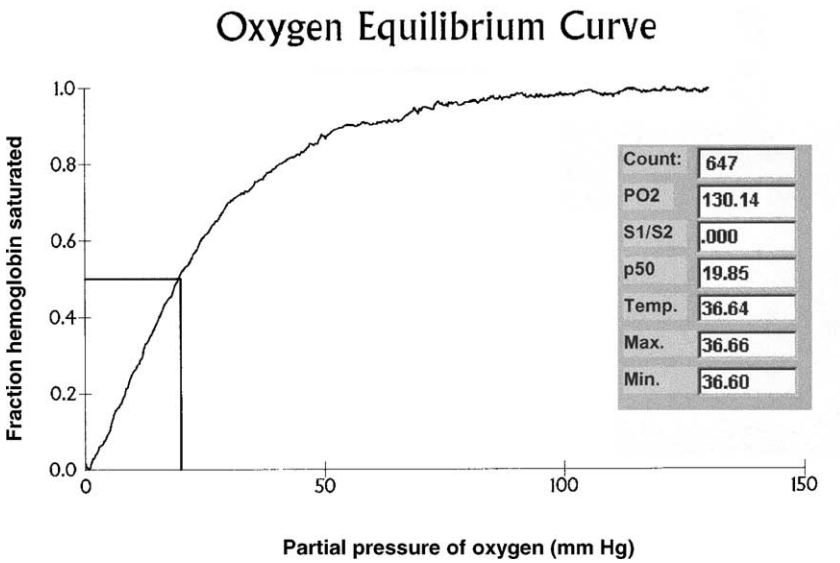

Fig. 2. Oxygen dissociation curve of PEG-neutral LEH.

distress to the animals (rats, $n = 7$ and rabbits, $n = 3$).

An advantage of using gamma ray emitting radionuclide (^{99m}Tc) was the capability of imaging the distribution of LEH in vivo without sacrificing the animal at intermediate data collection points. Fig. 3 shows the images of animals at 4, 24 and 48 h after injection of ^{99m}Tc-LEH. Since ^{99m}Tc decays with a half-life of 6 h, the acquisitions at 24 h and beyond produce relatively grainy pictures. Nevertheless, the scintigraphic images essentially provide us with the same information that was obtained by sacrificing the animal and counting various organs for radioactivity. Circulating LEH in animals can be estimated by amount of radioactivity accumulated in heart since it is mostly due to the blood pool. It is clear that even after 48 h, significant amount of LEH was still in blood – a property necessary for a long-acting oxygen carrier. Evidently, the long-circulation of LEH was the result of post-inserted PEG-DSPE. The two organs that accumulate the majority of LEH were organs of reticuloendothelial system (RES); however, spleen uptake in rabbits was not sufficient enough to appear prominently in acquired images. RES uptake of same preparation in rats was more pronounced as compared to that in rabbits. At the same time, rats cleared LEH from blood at a faster rate than the rabbits. There was significant radioactivity appearing in the kidneys and bladder within 30 min of infusion. While part of the kidney activity can be attributed to the blood pool, rest of it is due to the rapid renal clearance of radioactivity loosely bound to the liposomal membranes, because the radiolabeled compound (^{99m}Tc-HMPAO) is excreted rapidly from the kidneys.

The amount of LEH accumulated in various organs of rats and rabbits after 48 h of infusion is shown in Table 2. The major organs of accumulation of radioactivity were blood, spleen and liver in both rats and rabbits; other organs accumulated negligible amount of activity. There were some significant differences between rats and rabbits in terms of the extent of accumulation of LEH in blood, spleen and liver. Liver and spleen of rats accumulated more LEH than the liver and spleen of rabbits. Correspondingly, blood-borne LEH was less in rats than in rabbits, but on per gram tissue basis, the amount of LEH in rat blood was much more than that in rabbit blood. In fact, all the organs in rats accumulated considerably more than the rabbit on per gram tissue basis. Two other organs of significant accumulation were muscle and skin, and both appear to follow the pattern shown by the blood-borne activity.

