

study of pigs, we were able to reconfirm the biocompatibility of HbV. Pigs are often used for preclinical studies because of their similarity to humans in terms of their body size, anatomical structure and physiological function. However, due attention must be given when a pig receives a liposomal suspension because of the different biodistribution and cardiovascular responses.

REFERENCES

- Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. *Nat Rev Drug Discov* 2005;4:145–160.
- Laing RB, Milne LJ, Leen CL, Malcolm GP, Steers AJ. Anaphylactic reactions to liposomal amphotericin. *Lancet* 1994;344:682.
- Schneider P, Klein RM, Dietze L, Söhngen D, Leschke M, Heyll A. Anaphylactic reaction to liposomal amphotericin (AmBisome). *Br J Haematol* 1998;102:1108–1109.
- Szebeni J. Complement activation-related pseudoallergy: A new class of drug-induced acute immune toxicity. *Toxicology* 2005; 216:106–121.
- Szebeni J, Fontana JL, Wassef NM, Mongan PD, Morse DS, Dobbins DE, Stahl GL, Bünger R, Alving CR. Hemodynamic changes induced by liposomes and liposome-encapsulated hemoglobin in pigs: A model for pseudoallergic cardiopulmonary reactions to liposomes. Role of complement and inhibition by soluble CR1 and anti-C5a antibody. *Circulation* 1999;99:2302–2309.
- Sakai H, Sou K, Tsuchida E. Hemoglobin-vesicles as an artificial oxygen carrier. *Methods Enzymol* 2009;465:363–384.
- Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 2004; 32:539–545.
- Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, Tsuchida E, Kai T, Otagiri M. Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* 2009;136:232–239.
- Yamazaki M, Aeba R, Yozu R, Kobayashi K. Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation* 2006;114:I220–I225.
- Cabrales P, Sakai H, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Oxygen transport by low and normal oxygen affinity hemoglobin vesicles in extreme hemodilution. *Am J Physiol Heart Circ Physiol* 2005;288:H1885–H1892.
- Plock JA, Rafatmehrn N, Sinovic D, Schnider J, Sakai H, Tsuchida E, Banic A, Erni D. Hemoglobin vesicles improve wound healing and tissue survival in critically ischemic skin in mice. *Am J Physiol Heart Circ Physiol* 2009;297:H905–H910.
- Sakai H, Cabrales P, Tsai AG, Tsuchida E, Intaglietta M. Oxygen release from low and normal P₅₀ Hb vesicles in transiently occluded arterioles of the hamster window model. *Am J Physiol Heart Circ Physiol* 2005;288:H2897–H2903.
- Yamamoto M, Izumi Y, Horinouchi H, Teramura Y, Sakai H, Kohno M, Watanabe M, Kawamura M, Adachi T, Ikeda E, Takeoka S, Tsuchida E, Kobayashi K. Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. *J Surg Res* 2009;151:48–54.
- Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J Biol Chem* 2008;283:1508–1517.
- Sakai H, Okuda N, Sato A, Yamaue T, Takeoka S, Tsuchida E. Hemoglobin encapsulation in vesicles retards NO and CO binding and O₂ release when perfused through narrow gas-permeable tubes. *Am J Physiol Heart Circ Physiol* 2010;298:H956–H965.
- Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 2000;279:H908–H915.
- Takeoka S, Ohgushi T, Terase K, Ohmori T, Tsuchida E. Layer-controlled hemoglobin vesicles by interaction of hemoglobin with a phospholipid assembly. *Langmuir* 1996;12:1755–1759.
- Izumi Y, Sakai H, Kose T, Hamada K, Takeoka S, Yoshizu A, Horinouchi H, Kato R, Nishide H, Tsuchida E, Kobayashi K. Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J* 1997;43: 289–297.
- Abe H, Azuma H, Yamaguchi M, Fujihara M, Ikeda H, Sakai H, Takeoka S, Tsuchida E. Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. *Artif Cells Blood Substit Immobil Biotechnol* 2007;35:157–172.
- Sou K, Tsuchida E. Electrostatic interactions and complement activation on the surface of phospholipid vesicle containing acidic lipids: Effect of the structure of acidic groups. *Biochim Biophys Acta* 2008;1778:1035–1041.
- Abe H, Fujihara M, Azuma H, Ikeda H, Ikebuchi K, Takeoka S, Tsuchida E, Harashima H. Interaction of hemoglobin vesicles, a cellular-type artificial oxygen carrier, with human plasma: Effects on coagulation, kallikrein-kinin, and complement systems. *Artif Cells Blood Substit Immobil Biotechnol* 2006;34:1–10.
- Warner AE. Pulmonary intravascular macrophages. Role in acute lung injury. *Clin Chest Med* 1996;17:125–135.
- Brain JD, Molina RM, DeCamp MM, Warner AE. Pulmonary intravascular macrophages: Their contribution to the mononuclear phagocyte system in 13 species. *Am J Physiol Lung Cell Mol Physiol* 1999;276:L146–L154.
- Garty I, Tal I, Kaynan A. Tc-99 m colloid lung uptake in a rare case of toxoplasmosis with liver involvement. *Clin Nucl Med* 1984;9:310–313.
- Sou K. Electrostatics of carboxylated anionic vesicles for improving entrapment capacity. *Chem Phys Lipids* 2011;164:211–215.
- Goins B, Phillips WT, Klipper R, Rudolph AS. Role of complement in rats injected with liposome-encapsulated hemoglobin. *J Surg Res* 1997;68:99–105.
- Loughrey HC, Bally MB, Reinish LW, Cullis PR. The binding of phosphatidylglycerol liposomes to rat platelets is mediated by complement. *Thromb Haemostasis* 1990;64:172–176.
- Tsuchida E, Sou K, Nakagawa A, Sakai H, Komatsu T, Kobayashi K. Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry. *Bioconjug Chem* 2009; 20:1419–1440.
- Pape A, Kertscho H, Meier J, Horn O, Laout M, Steche M, Lossen M, Theisen A, Zwissler B, Habler O. Improved short-term survival with polyethylene glycol modified hemoglobin liposomes in critical normovolemic anemia. *Intensive Care Med* 2008;34:1534–1543.
- Bradley AJ, Maurer-Spurej E, Brooks DE, Devine DV. Unusual electrostatic effects on binding of C1q to anionic liposomes: Role of anionic phospholipid domains and their line tension. *Biochemistry* 1999;38:8112–8123.
- Chang TM, Lister CW. Assessment of blood substitutes: II. In-vitro complement activation of human plasma and blood for safety studies in research, development, industrial production and pre-clinical analysis. *Artif Cells Blood Substit Immobil Biotechnol* 1994;22:171–180.
- Bradley AJ, Devine DV, Ansell SM, Janzen J, Brooks DE. Inhibition of liposome-induced complement activation by incorporated poly(ethylene glycol)-lipids. *Arch Biochem Biophys* 1998;357: 185–194.
- Winkler GC. Pulmonary intravascular macrophages in domestic animal species: Review of structural and functional properties. *Am J Anat* 1988;181:217–234.
- Albertine KH, Staub NC. Vascular tracers alter hemodynamics and airway pressure in anesthetized sheep. *Microvasc Res* 1986;32: 279–288.
- Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: Influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* 2001;159:1079–1088.
- Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suetatsu M, Takaori M, Kobayashi K, Tsuchida E. Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* 2004;311:874–884.

37. Longworth KE, Westgate AM, Grady MK, Westcott JY, Staub NC. Development of pulmonary intravascular macrophage function in newborn lambs. *J Appl Physiol* 1992;73:2608–2615.
38. Miyamoto K, Schultz E, Heath T, Mitchell MD, Albertine KH, Staub NC. Pulmonary intravascular macrophages and hemodynamic effects of liposomes in sheep. *J Appl Physiol* 1988;64:1143–1152.
39. Miyamoto K, Nakano T, Aoi K, Aida A, Kawakami Y. Effects of differently composed liposomes on pulmonary arterial pressure in sheep—involvement of pulmonary intravascular macrophages. *Nihon Kyobu Shikkan Gakkai Zasshi* 1991;29:1268–1274.
40. Wassef NM, Johnson SH, Graeber GM, Swartz GM Jr, Schultz CL, Hailey JR, Johnson AJ, Taylor DG, Ridgway RL, Alving CR. Anaphylactoid reactions mediated by autoantibodies to cholesterol in miniature pigs. *J Immunol* 1989;143:2990–2995.
41. Sakai H, Hisamoto S, Fukutomi I, Sou K, Takeoka S, Tsuchida E. Detection of lipopolysaccharide in hemoglobin-vesicles by Limulus amoebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. *J Pharm Sci* 2004;93:310–321.
42. Szebeni J, Wassef NM, Rudolph AS, Alving CR. Complement activation by liposome-encapsulated hemoglobin in vitro: The role of endotoxin contamination. *Artif Cells Blood Substit Immobil Biotechnol* 1995;23:355–363.
43. Zeltner TB, Caduff JH, Gehr P, Pfenninger J, Burri PH. The post-natal development and growth of the human lung. I. Morphometry. *Respir Physiol* 1987;67:247–267.
44. Taguchi K, Miyasato M, Ujihira H, Watanabe H, Kadowaki D, Sakai H, Tsuchida E, Horinouchi H, Kobayashi K, Maruyama T, Otagiri M. Hepatically metabolized and -excreted artificial oxygen carrier, hemoglobin vesicles, can be safely used under conditions of hepatic impairment. *Toxicol Appl Pharmacol* 2010;248:234–241.



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Liposome-encapsulated hemoglobin (hemoglobin-vesicle) is not transferred from mother to fetus at the late stage of pregnancy in the rat model[☆]

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ABSTRACT

Aims: Liposome-encapsulated hemoglobin (hemoglobin vesicles: HbV; diameter 250 nm) is reconstructed from human hemoglobin and developed as an artificial oxygen carrier for use as a transfusion alternative. Previous studies using rodent models closely investigated the safety of daily repeated infusions (DRI) of HbV and reported that the reticuloendothelial system was physiologically capable of degrading HbV to maintain plasma clinical chemistry within normal ranges. The present study examined the effect of DRI of HbV on the pregnant rat mother and fetal development, focusing on placental transfer of HbV in pregnancy.

Main methods: Pregnant rats intravenously received HbV bolus injections at 2 ml/kg/day for the last 7 consecutive days till term. The cumulative infusion volume (14 ml/kg) was equal to 25% of the whole blood volume (56 ml/kg).

Key findings: Maternal DRI of HbV had no obvious side effects on the pregnant mother or on fetal development. Maternal vital signs, plasma clinical chemistry, and blood gas parameters were overall normal after DRI of HbV. In addition, maternal/fetal transfer of HbV was limited to the placenta and HbV did not reach the fetus. Histopathological examination with human hemoglobin antibody detected HbV accumulation in the maternal spleen, liver, kidney, and placenta, but not in the fetuses. These results were also confirmed by a pharmacokinetic study using ¹²⁵I-labeled HbV.

Significance: This safety study of HbV use in the pregnant mother and fetus will contribute to a possible application of HbV as a potential treatment for fetal hypoxia by supplying oxygen through the placenta.