Simultaneous to the gamma camera imaging of animals, blood samples were withdrawn at intermittent times during the 48-h period of study. These samples

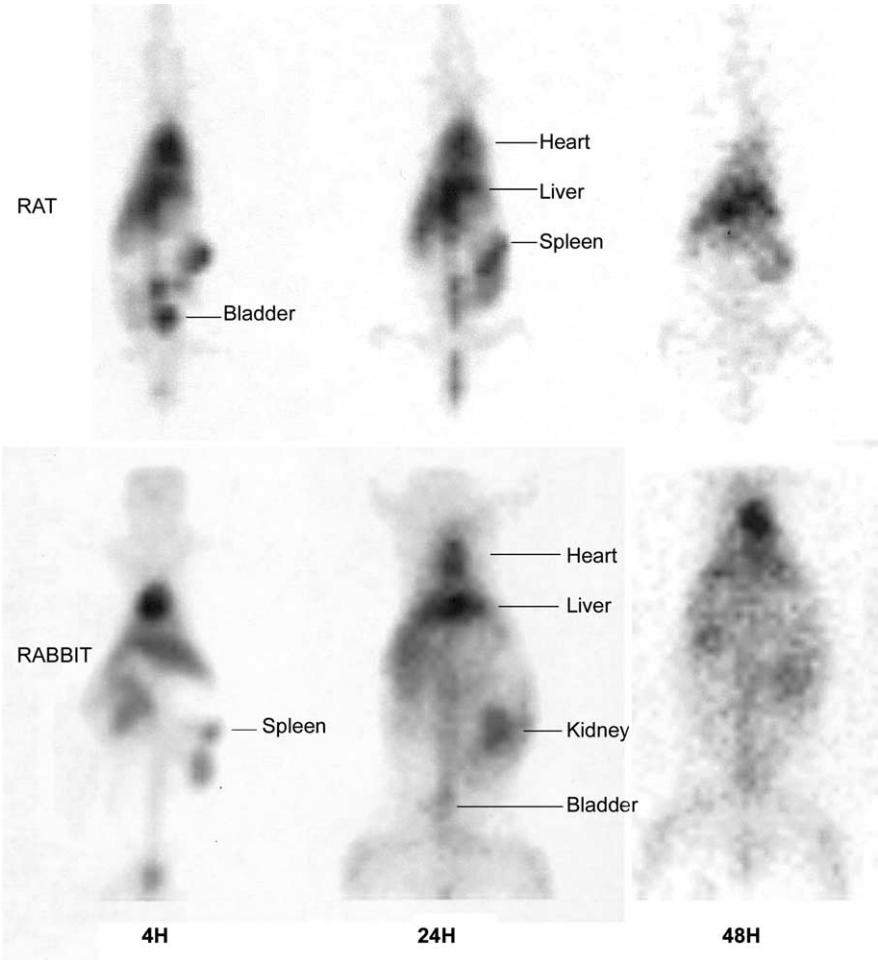

Fig. 3. Images acquired at 4, 24 and 48 h after infusion of ^{99m}Tc-LEH in the 25% hypovolemic exchange transfusion models. The upper panel is a rat model and the lower panel is a rabbit model. The important organs of LEH accumulation are labeled.

Table 2
Accumulation of ^{99m}Tc-LEH at 48 h in various organs of rats and rabbits after 25% hypovolemic exchange transfusion

Organ	Rats				Rabbits			
	ID/g tissue		ID/organ		ID/g tissue		ID/organ	
	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.	Mean	S.E.M.
Blood	0.77	0.09	17.65	1.87	0.24	0.02	30.88	0.38
Spleen	2.18	0.29	2.39	0.46	0.43	0.02	0.78	0.14
Liver	0.75	0.14	10.25	1.92	0.07	0.00	5.42	0.43
Kidney	0.66	0.18	1.53	0.40	0.16	0.02	2.42	0.28
Lung	0.65	0.18	1.37	0.37	0.13	0.05	1.25	0.28
Heart	0.36	0.20	0.43	0.23	0.03	0.00	0.16	0.01
Muscle	0.01	0.00	1.26	0.34	0.00	0.00	2.50	0.73
Bowel + stomach	0.19	0.04	3.84	0.93	ND	ND	ND	ND
Skin	ND	ND	ND	ND	0.00	0.00	1.22	0.41

ND = not determined.