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Introduction

Phospholipid vesicles or liposomes have been extensively studied as potential carriers of functional (macro) molecules for drug delivery

Abbreviations: Hb, hemoglobin; HbV, hemoglobin vesicle(s); Hct, hematocrit; PEG, polyethylene glycol; RBC, red blood cell count; WBC, leucocyte counts; PLT, platelet counts; DRI, daily repeated infusion(s); DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DHSG, 1,5-*O*-dihexadecyl-*N*-silycynyl-*l*-glutamate; MAP, mean arterial pressure; HR, heart rate; AST, aspartate aminotransferase; ALT, alanine aminotransferase; LDH, lactate dehydrogenase; CPK, creatine phosphokinase; TG, triglyceride; FFA, free fatty acid; UA, uric acid; BUN, urea nitrogen; CRE, creatinine; UIBC, unsaturated iron binding capacity; IP, inorganic phosphorus; PT, prothrombin time; APTT, activated partial thromboplastin time; SP-A, surfactant protein-A.

[☆] No placental transfer of HbV from mother to fetus.

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systems, with some already being approved for clinical use in antifungal or anticancer therapies (Lian and Ho, 2001). In addition to their use for transporting drugs, phospholipid vesicles encapsulating concentrated hemoglobin (Hb) have also been developed as artificial oxygen carriers. These so-called liposome-encapsulated Hb or Hb vesicles (HbV) have been shown in several metabolic studies to be able to transport levels of oxygen comparable with those of blood (Djordjevich et al., 1987; Chang et al., 1992; Izumi et al., 1997; Phillips et al., 1999; Sakai et al., 2004a). Possible applications for treatment of hypoxia-induced pathology such as brain ischemia have also been clarified in animal models (Komatsu et al., 2007).

In HbV safety studies using adult rodents, it has been clarified that the cellular structure and the size of the HbV are advantageous for maintaining a steady blood circulation without vasoconstriction and hypertension (Sakai et al., 2000a) and that the surface modification of HbV with polyethylene glycol (PEG) is beneficial not only for providing a longer circulation time (Phillips et al., 1999) but also for its ability to

suppress intervesicular aggregation of HbV for years during storage and also during the plasma phase in peripheral tissues after intravenous infusion (Sakai et al., 1998, 2000b). In the degradation and excretion processes of the HbV components also, the reticuloendothelial system has been reported to have sufficient physiological capacity, even at doses greater than putative clinical doses, to maintain plasma iron and bilirubin levels within normal ranges (Sakai et al., 2004b; Taguchi et al., 2009a, b).

To date, no safety study of HbV use in pregnant mothers and fetuses has been reported. The aim of the present study is to investigate the effects of HbV on the physiology of the pregnant mother and on placental/fetal homeostasis, as well as to identify any potential side effects, for possible future clinical applications of HbV during the perinatal period. These applications could include the provision of HbV to supply oxygen through the placenta as a treatment for fetal hypoxia. A rat placental model was selected due to its broad application in the study of placental development and reproduction. For the period for HbV application in pregnant rats, we chose the middle-late stage of pregnancy, in which the rat chorioallantoic placenta begins differentiating into two distinct zones: the maternal-facing junctional zone for the endocrine/invasive functions, and the adjacent fetal-facing labyrinthine responsible for maternal–fetal exchange (Soares, 1997). Since the rat placental barrier is not fully differentiated until the latter period of pregnancy, the last trimester was selected for this study of HbV infusion. From embryonic day 16 (E16) to E22, HbV was administered at a daily dose of 2 ml/kg, which had been previously investigated as a possible treatment for acute brain ischemia in adult rat models (Kakehata et al., 2010; Sakai et al., 2004c). The present study is the first to report the safety and possible side effects of HbV applications on the pregnant mother and fetus, and closely examines maternal–fetal transfer of HbV.

Materials and methods

Preparation of HbV suspension

The test fluid, the HbV suspension, was prepared under sterile conditions as reported previously (Sakai et al., 2000b; Sou et al., 2003). Human Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM pyridoxal 5'-phosphate (Aldrich Chemical Co., Milwaukee, WI) as an allosteric effector at a molar ratio of pyridoxal 5'-phosphate/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-O-dihexadecyl-N-succinyl-L-glutamate (DHSG) at a molar ratio of 5:5:1 (Nippon Fine Chemicals Co., Osaka, Japan), and 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-PEG5000 (NOF Co., Tokyo, Japan). Thus, the vesicular surface is covered with PEG chains. The molar composition of DPPC/cholesterol/DHSG/1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine-N-PEG5000 was 5:5:1:0.033. HbVs were suspended in normal saline, sterilized using filters (pore size, 0.45 μ m, Dismic; Toyo Roshi Co., Tokyo, Japan), and deoxygenated with bubbling N₂ for storage (Sakai et al., 2000b). The physicochemical parameters of the HbV are as follows: particle diameter, 252 \pm 53 nm; [Hb], 10 g/dl; [lipids], 6–7 g/dl; and oxygen affinity (P₅₀), 25–28 Torr. The endotoxin content was measured by a modified *Limulus* amoebocyte lysate gel-clotting analysis (Wako Pure Chemicals, Tokyo, Japan) and was less than 0.2 endotoxin unit/ml.

Daily repeated infusion (DRI) of HbV

Animal care and use were reviewed and approved by the Committee for Animal Research of Tohoku University (approval#2010-73 medical school), Kumamoto University (approval#C22-161) and the National Center of Neurology and Psychiatry (approval#2011023). The experiments were carried out using timed pregnant Wistar rats

(vaginal smear positive, day 0; term, day 23) purchased from Japan SLC, Inc. (Hamamatsu, Shizuoka, Japan). All the rats were provided with food and water ad libitum in a temperature-controlled room on a 12-h light/dark cycle.

On day 12 of pregnancy, rats were anesthetized by isoflurane inhalation and then pentobarbital sodium i.p. and the catheter was surgically secured in the internal jugular vein. From days 16 to 22 of pregnancy, rats received daily repeated injections (DRIs) of HbV (n=5) or saline (n=5) via the catheter at a dose rate of 2 ml/kg body weight with an injection rate of 1 ml/min. The total volume of the infused HbV into each rat over 7 days reached 14 ml/kg, which was equal to 25% of the actual blood volume of the rat (56 ml/kg). The total infused HbV is calculated to be 1400 mg Hb/kg (2 ml/kg/day \times 7 days), because [Hb] of the HbV fluid is adjusted to 10 g/dl. The rats were sacrificed on day 23 of pregnancy, 1 day after the final 7th infusion.

Radio-telemetry monitoring of maternal blood pressure and heart rate

The Dataquest IV telemetry system (Data Sciences International, St. Paul, MN, USA) was used for measurement of systolic pressure, diastolic pressure, mean arterial pressure, and heart rate. The monitoring system consists of a transmitter (radio frequency transducer model TA11PA), receiver panel, consolidation matrix, and personal computer with accompanying software. Before the device was implanted, calibrations were verified to be accurate within 63 mm Hg. On day 10 of pregnancy, rats were anesthetized by isoflurane inhalation and then pentobarbital sodium, and the flexible catheter of the transmitter was surgically secured in the abdominal aorta just below the renal arteries and pointing upstream (against the flow). The transmitter was sutured to the abdominal wall. Rats were housed in individual cages after the operation. Each cage was placed over the receiver panel, which was connected to the personal computer for data acquisition. The rats were unrestrained and free to move within their cages. Hemodynamic data were sampled every 5 min for 10 s. Preliminary experiments showed that blood pressure and heart rate took up to 4 days to stabilize postoperatively. Therefore, HbV treatment was commenced 4 days after surgery and telemetry data were collected until the day of necropsy.

Hematological test of maternal blood

Mother rats were sacrificed on day 23 of pregnancy, 1 day after the 7th day of DRI, and a one-time collection of 5 ml of blood was made from the abdominal aorta for hematological examination, blood gas analysis, plasma chemical examination, blood coagulation test and fibrinogen concentration measurement. 500 μ l of the drawn blood was collected in an EDTA tube and a complete blood count was run by a commercial laboratory (Mitsubisi Chemical Medience, Tokyo, Japan). In detail, total leucocyte counts (WBCs) were determined by flow cytometry; red blood cell counts (RBCs) and platelet counts (PLTs) were determined by electronic impedance counting method; total hemoglobin was determined by sodium lauryl sulfate-hemoglobin (SLS-Hb) method; and the hematocrit (Hct) value was determined by RBC pulse wave-height detection method.

Maternal hemodynamic and blood gas parameters, blood glucose level, and urinalysis

For the blood gas analysis, 200 μ l of the 5 ml maternal blood drawn on day 23 of pregnancy was injected into the cartridge of a pH/blood gas analyzer (HPM3600A i-STAT hand held blood analyzer; Hewlett Packard, Les Ulis, France) for analysis of the arterial blood O₂ tension, arterial blood carbon dioxide tension, pH, base excess, and lactate. Urinalysis was performed by dip-stick testing (UA-L08M; Terumo Co., Tokyo, Japan) as a qualitative measurement. A urine specimen of each rat was

collected in a transparent plastic bag, and a test stick was dipped into the collected urine. For each measured item, the level was judged by visual examination of the color identification after a specific time of exposure according to the instructions. Items were measured in the following order; protein (10 s), pH (10 s), occult blood (20 s), ketone body (20 s), urobilinogen (20 s), glucose (30 s), nitrite (30 s), and bilirubin (40 s).

Plasma clinical chemistry in maternal blood

Two ml of the 5 ml maternal blood drawn on day 23 of pregnancy was centrifuged to obtain plasma, as described in previous studies (Sakai et al., 2003, 2004d). The obtained transparent plasma specimens were stored in a freezer at -80°C until the clinical chemistry tests were performed at a commercial laboratory (Nagahama Life Science Laboratory, Oriental Yeast, Tokyo, Japan). The selected analytes were total protein, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), amylase, creatine phosphokinase (CPK), lipase, total cholesterol, cholesterol ester, free cholesterol, triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, uric acid (UA), urea nitrogen (BUN), creatinine (CRE), Na^{+} , K^{+} , Cl^{-} , Ca^{2+} , inorganic phosphorus (IP), unsaturated iron binding capacity (UIBC), and Fe^{3+} .

Maternal blood coagulation test and fibrinogen concentration

To test for blood coagulation following a 7 day DRI of HbV or saline, 1.8 ml of the 5 ml maternal blood drawn on day 23 of pregnancy was immediately mixed with 0.2 ml of 3.8% sodium citrate solution. The plasma fraction, obtained by gentle centrifugation, was analyzed for prothrombin time (PT), activated partial thromboplastin time (APTT), and fibrinogen at a commercial laboratory (Mitsubishi Chemical Medience, Tokyo, Japan).

Histopathological examination in maternal and fetal organs

On day 23 of pregnancy, the animals were finally sacrificed by acute bleeding from the abdominal aorta, and the liver, spleen, kidneys, placenta and pups were resected for weight measurements, while the remaining organs were obtained for histopathological study. They were fixed in 10% buffered formalin (Wako Pure Chemicals) immediately after removal, and the paraffin sections were stained with hematoxylin and eosin, and Berlin blue.

Another immunohistochemistry was also performed to detect human Hb from the injected HbV in the spleen, liver and placenta of the maternal pregnant rats and fetal liver. Four-micrometer-thick paraffin sections were mounted on 3-aminopropyl triethoxysilane-coated glass slides. The sections were treated with 0.03% H_2O_2 in methanol for 10 min at room temperature to block endogenous peroxidase activity. For antigen retrieval, the sections were also treated with proteinase K (0.4 mg/ml; Dako Cytomation California Inc., Carpinteria, CA) for 10 min at room temperature. The sections were treated with 5% normal swine serum for 30 min at room temperature and reacted with rabbit polyclonal antibodies against human Hb (1:500 dilution; Dako Cytomation A/S, Glostrup, Denmark) for 60 min at room temperature. They were further incubated with alkaline phosphatase-conjugated swine antibodies against rabbit immunoglobulins (1:100 dilution; Dako Cytomation A/S). Color development was performed using a New Fuchsin Substrate kit (Nichirei Co.), and the sections were counterstained with hematoxylin.