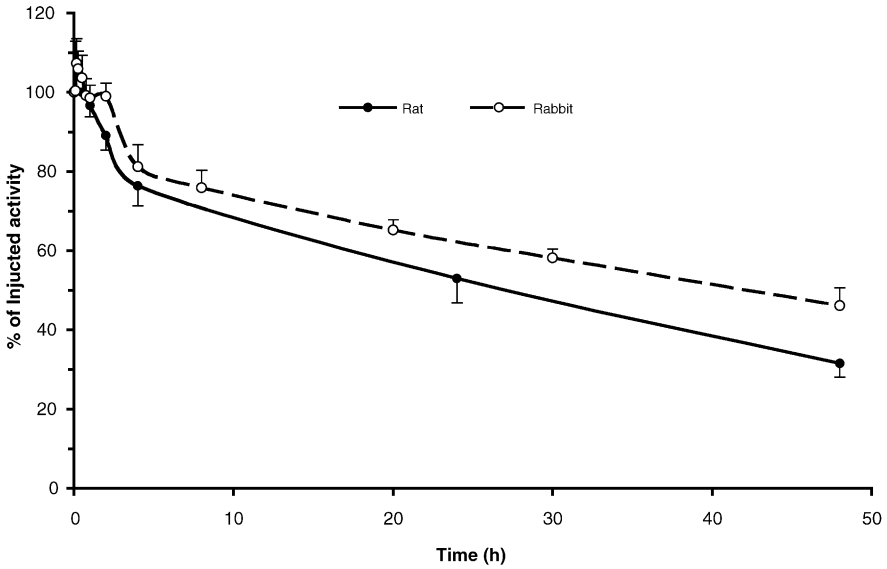

Fig. 4. The circulation kinetics of ^{99m}Tc-LEH in the models of 25% hypovolemic exchange transfusion

were counted for radioactivity reflecting LEH in circulation. Fig. 4 shows the circulation profiles of the LEH preparations in blood of both rats and rabbits. The amounts of radioactivity still circulating at 48 h were 31% for rats and 46% for rabbits. LEH circulation in both rats and rabbits followed a biphasic pattern – a rapid decrease during the first 4 h followed by a more gradual drop during the rest of the study. The estimated $T_{1/2}$ of LEH in circulation after the 25% exchange transfusion was about 30 h in rats and 39.8 h in rabbits, respectively.

4. Discussion

Hemoglobin vesicles or LEH are being examined as an oxygen carrier that has membrane structure to maintain hemoglobin in an enclosed compartment (Rudolph, 1995; Sakai et al., 1996; Phillips et al., 1999). During the last two decades, a clear understanding has emerged that the encapsulated hemoglobin is more safe and effective for resuscitation in comparison to hydroxyethyl starch (Takaori and Fukui, 1996), lactated Ringer's solution (Rabinovici et al., 1993), 6% albumin (Ogata et al., 1997), saline (Kobayashi et al., 1997) and even blood (Usuba et al., 1994). Volume resuscitation with these fluids may temporarily sustain blood pressure, cardiac output, heart rate and blood flow to critical organs, but more subtle physiological parameters such as base excess, intestinal mucosal pH and oxygen tension are maintained for prolonged duration only by red blood cells or LEH (Takeoka et al., 2004).

The desired intravascular persistence of an oxygen therapeutic agent should be at least equal to the time to regenerate red blood cells (Sehegal et al., 1983). Reticulocytosis starts within 24 h of significant blood loss and peaks in about a week (Hughes et al., 1995). Table 3 gives a fundamental idea of the circulation times of several HBOC preparations in various stages of testing. This report is the first report that demonstrates long-term (48 h) circulation kinetics of post-inserted neutral PEG-LEH in models of hypovolemic exchange transfusion. Previously, we reported a circulation $T_{1/2}$ of 19.3 h when a small dose (∼16 mg of lipid) of PEG-neutral LEH was injected in rabbits (Awasthi et al., 2004). We hypothesized that infusion of a larger dose would enhance the circulation $T_{1/2}$ to a level (>50 h) previously reported (Phillips et al., 1999). From the circulation data (Fig. 4), it is clear that the clearance of LEH from blood within the first 4 h of injection is rapid, and accounts for more than 35% of the total clearance in both species; after 4 h, the clearance is gradual and follows a linear fit. A fast renal elimination of loosely bound radiolabel is seen within the first 60 min of

Table 3
Intravascular persistence of various hemoglobin-based oxygen carriers

Product	Intravascular retention	Treatment (animal)	Reference
Pyridoxylated polyhemoglobin	$T_{1/2}$ < 10 h	25% isolvolemic ET (rat)	Keipert and Chang (1987)
	$T_{1/2}$ 30 h	100% isovolemic ET (rat)	
Zero-linked polymerized adipoyl-cross-linked bovine hemoglobin	$T_{1/2}$ = 6.7 h	?? (rat)	Matheson et al. (2002)
Unmodified hemoglobin	5% in circulation after 45 min	50% isovolemic ET (rat)	Lieberthal et al. (2002)
Diaspirin-cross-linked hemoglobin	10% in circulation after 45 min	50% isovolemic ET (rat)	Lieberthal et al. (2002)
O-raffinose-cross-linked hemoglobin, hemolink (Hemosol, Canada)	11% remained after 45 min	50% isovolemic ET (rat)	Lieberthal et al. (2002)
	$T_{1/2}$ 25 h	25% Topload (dog)	Wicks et al. (2003)
	$T_{1/2}$ 10-11 h	50% IET (rat)	Hsia et al. (1992)
MP4 hemoglobin	$T_{1/2}$ 23.4 h	110% Topload (rat)	Vandegriff et al. (2003)
Recombinant hemoglobin	∼30% in circulation after 24 h	40% hypovolemic ET (swine)	Malhotra et al. (2003)
Bovine polymerized hemoglobin, hemopure (Biopure, CA)	$T_{1/2}$ 20 h	15% phlebotomy (human)	Hughes et al. (1995)
Polyheme (Northfield Labs, Evanston, IL)	$T_{1/2}$ 24 h	?? (human) (?)	Gould et al. (2002) Gould et al. (1997)