Pharmacokinetic analysis of maternal/fetal transfer of HbV

Administration and collection of blood and organs from rats

HbV labeled with iodine 125 (^{125}I -HbV) was prepared as previously described (Taguchi et al., 2009a, b). Before the pharmacokinetic

studies using ^{125}I -HbV, five pregnant Wistar rats were given water containing 5 mM sodium iodide (NaI) to avoid specific accumulation of the isotope in the glandula thyreoidea throughout the experiment. All rats were anesthetized with isoflurane and received a single injection of ^{125}I -HbV in the tail vein (1400 mg of Hb/kg), which is equivalent to the total volume of a 7 day DRI. Twelve hours after ^{125}I -HbV injection, blood was collected from the inferior vena cava, and plasma was obtained by centrifugation (3000 g, 5 min). After collection of blood, the animals were sacrificed for collection of organs, which were then rinsed with saline.

Measurement of ^{125}I radioactivity

To remove degraded protein and free ^{125}I , 1% bovine serum albumin (BSA) and 40% trichloroacetic acid (TCA) were added to the plasma, and pellets were obtained by centrifugation (1000 g, 10 min). The organs were weighed on an electronic balance. ^{125}I radioactivity was counted using a liquid scintillation counter (ARC-5000; Aloka, Tokyo, Japan).

Surfactant protein-A (SP-A) immunoblot analysis of fetal lung for assessing fetal maturation

Rat amniotic fluid (AF) was aspirated from exposed amniotic sacs of the rats on day 23 of pregnancy using a sterile 22-gauge half-inch needle. The AF was fractionated in gradient polyacrylamide gels (Invitrogen) and transferred onto Hybond-P (Amersham Pharmacia), as described previously (Condon et al., 2004). Blots were probed using a rabbit monoclonal antibody for SP-A (kindly donated by Dr. Kuroki, Sapporo Medical School, Japan); and with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:5,000) (Amersham Pharmacia) as the secondary antibody. Immunoreactive bands were visualized by using ECL-detection (Amersham Pharmacia).

Data analysis

Differences between the control and the treatment groups were analyzed using a one-way analysis of variance followed by Fisher's protected least significant difference test. The changes were considered statistically significant if $p < 0.05$.

Results

Body weight of pregnant rat mother and fetus

The body weight of pregnant rats in the saline control (baseline: 0 day, 243.2 ± 26.0 g) and HbV groups (baseline: 0 day, 265.6 ± 12.2 g) showed significant increases after the 7-day DRI period and reached 299.8 ± 19.8 g and 315.8 ± 38.5 g, respectively (Fig. 1a; one-way ANOVA followed by Tukey's test, $p < 0.01$); no statistical difference was noted in maternal body weights between the saline and HbV group on both 0 day (E16) (saline 243.2 ± 26.0 g vs HbV 265.6 ± 12.2 g) and 7 days (E23) (299.8 ± 19.8 g vs 315.8 ± 38.5 g) of HbV DRI (Fig. 1a; one-way ANOVA).

Organ weights

A previous study indicated that spleen and liver were the main organs that trap and degrade HbV. As shown in Fig. 1d and e, however, no significant weight differences between the saline and HbV groups were observed, indicating no splenomegaly or hepatomegaly after the 7 day DRI. No weight difference between the saline and HbV groups was detected after the 7 day DRI in other organs including the kidney (Fig. 1f, *t*-test, $p > 0.05$).

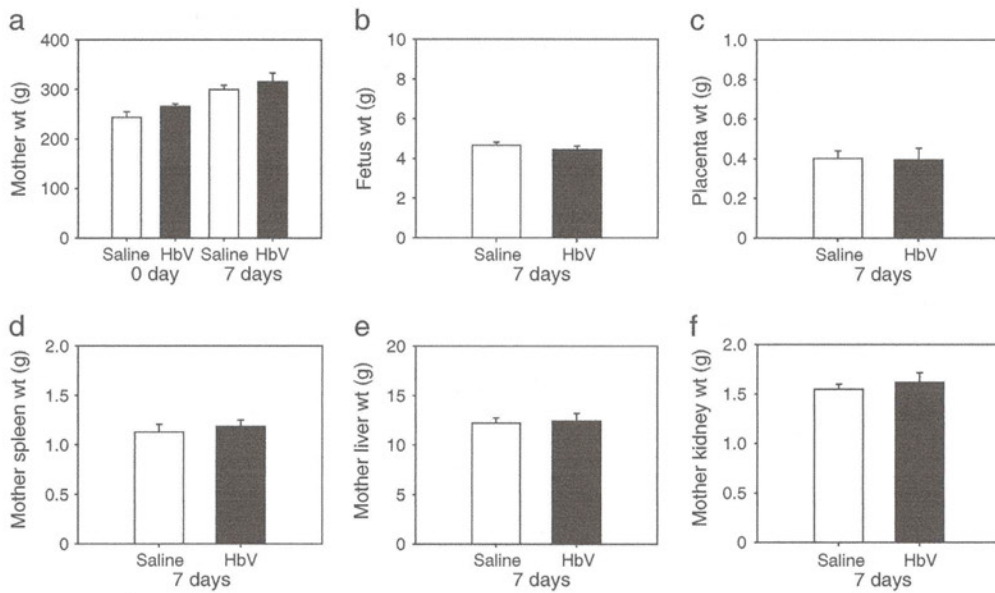


Fig. 1. The weights of pregnant rats and fetuses and maternal organ weights after the 7 day DRI (daily repeated infusions) of HbV or saline. (a) Time course of the gains in body weights of pregnant mother rats before and after DRI of HbV or saline for 7 days at a dose rate of 2 ml/kg/day (n = 5 for each group; value: average ± s.d.). The weights of fetuses (b) and the placentas (c) after the 7 day DRI of HbV or saline (n = 5 for each group; value: average ± s.d.). Organ weights (spleen (d), liver (e), and kidney (f)) of pregnant rats after the 7 day DRI of HbV or saline (n = 5 for each group; value: average ± s.d.).

Hematological tests

No significant difference was detected in Hct, WBC, RBC and PLT between the saline and HbV groups (Fig. 2) although the WBC and PLT in the HbV groups were slightly greater than those in the saline

group with no statistical significance: Hcts (%) were 32.0 ± 1.5 for the saline group and 32.8 ± 1.2 for the HbV group; WBCs ($\times 10^2/\mu\text{l}$) were 109.20 ± 8.23 for the saline group and 136.20 ± 18.90 for the HbV group; RBCs ($\times 10^4/\mu\text{l}$) were 518.8 ± 27.3 mg/dl for the saline group and 553.0 ± 18.3 mg/dl for the HbV group; and PLTs

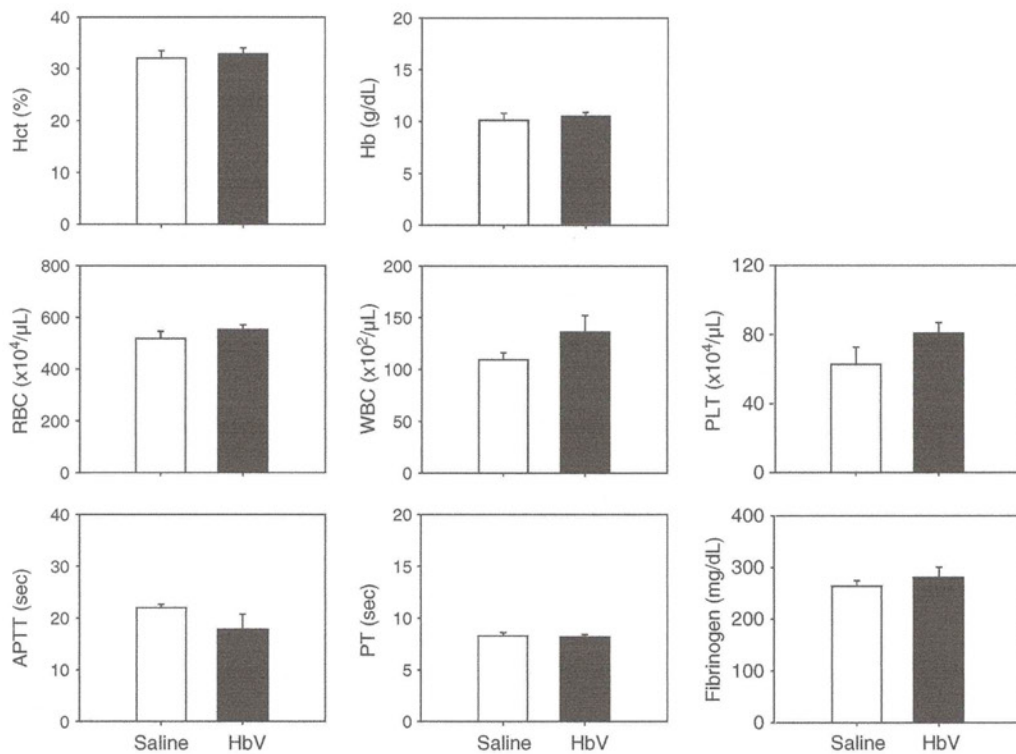


Fig. 2. Hct, blood cell counts, and blood coagulation parameters of pregnant rats after the 7 day DRI of HbV or saline at a dose rate of 2 ml/kg/day (n = 5 for each group; value: average ± s.d.).

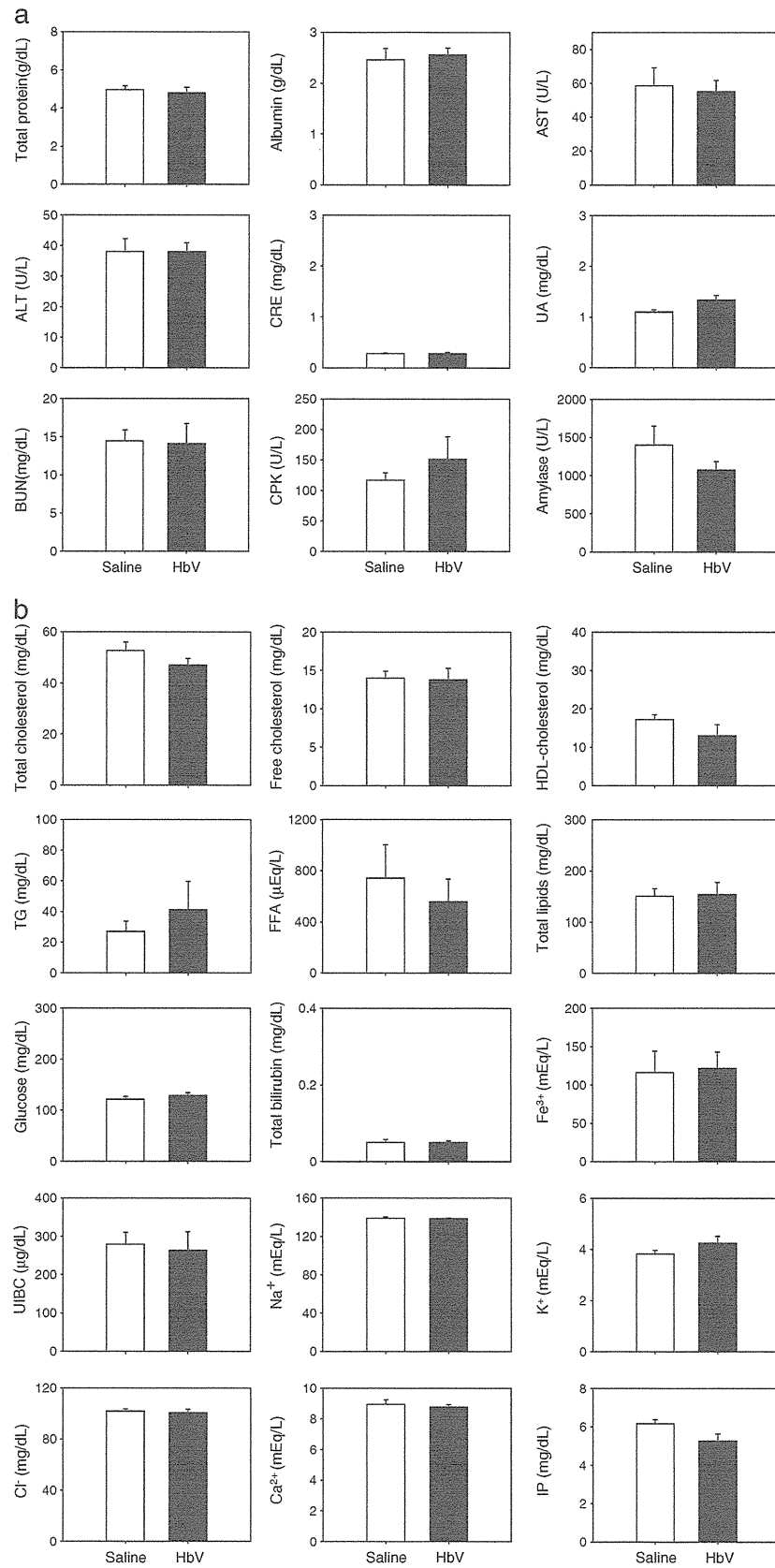


Fig. 3. a and b. Plasma clinical chemistry tests of pregnant rats after the 7 day DRI of HbV or saline (n=5 for each group; value: average ± s.d.).

($\times 10^4/\mu\text{l}$) were 62.7 ± 9.9 mg/dl for the saline group and 80.8 ± 6.1 mg/dl for the HbV group.

Coagulation test

No significant difference was detected in APTT, PT, and fibrinogen concentration between the saline and HbV groups although relatively large individual variations were observed in APTT: APTTs were 22.0 ± 1.4 s for the saline group and 17.8 ± 6.5 s for the HbV group; PTs were 8.3 ± 0.7 s for the saline group and 8.2 ± 0.6 s for the HbV group; and fibrinogen concentrations were 264 ± 23 mg/dl for the saline group and 281 ± 45 mg/dl for the HbV group (Fig. 2).

Blood pressure, heart rate, and blood gas parameters

Table 1 summarizes the blood gas parameters, blood glucose level, mean arterial pressure (MAP), and heart rate (HR) after the 7 day DRI of HbV or saline. There was no significant difference in any of the parameters between the saline and HbV groups. All parameters were within normal ranges.

Urinalysis

Table 2 summarizes the results of the urinalysis. The color of the urine was normal for all groups and there were no signs of hemoglobinuria. Both the HbV and saline groups showed a low level of the proteinuria, which is consistent with a previous report (Sakai et al., 2004b). Urobilinogen and bilirubin were within the normal range in spite of the large amount of HbV infused.

Plasma clinical chemistry

The HbV particles in the plasma are easily removed from the plasma by ultracentrifugation (50,000 g; 20 min) (Sakai et al., 2003), and any interference effect of HbV is avoided in the colorimetric and turbidimetric analyses in the plasma clinical chemistry. The parameters for the liver function (total protein, albumin, AST, ALT) did not show any significant differences between the saline and HbV groups ($p > 0.05$) (Fig. 3a).

The parameters affecting the function of the kidneys (CRE, uric acid, and BUN) and those reflecting the heart and skeletal muscle (CPK) varied within normal ranges and no statistical differences were detected between the saline and HbV groups ($p > 0.05$). The parameters of pancreatic function, amylase activity, also did not show any significant changes between the saline and HbV groups ($p > 0.05$) (Fig. 3a).

The concentrations of the cholesterol component (total, free and HDL-cholesterols), lipids (triacylglycerol, free fatty acid, total lipids) and glucose were not significantly different between the saline and HbV groups after the 7 day DRI (Fig. 3b). No differences were also observed in bilirubin and ferric iron (Fe^{3+}), which should be released from Hb decomposition. UIBC (unsaturated iron binding capacity) also did not differ between the saline and HbV groups. The electrolyte concentrations varied within the normal range.

Table 1

Blood gas parameters, blood glucose level, MAP, and HR after the 7 day DRI of HbV or saline (value: average \pm s.d.).

Parameters	Saline	HbV
pH	7.44 ± 0.02	7.45 ± 0.05
PaCO ₂ (Torr)	36 ± 2	35 ± 5
PaO ₂ (Torr)	93 ± 12	94 ± 20
Lactate (mM)	1.78 ± 0.59	2.14 ± 0.73
Base excess (mM)	0.6 ± 2.1	-0.4 ± 1.8
Glucose (mg/dl)	91 ± 10	116 ± 20
MAP (mm Hg)	103 ± 3	112 ± 9
HR (beats/min)	388 ± 34	405 ± 48

Table 2

Urinalysis for the HbV and saline control groups after the 7 day DRI of HbV or saline (n = 5 for each group.).

Parameters	Levels	N = 5/5	
		After the 7 day DRI	
		Saline	HbV
Protein	–	1	0
	±	1	3
	+	3	2
	++	0	0
	+++	0	0
pH	5	0	0
	6	1	1
	7	2	0
	8	2	3
	9	0	1
Occult blood	–	5	5
	±	0	0
	+	0	0
	++	0	0
	+++	0	0
Ketone body	–	4	2
	±	1	3
	+	0	0
	++	0	0
	+++	0	0
Urobilinogen	–	0	0
	±	5	5
	+	0	0
	++	0	0
	+++	0	0
Glucose	–	5	5
	±	0	0
	+	0	0
	++	0	0
	+++	0	0
Nitrite	–	5	5
	+	0	0
	–	5	5
Bilirubin	±	0	0
	+	0	0
	++	0	0
	+++	0	0
	–	0	0

Histopathological examination

After 7 days of DRI, immunohistochemical analysis using anti-human Hb-antibody causing human Hb in HbV to show up as pink areas detected human Hb in the maternal liver, spleen, kidney and in the junctional zone or labyrinth of the placenta, but could not detect HbV in any organs of the fetus such as the fetal liver (Fig. 4). In addition, Berlin blue staining, which detects hemosiderin, indicated Hb decomposition in the spleen, liver, kidney and junctional zone of the placenta, but could not detect hemosiderin in the labyrinth of the placenta or fetal liver (Fig. 5).

Pharmacokinetic analysis of maternal/fetal transfer of HbV

The fate of the ^{125}I -HbV administered to pregnant rats was evaluated as residual TCA-precipitable radioactivity in the plasma. Fig. 6 shows the tissue distribution in each organ 12 h after injection of ^{125}I -HbV. ^{125}I -HbV was distributed mainly in the plasma, spleen, and liver in rat mothers while the distribution of ^{125}I -HbV was significantly low in most other organs. Significantly low distributions of ^{125}I -HbV were also observed in the fetal organs such as the fetal liver, suggesting no maternal/fetal transfer of HbV but possible transfer of ^{125}I detached from HbV.

Augmented secretion of SP-A into AF during late gestation

SP-A gene expression serves as an excellent marker of fetal lung maturity (Mendelson and Boggaram, 1991). No significant difference

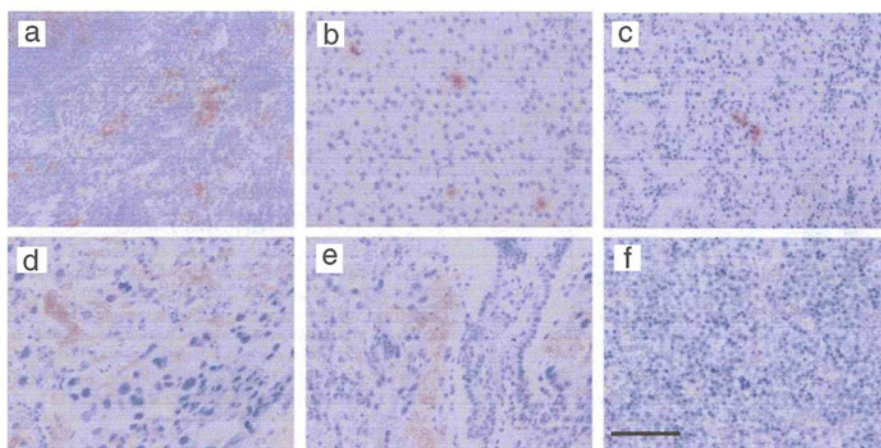


Fig. 4. Immunohistochemical staining for human Hb antibody in HbV in the spleen (a), liver (b), kidney (c), junctional zone (d) and labyrinth (e) of the placenta of pregnant rats and fetal liver (f) after the 7 day DRI of HbV. The tissues were stained with human Hb antibody. The pink-colored areas indicate the presence of a large amount of HbV. Scale bar, 100 μ m.

in SP-A protein was detected between the saline and the HbV group (Fig. 7) on day 23 of pregnancy, the day of the expected birth (one-way ANOVA).

Discussion

The present study examined the effects of maternal intravenous administration of HbV by 2 ml/kg/day for 7 days on pregnant rats and fetuses. Our primary finding is that all the pregnant rats and fetuses tolerated DRI (daily repeated infusion) of HbV well with no deteriorative signs in maternal organ functions or fetal development. In addition, we have demonstrated that the placental transfer of HbV did not occur after the 7 day DRI of HbV at the late stage of pregnancy.

In the pregnant rat mothers, the body weights increased in the same manner from E16 to E23 between the saline and HbV groups during DRI and no significant difference in maternal weights between the groups was detected. In maternal general conditions, HbV infusion induced no signs of hypertension or blood hyperviscosity. In the complete blood counts, none of the parameters showed any difference between the groups. The leukocyte cell and platelet counts slightly increased for the HbV group with no statistical difference, and CRP, an inflammation-induced peptide, did not increase and remained

negative. No splenomegaly or hepatomegaly was recognized after the 7 day DRI in the present study. A large amount of HbV accumulated in the red pulp zone of the spleen and in Kupffer cells of the liver, however levels of hepatic enzyme concentrations were found to be normal. In renal functions, no abnormal values were noted for CRE, UA, or BUN, although urinalysis showed a slight increase in protein levels. The mesangial cells in the renal glomerulus entrapped HbV in their intracellular spaces as detected by anti-human Hb-antibody staining. In lipid metabolism, the plasma lipid components did not differ between the two groups although relatively high individual variations were found in TG and FFA.