? Not known.

injection, and it appears in urinary bladder. At the same time, during the first few hours, the RES accumulates circulating LEH up to its phagocytic capacity and thereafter, clears circulating LEH in a more gradual manner. Thus, when data time points earlier than 4 h are disregarded, linear normal fit ($y = mx + c$) with $R^2 > 0.99$ is obtained for both the animal models with clearance $T_{1/2}$ of 53 and 39 h for rabbits and rats, respectively. Based on a comparison of liposome-encapsulated doxorubicin in small animals and humans, it has been roughly estimated that a circulation $T_{1/2}$ of 12–20 h in rats or mice translates into 40–60 h in humans (Woodle and Working, 1995). On a same scale, a 39-h $T_{1/2}$ of LEH in rat would translate into a $T_{1/2}$ of 5 days in humans.

Once encapsulated, hemoglobin follows the kinetics of liposome distribution. In general, circulation $T_{1/2}$ of conventional liposomes decreases with increasing size, negative charge density and fluidity of the bilayer. However, both liposome size and surface charge over-ride the state of bilayer stability in determining the liposome clearance (Gregoriadis, 1995). Surface charge also has a major influence on the toxicity of liposomes administered intravenously. Therefore, we formulated LEH with neutral lipids and PEGylated to control the size distribution and in vivo behavior of the LEH (Awasthi et al., 2003, 2004). Although the pattern of accumulation of LEH in various organs is the same in the two species, interestingly, there were few major differences in the extent of LEH accumulation in various organs. Rats were found to clear LEH more rapidly than rabbits. Studies comparing handling of liposomes by various species have also found that rats clear liposomes faster than the rabbits (Laverman et al., 2000).

Besides circulation half-life, another important property that needs to be preserved in any red cell substitute is an appropriate functional half-life of hemoglobin. HBOCs are inherently limited by a rapid conversion of oxygen-carrying ferrous hemoglobin to non-functional ferric hemoglobin after administration. Thus, exogenously administered purified hemoglobin converts to methemoglobin at a rate of >1–2% per hour (Ogata et al., 1997). As a consequence of short circulation and functional half-life, HBOC preparations can provide effective resuscitation for a few hours without repeated administration. LEH has a potential to circumvent this problem associated with acellular hemoglobin preparations. While PEGylation extends clearance half-life (Awasthi et al., 2003, 2004), co-encapsulation of antioxidant-reduction enzyme system reduces the rate of methemoglobin formation and enhances the functional half-life of hemoglobin in circulation (Ogata et al., 1997). Acellular hemoglobin has intrinsic toxicity, secondary to the harmful reactive oxygen species that are readily produced during auto-oxidation (Kawano and Hosoya, 2002), and therefore, has been called oxidative neurotoxin. It may contribute to the cell injury after CNS trauma and hemorrhagic

shock (Regan and Rogers, 2003). Free hemoglobin also acts as a proinflammatory protein that results in significant toxicities if administered for resuscitation in traumatic brain injury (Gibson et al., 2002). In light of these toxicities, one major concern with the HBOCs has been the ischemia-reperfusion injury common in severe sustained hemorrhagic shock, stroke, MI, organ transplantation and head trauma (Chang, 2003). In addition, acellular hemoglobin tends to bind nitric oxide (NO), the endothelial regulator of vascular smooth muscle. Another factor that may play critical role in vasoconstriction is the hydrogen peroxide produced during hemoglobin performance (Yang et al., 2003) and it may freely diffuse through the endothelium and affect vascular tone secondary to its reaction with NO and production of hydroxyl radical (Nappi and Vass, 1998). An attempt to conjugate superoxide dismutase and catalase to the hemoglobin molecule has been made and is being investigated (Quebec and Chang, 1995; D'Agnillo and Chang, 1998). It is possible to co-encapsulate catalase with hemoglobin for prompt and efficient catalysis of H_2O_2.

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