In fetuses, like pregnant mother rats, body weights did not differ between the saline and HbV groups after DRI nor was any significant difference detected between the placenta weights of the two respective groups. Fetal maturation assessed by SP-A in the amniotic fluid showed no significant difference between the HbV and the saline group, indicating that HbV may not have a negative influence over fetal lung maturation. Corresponding to fetal lung maturity, SP-A mRNA is first detectable in rodent fetal lung at the late stage of pregnancy and increases to term (Mendelson and Boggaram, 1991; Alcorn et al., 1999). SP-A protein in rat amniotic fluid was reported to be evident at 18 days of pregnancy and increased markedly through term

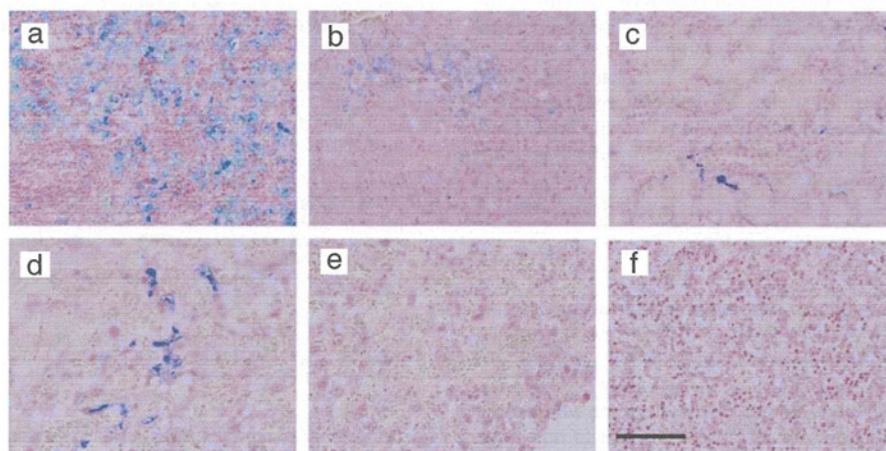


Fig. 5. Immunohistochemical staining for hemosiderin by Berlin blue in the spleen (a), liver (b), kidney (c), junctional zone (d) and labyrinth (e) of the placenta of pregnant rats and fetal liver (f) after the 7 day DRI of HbV. Scale bar, 100 μ m.

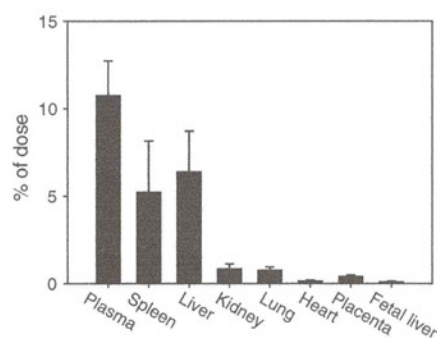


Fig. 6. Tissue distributions of ¹²⁵I-HbV at 12 h after administration to pregnant rats. Rats received a single injection of ¹²⁵I-HbV from the tail vein of a dose of 1400 mg/kg. 12 h after injection, each organ was collected (n = 5 for each group; value: average ± s.d.).

(Sakai et al., 1994). Since fetal lung maturation is one of the most important indicators reflecting fetal development, these data suggest that rat fetuses generally developed well during the 7 days of HbV infusion.

Regarding placental transfer of HbV, the present in vivo examination using ¹²⁵I-HbV demonstrated that significantly low signals were detected from fetal liver, indicating that HbV did not cross the placenta after a dose of 1400 mg/kg, which is equivalent to the total applied during the full 7 days of DRI. In the full-term placenta of the rat, horseradish peroxidase, a tracer of the diameter of approximately 0.6 nm (Osicka and Comper, 1998), does not penetrate beyond the layer II, the main barrier of the rat placenta (Metz et al., 1978; Tomi et al., 2011). Since the hemoglobin vesicle (HbV; diameter 252 ± 53 nm) has an approximately 400 times larger molecular size than the horseradish peroxidase, the HbV are not likely to be transferred from maternal to fetal compartments through the rat placenta. The result of the ¹²⁵I-HbV study showing no placental transfer of HbV was also supported by immunohistochemical analysis using anti-human Hb-antibody, which detected no HbVs in the fetal organs such as the fetal liver. This is also consistent with the results of Berlin Blue staining portions, which reflect the presence of excessive amounts of hemosiderin, most likely to be from HbV, in the maternal spleen, liver, kidney and placenta but not in the fetal liver. In pregnant rat mothers, a relatively large amount of the ¹²⁵I-HbV dose was distributed in the plasma, spleen and liver, which is consistent with a previous report (Taguchi et al., 2009a, b; Taguchi et al., 2011). Since HbV possesses a liposome structure, they are expected to be captured by the MPS (mononuclear phagocyte system) in the spleen and liver (Kiwada et al., 1998). Although phospholipid and other hemoglobin-deprived proteins decomposed from HbV are reported to be safely metabolized in the MPS (Sakai et al., 2008; Taguchi et al., 2009a), further studies are still required to examine the effects of these decomposed substances on the fetus, which pass through the complex placental transport system which includes enzymes, transporters and channels.

Conclusion

In conclusion, we have shown that the pregnant rats and fetuses tolerated daily repeated infusion (DRI) of HbV with no detrimental signs to pregnancy or organ functions and also that no detectable placental transfer of HbV occurred during the late stage of pregnancy. Although further investigations are required to elucidate whether the same effects occur in humans, our results are the first to report the safety of Hb-based oxygen carriers including HbV for application to pregnant mothers and fetuses using an animal model, and have also provided important information for possible HbV clinical applications

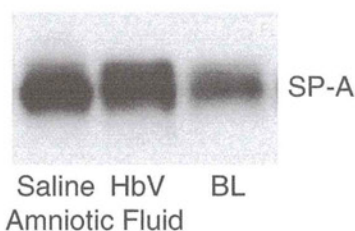


Fig. 7. Fetal lung maturation assessed by surfactant protein-A (SP-A). Amniotic fluid isolated from pregnant rats at 23 days of pregnancy was analyzed for SP-A by immunoblotting. Shown is a representative immunoblot of amniotic fluid from the saline and the HbV group, and of bronchoalveolar lavage (BL) from an intact rat as a positive control. No clear difference in the amount of SP-A between the saline and the HbV groups was observed, suggesting that the fetuses in the HbV group had as matured lungs as those in the saline group (n = 5 for each group).

for addressing fetal hypoxic conditions induced by pathogenic placenta during pregnancy.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgment

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References

- Alcorn JL, Hammer RE, Graves KR, Smith ME, Maika SD, Michael LF, et al. Analysis of genomic regions involved in regulation of the rabbit surfactant protein A gene in transgenic mice. *Am J Physiol* 1999;277:L349–61.
- Chang TM, Lister C, Nishiyama, Varma R. Immunological effects of hemoglobin, encapsulated hemoglobin, polyhemoglobin and conjugated hemoglobin using different immunization schedules. *Biomater Artif Cells Immobil Biotechnol* 1992;20:611–8.
- Condon JC, Jayasuria P, Faust JM, Mendelson CR. Surfactant protein secreted by the maturing mouse fetal lung acts as a hormone that signals the initiation of parturition. *PNAS* 2004;101:4978–83.
- Djordjevic L, Mayoral J, Miller IF, Ivankovich AD. Cardiorespiratory effects of exchanging transfusions with synthetic erythrocytes in rats. *Crit Care Med* 1987;15:318–23.
- Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, et al. Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. *Crit Care Med* 1997;24:1869–73.
- Takehata J, Yamaguchi T, Togashi H, Sakuma I, Otani H, Morimoto Y, et al. Therapeutic potentials of an artificial oxygen-carrier, liposome-encapsulated hemoglobin, for ischemia/reperfusion-induced cerebral dysfunction in rats. *J Pharmacol Sci* 2010;114:189–97.
- Kiwada H, Matsuo H, Harashina H. Identification of proteins mediating clearance of liposomes using a liver perfusion system. *Adv Drug Deliv Rev* 1998;32:61–79.
- Komatsu H, Furuya T, Sato N, Ohta K, Matsuura A, Ohmura T, et al. Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. *Neurosci Lett* 2007;421:121–5.
- Lian T, Ho RJ. Trends and developments in liposome drug delivery systems. *J Pharm Sci* 2001;90:667–80.
- Mendelson CR, Boggaram V. Hormonal control of the surfactant system in fetal lung. *Annu Rev Physiol* 1991;53:415–40.
- Metz J, Aoki A, Forssmann WG. Studies on the ultrastructure and permeability of the hemotrichorial placenta. I. Intercellular junctions of layer I and tracer administration into the maternal compartment. *Cell Tissue Res* 1978;192:391–407.

- Osicka TM, Comper WD. Tubular inhibition destroys charge selectivity for anionic and neutral horseradish peroxidase. *Biochim Biophys Acta* 1998;1381:170–8.
- Phillips WT, Klipper RW, Awasthi VD, Rudolph AS, Cliff R, Kwasiborski V, et al. Polyethylene glycol-modified liposome-encapsulated hemoglobin: a long circulating red cell substitute. *J Pharmacol Exp Ther* 1999;288:665–70.
- Sakai K, Kohri T, Kweon MN, Kishino Y. A non-glycosylated form of pulmonary surfactant protein A appears in rat amniotic fluid. *Eur Respir J* 1994;7:88–93.
- Sakai H, Tsai AG, Kerger H, Park SI, Takeoka S, Nishide H, et al. Subcutaneous microvascular responses to hemodilution with a red cell substitute consisting of polyethyleneglycol-modified vesicles encapsulating hemoglobin. *J Biomed Mater Res* 1998;40:66–78.
- Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, et al. Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension in conscious hamster model. *Am J Physiol* 2000a;279:H908–15.
- Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. Polyethyleneglycol-conjugation and deoxygenation enable long-term preservation of hemoglobin vesicles as O₂ carriers in a liquid state. *Bioconjug Chem* 2000b;11:425–32.
- Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, et al. Pretreatment of serum containing Hb-vesicles (oxygen carriers) to avoid their interference in laboratory tests. *Clin Chem Lab Med* 2003;41:222–31.
- Sakai H, Horinouchi H, Masada Y, Takeoka S, Ikeda E, Takaori M, et al. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials* 2004a;25:4317–25.
- Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, et al. Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* 2004b;311:874–84.
- Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, et al. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 2004c;32:539–45.
- Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, et al. Detection of lipopolysaccharide in hemoglobin-vesicles by *Limulus* amoebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment with a surfactant. *J Pharm Sci* 2004d;93:310–21.
- Sakai H, Okamoto M, Ikeda E, Horinouchi H, Kobayashi K, Tsuchida E. Histopathological changes of rat brain after direct injection of Hb-vesicles (artificial oxygen carriers) and neurological impact in an intracerebral hemorrhage model. *J Biomed Mater Res A* 2008;90:1107–19.
- Soares MJ. Molecular mechanisms of placental development. In: Battaglia FC, editor. *Placental function and fetal nutrition*, 39. Philadelphia, PA: Nestec Ltd. Vevey/Lippincott-Raven Publishers; 1997. p. 31–45.
- Sou K, Naito Y, Endo T, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. *Biotechnol Prog* 2003;19:1547–52.
- Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, et al. Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* 2009a;136:232–9.
- Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, et al. Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* 2009b;37:1456–63.
- Taguchi K, Iwao Y, Watanabe H, Kadowaki D, Sakai H, Kobayashi K, et al. Repeated injection of high doses of hemoglobin-encapsulated liposomes (hemoglobin vesicles) induces accelerated blood clearance in a hemorrhagic shock rat model. *Drug Metab Dispos* 2011;39:484–9.
- Tomi M, Nishimura T, Nakashima E. Mother-to-fetus transfer of antiviral drugs and the involvement of transporters at the placental barrier. *J Pharm Sci* 2011;100:3708–18.



A Fourteen-Day Observation and Pharmacokinetic Evaluation after a Massive Intravenous Infusion of Hemoglobin-Vesicles (Artificial Oxygen Carriers) in Cynomolgus Monkeys

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Abstract

Hemoglobin-vesicles (HbV) are a cellular type hemoglobin-based oxygen carrier in which a concentrated hemoglobin solution is encapsulated within a phospholipid vesicle (liposome). Although it was previously revealed that HbV possesses a higher biocompatibility, low toxicity and no accumulation in the body in an animal model, these assessments were limited to the use of rodents, including mice, rats and rabbits as models. The aim of this study was to observe the effects of the administration of HbV in a nonhuman primate. For this purpose, cynomolgus monkeys were used as the model and the systematic response, serum biochemical analysis and pharmacokinetic properties were monitored for 14 days after a massive intravenous injection of HbV at a putative dose (1400 mg Hb/kg, 17.5 mL/kg). All of the monkeys tolerated the massive amount of injected HbV and survived, and no abnormal behavior was observed. The systematic response and serum biochemical analysis were overall normal, except for a transient elevation in alanine aminotransferase levels. In addition, the levels of phospholipids, total cholesterol and total bilirubin, metabolites of hemoglobin and lipid components of HbV, were increased after HbV administration. In the pharmacokinetic study, HbV was retained for a sufficient period to permit it to function as an alternative to red blood cells and showed good metabolic properties without accumulation in the bloodstream. In conclusion, this is the first report of biological reactions and a pharmacokinetic evaluation for a 14 day period after a massive intravenous injection of HbV in a primate. The results obtained in this study provide useful information, not only for the development of further optimized HbV but also for designing relevant and rational protocols for clinical trials.

Keywords: Hemoglobin; Liposome; Artificial blood; Cynomolgus monkey

Abbreviations: RBC: Red Blood Cells; HBOCs: Hemoglobin-Based Oxygen Carriers; HbV: Hemoglobin-Vesicles; Hb: Hemoglobin; PEG: Polyethylene glycol; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-Phosphatidylcholine; DHSG: 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate; PaO₂: arterial blood oxygen tension; PaCO₂: arterial blood carbon dioxide tension; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; γ -GTP: γ -glutamyltransferase; ALP: Alkaline Phosphatase; BUN: Blood Urea Nitrogen; TG: Triglyceride; HDL-Cholesterol: High-Density Lipoprotein; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MPS: Mononuclear Phagocyte System

Introduction

There is now little doubt that the transfusion of red blood cells (RBC) is an indispensable procedure in the treatment of patients with massive hemorrhages. However, conventional RBC transfusions still have the potential for blood-type mismatching, infections by unrecognized pathogens, hepatitis, human immunodeficiency virus or West Nile virus etc. In addition, it is difficult to maintain a steady supply of RBC at a time of a disaster during military conflicts and problems associated with its short 2-3 week preservation period. To overcome these problems, various artificial oxygen carriers, such as perfluorocarbon-based oxygen carriers, synthetic Fe²⁺ porphyrin-based materials, acellular type hemoglobin-based oxygen carriers (HBOCs) and cellular type HBOCs, have been under development worldwide [1-4]. Pre-clinical and clinical trials dealing with the above systems

indicate that perfluorocarbon-based oxygen carriers can induce chronic pneumonitis due to their insufficient excretion from the body and their accumulation in the lung [5], and some acellular type HBOCs lead to the development of myocardial lesions and an increase in mortality rates in humans [6]. Therefore, perfluorocarbon-based oxygen carriers and acellular type HBOCs can be excluded as possible candidates for artificial oxygen carriers even though they proceeded to the stage of clinical trials.

Hemoglobin-vesicles (HbV) are a type of cellular type HBOCs in which a concentrated human hemoglobin (Hb) solution is encapsulated in a liposome, the surface of which is covered with polyethylene glycol (PEG) [7]. The cellular structure of HbV most closely mimics the characteristics of a natural RBC such as the cell membrane function, which physically prevents Hb from coming into direct contact with the components of the blood and vasculature during its circulation. In addition, HbV has been shown to possess several superior characteristics

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to RBC transfusions including the absence of viral contamination [8], a long-term storage period of over 2 years at room temperature [9] and no need of cross-matching etc. Furthermore, the transport of oxygen by HbV is equivalent to RBC, after adjusting for the amount of allosteric effector [10], and HbV and RBC have comparable pharmacological effects in hemorrhagic shock animal models [11-13]. Based on these facts, HbV appears to have potential for use as an alternative to RBCs, and has considerable promise for use in clinical settings.

Since the dosage of HbV for use as a substitute for RBC is significantly greater than that for other commercial drugs, comparatively massive amounts of HbV and its associated components, including Hb and lipids, are introduced into the body. Because of the fact that massive amounts of HbV are used, Hb and lipids derived by HbV during metabolism and disposition could result in the accumulation of such components in blood or organs, and might cause a variety of adverse effects, such as hypertension, renal disease, arterial sclerosis and hyperlipidemia [14,15]. Therefore, it becomes necessary to develop an in-depth understanding of the biological reactions associated with a massive HbV administration at the preclinical trial stage. To date, several preclinical trials have evaluated the histology, biochemical analysis and pharmacokinetic properties after the administration of a putative dose of HbV. The results show that HbV possesses a low toxicity and is promptly metabolized (no accumulation in the body) even after a massive infusion [16-21]. Although pre-clinical studies (toxicity, pharmacology and pharmacokinetic etc.) have been carried out in multiple species including rodents, domestic animals and nonhuman primates, all of the aforementioned *in vivo* studies of HbV were limited to the use of rodents, such as mice, rats and rabbits. In considering nonhuman primates as animal models, rhesus monkeys and cynomolgus monkeys are particularly good models for observing the biological reactions of drugs at the preclinical trial stage. In addition, the results of such studies can be helpful in designing the most relevant and rational protocols for clinical studies. Therefore, before HbV proceeds to the stage of clinical trials, biological reactions after the administration of a massive amount of HbV need to be done using nonhuman primates.

Based on this background, we conducted the further evaluations of systemic response, serum biochemical analysis and pharmacokinetic properties for 14 days after massive intravenous injection of HbV at a putative dose in cynomolgus monkeys.

Materials and Methods

All studies were conducted at the Shin Nippon Biomedical Laboratories (Kagoshima, Japan). The study protocol was approved by the Shin Nippon Biomedical Laboratories Animal Care and Use Committee (IACUC707-004). All experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Shin Nippon Biomedical Laboratories.

Preparation of HbV particles and HbV test solution

HbV particles were prepared under sterile conditions, as previously reported [22]. Briefly, an Hb solution was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM of Pyridoxal 5'-phosphate as an allosteric effector to regulate oxygen affinity (P_{50}). The lipid bilayer was a mixture of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1, 5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (DHSG) at a molar ratio of 5/5/1, and 1, 2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG (0.3 mol%). The HbV particles were suspended in a physiological salt

solution, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and nitrogen gas was bubbled through the solution for storage. The properties of the HbV solution used in this study (Lot #: 1010) are shown in Table 1. Before the experiments, the HbV solution was mixed with a 25% recombinant human serum albumin solution (Nipro Corp., Osaka, Japan) to adjust the albumin concentration of the vesicle-suspension medium to 5 g/dL. Under these conditions, the colloid osmotic pressure of the suspension is maintained constant at approximately 20 mm Hg [22]. This solution was used for all experiments as the HbV test solution.

Animal and HbV test solution injection

Four cynomolgus male monkeys (5.57-5.93 kg) were treated with HbV test solution. From at least 10 days before the HbV administration, all animals were acclimatized to experimental conditions, such as fixture to a positioner, administration, blood sampling, and were maintained in a temperature-controlled room with a 12-hrs dark/light cycle with *ad libitum* access to water. The food intake was restricted to approximately 108 g per day with treats twice per week.

On day 0, the monkeys without anesthesia were fixed to the positioner. Subsequently, intravenous cannula were introduced into the cephalic vein of the forearm for infusion, and each animal received a single intravenous infusion of the HbV test solution, administered as a transfusion of 17.5 mL/kg (1400 mg Hb/kg), representing approximately 20% of total blood volume, at a rate of 1 mL/min as well as previous studies [4,19,21].

Measurement of blood pressure

Under unanesthetized conditions, monkeys were fitted with a blood pressure cuff on the upper arm, and both systolic and diastolic blood pressure were measured using a non-invasive blood pressure monitor (BP-8800NC, Omron Colin, Tokyo, Japan) at 7 days before administration (baseline). And 1, 6 hour, 1, 3, 7, 14 days after HbV test solution administration.

Blood sampling and measurement of blood gas, hematology and serum chemistry

At 7 days before administration (baseline), 1, 3, 7 and 14 day after the HbV test solution administration, arterial blood samples were collected from the femoral artery. Immediately after withdrawal, the arterial blood oxygen tension (P_{aO_2}), arterial blood carbon dioxide tension (P_{aCO_2}), the pH of all arterial blood samples were measured using a blood gas analyzer (i-STAT 300F; Abbott Point of Care Inc., Princeton, NJ). The venous blood samples were collected from the femoral vein at 7 days before administration (baseline), 7 and 14 day after the HbV test solution administration for the evaluation of serum chemistry. The venous blood was centrifuged (1710 g, 10 min) to obtain serum. The serum samples were then ultracentrifuged to remove HbV (50000 g, 30 min), because HbV interferes with some of the laboratory tests [23]. All serum samples were analyzed by a JCA-BM6070 or JCA-BM6050 (JEOL Ltd., Tokyo, Japan) instrument. The analyses performed were total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyltransferase (γ -GTP), alkaline

Solution	Hemoglobin (g/dL)	Lipids (g/dL)	P_{50} (mm Hg)	Diameter (nm)	Methemoglobin (%)
HbV	10.1	9.8	20.2	252.8	7.4

P_{50} , arterial blood oxygen tension at which hemoglobin is half-saturated with oxygen

Table 1: Solution properties of Hemoglobin-vesicles (HbV).

phosphatase (ALP), blood urea nitrogen (BUN), creatinine, uremic acid, lipase, amylase, triglyceride (TG), phospholipids, total cholesterol, high-density lipoprotein (HDL)-cholesterol, total bilirubin, sodium, potassium, calcium, chloride, glucose and inorganic phosphate.

Pharmacokinetic study

Under unanesthetized conditions, blood samples were collected from the femoral vein at multiple time points after administration of the HbV test solution (7 days before administration (baseline), immediately after finishing the administration (~1 min), 10 min, 30 min, 1, 3, 6, 12 hrs, 1, 2, 3, 7 and 14 days after administration) and the plasma was separated by centrifugation (1710 g, 15 min). The concentration of HbV in the plasma was evaluated by measuring the concentration of Hb in plasma, because HbV remains in the plasma phase after centrifugation. The Hb concentration of HbV in plasma was determined by a cyanomethemoglobin method (hemoglobin B test kit Wako: Wako Chemicals, Saitama, Japan).

Data analysis

Friedman's test was used for comparison of baseline and subsequent values. Data are means \pm SD for the indicated number of animals. A probability value of $p < 0.05$ was considered to indicate statistical significance.

Results and Discussion

Body weight and behavior

All monkeys survived up to 14 days after HbV administration and none were on the verge of death. Body weight before administration was 5773 ± 161 g, which increased slightly to 5893 ± 206 g at 14 days after HbV administration. These data indicate that the effect of HbV on physiological functions and the suppression of growth are negligible. Moreover, no abnormal behavior was observed, i.e., no changes in appearance of any reduction in appetite after HbV administration. It can therefore be concluded that HbV is not toxic to the cerebral nervous system.

Arterial pressure

Table 2 shows average values for systolic blood pressure and diastolic blood pressure for the 14 day follow-up period for the animals. Similar to reported data for the previous study [24], no significant changes were observed in either systolic or diastolic blood pressure immediately after HbV administration and during the experiment. It has been reported that acellular type HBOCs such as polymerized Hb or intramolecular cross-linked Hb can induce hypertension due to scavenging of the endogenous vasorelaxation factors nitric oxide and carbon monoxide [25-27]. On the other hand, the similarity of the HbV structure to RBC prevents the Hb from contact with endothelial cells,

which retard the reaction with endogenous nitric oxide and carbon monoxide [28,29]. Therefore, changes in systolic and diastolic blood pressure after HbV administration would not be expected.

Blood gas parameters and electrolytes

Table 3 summarizes the blood gas parameters for the 14 day follow-up period. No abnormal values or significant differences between before and after HbV administration were observed. In addition, no changes in electrolytes were observed during the experiment except for inorganic phosphate (Table 4). Although inorganic phosphate levels were significantly different at 14 day after HbV administration as compared to before HbV administration, these changes were within the normal range. Previous reports also showed, in the case of the healthy rat, that HbV administration did not affect blood gas parameters and electrolytes even though they infused HbV (950 mg Hb/kg/day) for 14 consecutive days, which was equal to 2.5 times the actual blood volume [18]. These collective findings indicate that HbV administration has no effect on blood gas parameters and electrolytes in all mammalian species.

Serum laboratory test

In a previous study, it was reported that an HbV suspension showed considerable interference effects in some analysis including colorimetric and turbidimetric analyses and chemical reactions that occur in some assays [21]. However, they can be easily removed from the plasma by ultracentrifugation, which substantially diminished these interfering components in clinical chemistry [23]. Therefore, no interference by HbV was detected in 22 serum laboratory tests, including electrolytes, as shown in Table 4, since it was removed from the serum by ultracentrifugation.

The parameters reflecting liver function (total protein, albumin, AST, ALT, γ -GTP and ALP) are shown in Figure 1. Among these parameters, no changes in total proteins, AST, γ -GTP and ALP were found at 7 and 14 days after HbV administration. While ALT was slightly increased at 7 day after HbV administration, their values returned to original levels within 14 day after HbV injection. Sakai et al. (2004) previously reported, in a healthy rat model, that ALT increased slightly after an HbV infusion at a dose of 2000 mg Hb/kg [16]. The liver is one of the main organs of the metabolism of HbV, because HbV particles are ultimately captured by the mononuclear phagocyte system (MPS), such as Kupffer cells [17-19]. Therefore, an extra load on the liver during the metabolism of the massive amounts of HbV might result in elevated ALT. In addition, albumin was also slightly changed at 14 days after HbV injection. However, it is possible that the effect of HbV administration on liver function was of no consequence, because these changes of ALT and albumin were transient and/or normal range and no changes in other parameters reflecting liver function were found (Figure 1).

	Pre	after HbV administration					
		(hour)		(day)			
		1	6	1	3	7	14
Systolic blood pressure (mmHg)							
Ave.	118.5 \pm 8.3	124.5 \pm 4.7	123.3 \pm 15.4	109.3 \pm 11.1	110.5 \pm 14.3	104.3 \pm 10.3	109 \pm 10.1
(range)	(110-130)	(120-129)	(103-139)	(99-125)	(94-129)	(93-114)	(101-122)
Diastolic blood pressure (mmHg)							
Ave.	64.3 \pm 4.9	66.8 \pm 5.7	66.3 \pm 6.6	56.3 \pm 9.1	63.3 \pm 10.0	56.5 \pm 5.1	55.3 \pm 6.1
(range)	(58-70)	(60-72)	(57-71)	(48-69)	(53-77)	(53-64)	(50-64)

Data are mean \pm S.D. (n=4)

Table 2: Changes in mean systolic and diastolic blood pressure after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

	pre	Day after HbV administration			
		1	3	7	14
PO ₂ (mmHg)					
Ave.	96.3±4.9	93.3±2.2	93.8±6.2	98.5±6.0	90.8±17.7
(range)	(90-101)	(90-95)	(87-100)	(92-106)	(66-107)
PCO ₂ (mmHg)					
Ave.	29.7±2.4	31.9±2.3	31.0±2.1	30.5±2.9	28.5±1.8
(range)	(27.1-32.9)	(29.4-34.9)	(28.0-32.8)	(26.3-32.9)	(26.3-30.6)
pH					
Ave.	7.36±0.09	7.44±0.07	7.47±0.02	7.44±0.02	7.38±0.08
(range)	(7.26-7.46)	(7.36-7.52)	(7.44-7.49)	(7.41-7.45)	(7.27-7.47)

Data are mean ± S.D. (n=4)

Table 3: Changes in arterial blood oxygen tension (PaO₂), arterial blood carbon dioxide tension (PaCO₂) and pH after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

	pre	Day after HbV administration	
		7	14
Sodium (mEq/dL)			
Ave.	153.9±1.5	146.9±2.1	148.7±2.4
(range)	(152-155)	(145-152)	(144-150)
Potassium (mEq/dL)			
Ave.	4.3±0.5	3.9±0.2	4.4±0.4
(range)	(3.6-4.7)	(4.1-5.1)	(3.7-4.4)
Calcium (mEq/dL)			
Ave.	10.3±1.5	10.8±1.7	9.9±1.4
(range)	(8.3-12.0)	(8.3-11.3)	(9.4-12.9)
Chloride (mEq/dL)			
Ave.	112.2±2.0	108.8±1.8	112.4±3.6
(range)	(111-115)	(107-115)	(106-111)
Glucose (mg/dL)			
Ave.	91.8±24.4	91.5±10.2	91.0±24.9
(range)	(72-122)	(70-127)	(80-102)
Inorganic phosphate (mg/dL)			
Ave.	5.2±0.9	5.7±0.7	6.0±0.8**
(range)	(4.0-5.9)	(5.0-6.6)	(4.7-6.4)

** p<0.01 vs. pre-treatment. Data are mean ± S.D. (n=4)

Table 4: Summary of electrolytes after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

It is well-known that the Hb derived from hemolysis causes renal toxicity by the dissociation of tetrameric Hb subunits into two dimers, extravasation, and precipitation in tubules [15]. In the present study, BUN, creatinine and uremic acid levels, which reflect renal function, were slightly changed during the 14 days after the HbV injection, but these changes were still within the normal ranges (Figure 2). This could be due to the characteristics of HbV in which its structure is maintained intact in the circulation, while Hb derived from HbV was completely degraded by MPS. In fact, several results observed in this study (as well as in previous reports) support this conclusion: (i) there was no evidence of the presence of Hb in the supernatant after ultracentrifugation and hemoglobinuria in this study (data not shown), (ii) HbV is circulated in the form of stable HbV in the blood circulation until metabolized by MPS [19].

Amylase and lipase activity was measured to analyze pancreatic function (Figure 2). Amylase levels were essentially unchanged. On the other hand, lipase levels were temporarily increased seven days after HbV, but returned to the basal level within 14 days. The same tendency was observed in a previous report using healthy rats [16]. This elevated lipase activity could be due to the damage to the pancreas by HbV. However, when the pancreas is damaged (e.g. acute necrotizing pancreatitis), lipase activity typically becomes dramatically elevated from 10 to 475-540 IU/L [30]. Thus, the small elevation in lipase levels

observed here can likely be attributed to the induction of pancreatic enzymes by the presence of a large amount of lipids associated with HbV. This speculation was also supported by our present results that only lipase activity increased followed by elevation of TG and phospholipid shown in Figure 4, but amylase did not.

The concentration of the TG and HDL-cholesterol were increased slightly, and the levels of phospholipids and total cholesterol, metabolites of the lipid components of HbV, were significantly increased after HbV administration (Figure 3). In addition, total bilirubin, which is related to the metabolic routes of Hb, were temporarily increased at 7 days after HbV administration, but returned to basal levels within 14 days after the HbV administration (Figure 3). They are likely derived from the HbV particles because they contain a large amount of cholesterol (1200 mg/dL), DPPC (1840 mg/dL) and Hb (10000 mg/dL). During the metabolism of Hb derived from RBC, bilirubin is released and excreted in the bile. In this study, total bilirubin increased at 7 day after HbV administration, indicating that Hb derived from HbV is metabolized and excreted via the normal pathway. On the other hand, cholesterol of the vesicles should reappear in the blood mainly in the form of a lipoprotein-cholesterol complex after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by hepatocytes [31]. Furthermore, it was reported that phospholipids in liposomes are metabolized in MPS and reused as cell membranes or are excreted into the bile [32,33]. In a study using healthy mice and rats, we demonstrated that the outer lipid components, especially cholesterol, were mainly eliminated in the feces via biliary excretion after metabolism by MPS [19]. Therefore, it would be desirable to understand whether the lipid components in HbV behaved the same as endogenous lipid after the metabolism of HbV in MPS.

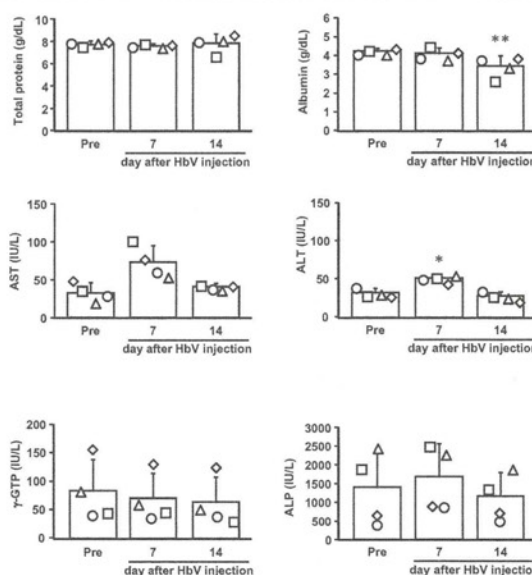


Figure 1: Serum laboratory tests representing liver function after the administration of hemoglobin-vesicles at a dose of 1400 mg Hb/kg in cynomolgus monkeys.

Data are mean ± SD. (n=4) * p<0.05, ** p<0.01 vs. pre-treatment. The individual values are representing as following symbols (○; No.1, □; No.2, △; No.3, ◇; No.4). AST; aspartate aminotransferase, ALT; alanine aminotransferase, γ-GTP; γ-glutamyltransferase, ALP; alkaline phosphatase.

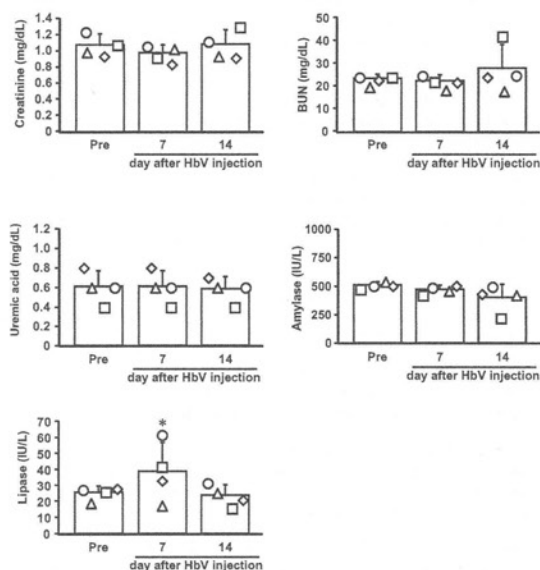


Figure 2: Serum laboratory tests representing renal and pancreatic function after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.
Data are mean \pm SD. (n=4) * p<0.05 vs. pre-treatment. The individual values are representing as following symbols (\circ ; No.1, \square ; No.2, Δ ; No.3, \diamond ; No.4). BUN; blood urea nitrogen.

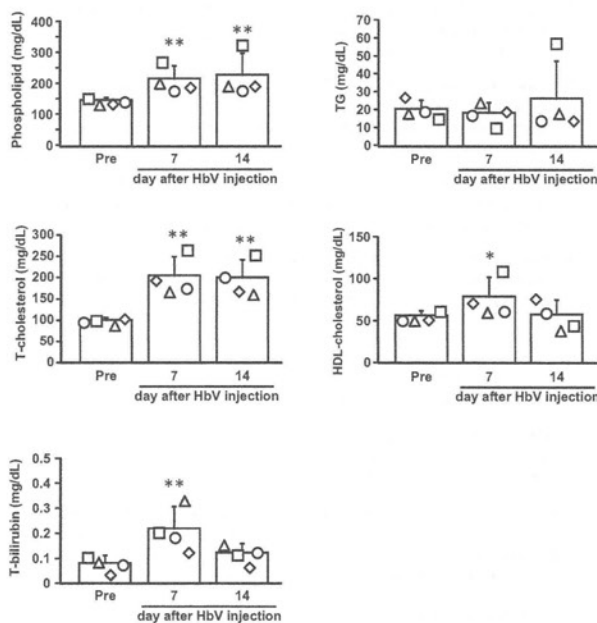


Figure 3: Serum laboratory tests representing the metabolism of lipid and hemoglobin after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.
Data are mean \pm SD. (n=4) * p<0.05, ** p<0.01 vs. pre-treatment. The individual values are representing as following symbols (\circ ; No.1, \square ; No.2, Δ ; No.3, \diamond ; No.4). TG; triglyceride, T-cholesterol; total cholesterol, HDL-cholesterol; high-density lipoprotein cholesterol, T-bilirubin; total bilirubin.

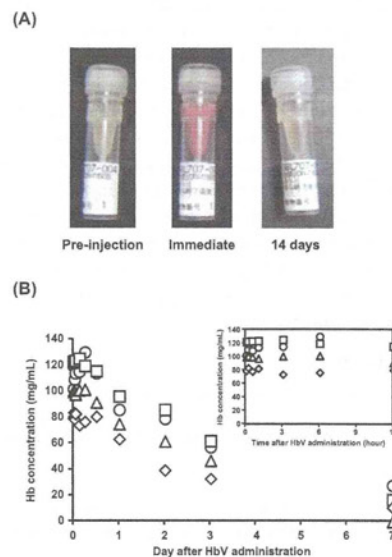


Figure 4: (A) The appearance of plasma before hemoglobin-vesicle administration (left), immediately after finishing administration (middle) and 14 days after hemoglobin-vesicle administration (right). (B) Time course for plasma concentration of hemoglobin concentration in plasma after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.
The individual values are representing as following symbols (\circ ; No.1, \square ; No.2, Δ ; No.3, \diamond ; No.4).

Pharmacokinetics

Since HbV is known to be dispersed in the plasma, we measured the Hb concentration in plasma using a cyanomethemoglobin method to examine the retention of HbV in the circulation in cynomolgus monkeys. Figure 4B shows the time course for the concentration of Hb in plasma after the administration of HbV at a dose of 1400 mg Hb/kg in cynomolgus monkeys. Although the plasma concentration of Hb varies widely among individuals, HbV is sufficiently retained in the blood in the monkey (Half-life; 47-72 hour). On the other hand, no Hb was detected in plasma when analyzed by the cyanomethemoglobin method (data not shown) and its appearance (Figure 4A) at 14 days after HbV administration was the same as that before HbV administration. Therefore, HbV appears to be completely cleared from plasma within 14 days after HbV administration. Interestingly, in the present study using nonhuman primates, a saturation phenomenon for HbV elimination was observed for the first 12 hour after HbV administration (Figure 4B), and the plasma HbV concentration started to decrease following a 1-compartment model at 12 hour after HbV administration. HbV in the bloodstream is finally captured and metabolized by phagocytes in the MPS, especially Kupffer cells in the liver [17,19]. Therefore, the saturation phenomenon observed in this study appears to be due to the saturation of Kupffer cells. These data indicate that HbV possesses both good retention in the blood and metabolic properties even in a nonhuman primate.

Conclusions

In addition to functioning as a substitute for RBCs, HbV would be expected to have a variety of other applications, based on its oxygen transport characteristics, such as in cardiopulmonary bypass priming solutions [34], wound healing in critically ischemic skin [35], acute

ischemic strokes [36] and as a radiation therapy agent [37]. Therefore, HbV has considerable promise for use in the clinic in the future. This study is the first report about the observation of biological reaction and pharmacokinetic evaluation after massive intravenous injection of HbV at a putative dose in a nonhuman primate. As a result of this study, no severe adverse effects were observed in terms of systemic response and in serum biochemical analyses. In addition, pharmacokinetic evaluation showed that HbV is retained in the blood long enough to function as an RBC substitute in nonhuman primates as well as rodents [19,38-40]. The results obtained in present study not only clarify the effect of HbV on physiological responses in primates but also provide helpful information related to designing the most relevant and rational protocol for a clinical study.

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References

- Spahn DR, Kocian R (2003) The place of artificial oxygen carriers in reducing allogeneic blood transfusions and augmenting tissue oxygenation. *Can J Anaesth* 50: S41-S47.
- Stowell CP (2005) What happened to blood substitutes? *Transfus Clin Biol* 12: 374-379.
- Komatsu T, Nakagawa A, Qu X (2009) Structural and mutagenic approach to create human serum albumin-based oxygen carrier and photosensitizer. *Drug Metab Pharmacokin* 24: 287-299.
- Taguchi K, Maruyama T, Otagiri M (2011) Pharmacokinetic properties of hemoglobin vesicles as a substitute for red blood cells. *Drug Metab Rev* 43: 362-373.
- Nosé Y (2004) Is there a role for blood substitutes in civilian medicine: a drug for emergency shock cases? *Artif Organs* 28: 807-812.
- Natanson C, Kern SJ, Lurie P, Banks SM, Wolfe SM (2008) Cell-free hemoglobin-based blood substitutes and risk of myocardial infarction and death: a meta-analysis. *JAMA* 299: 2304-2312.
- Sakai H, Sou K, Horinouchi H, Kobayashi K, Tsuchida E (2009) Review of hemoglobin-vesicles as artificial oxygen carriers. *Artif Organs* 33: 139-145.
- Abe H, Ikebuchi K, Hirayama J, Fujihara M, Takeoka S, et al. (2001) Virus inactivation in hemoglobin solution by heat treatment. *Artif Cells Blood Substit Immobil Biotechnol* 29: 381-388.
- Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E (2000) Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug Chem* 11: 425-432.
- Sakai H, Suzuki Y, Kinoshita M, Takeoka S, Maeda N, et al. (2003) O₂ release from Hb vesicles evaluated using an artificial, narrow O₂-permeable tube: comparison with RBCs and acellular Hbs. *Am J Physiol Heart Circ Physiol* 285: H2543-H2551.
- Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, et al. (2004) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 32: 539-545.
- Sakai H, Seishi Y, Obata Y, Takeoka S, Horinouchi H, et al. (2009) Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. *Shock* 31: 192-200.
- Terajima K, Tsueshita T, Sakamoto A, Ogawa R (2006) Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. *Shock* 25: 184-189.
- Grone EF, Grone HJ (2008) Does hyperlipidemia injure the kidney? *Nat Clin Pract Nephrol* 4: 424-425.
- Parry E (1988) Blood Substitute: Preparation. *Physiology and Medical Applications*.
- Sakai H, Horinouchi H, Masada Y, Takeoka S, Ikeda E, et al. (2004) Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials* 25: 4317-4325.
- Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, et al. (2001) Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* 159: 1079-1088.
- Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, et al. (2004) Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. *J Pharmacol Exp Ther* 311: 874-884.
- Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, et al. (2009) Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* 37: 1456-1463.
- Sakai H, Suzuki Y, Sou K, Kano M (2012) Cardiopulmonary hemodynamic responses to the small injection of hemoglobin vesicles (artificial oxygen carriers) in miniature pigs. *J Biomed Mater Res A*.
- Taguchi K, Miyasato M, Ujihira H, Watanabe H, Kadowaki D, et al. (2010) Hepatically-metabolized and -excreted artificial oxygen carrier, hemoglobin vesicles, can be safely used under conditions of hepatic impairment. *Toxicol Appl Pharmacol* 248: 234-241.
- Sakai H, Takeoka S, Park SI, Kose T, Nishide H, et al. (1997) Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. *Bioconjug Chem* 8: 23-30.
- Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, et al. (2003) Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. *Clin Chem Lab Med* 41: 222-231.
- Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, et al. (2000) Molecular dimensions of Hb-based O₂ carriers determine constriction of resistance arteries and hypertension. *Am J Physiol Heart Circ Physiol* 279: H908-H915.
- Loeb AL, McIntosh LJ, Raj NR, Longnecker DE (1998) Resuscitation after hemorrhage using recombinant human hemoglobin (rHb1.1) in rats: effects on nitric oxide and prostanoid systems. *Crit Care Med* 26: 1071-1080.
- Moon PF, Bliss SP, Posner LP, Erb HN, Nathanielsz PW (2001) Fetal oxygen content is restored after maternal hemorrhage and fluid replacement with polymerized bovine hemoglobin, but not with hetastarch, in pregnant sheep. *Anesth Analg* 93: 142-150.
- Sprung J, Mackenzie CF, Barnas GM, Williams JE, Parr M, et al. (1995) Oxygen transport and cardiovascular effects of resuscitation from severe hemorrhagic shock using hemoglobin solutions. *Crit Care Med* 23: 1540-1553.
- Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J Biol Chem* 283: 1508-1517.
- Sakai H, Sato A, Sobolewski P, Takeoka S, Frangos JA, et al. (2008) NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers. *Biochim Biophys Acta* 1784: 1441-1447.
- Hofbauer B, Friess H, Weber A, Baczako K, Kislign P, et al. (1996) Hyperlipaemia intensifies the course of acute oedematous and acute necrotising pancreatitis in the rat. *Gut* 38: 753-758.
- Kuipers F, Spanjer HH, Havinga R, Scherphof GL, Vonk RJ (1986) Lipoproteins and liposomes as in vivo cholesterol vehicles in the rat: preferential use of cholesterol carried by small unilamellar liposomes for the formation of muricholic acids. *Biochim Biophys Acta* 876: 559-566.
- Dijkstra J, van Galen M, Regts D, Scherphof G (1985) Uptake and processing of liposomal phospholipids by Kupffer cells in vitro. *Eur J Biochem* 148: 391-397.
- Verkade HJ, Derksen JT, Gerding A, Scherphof GL, Vonk RJ, et al. (1991) Differential hepatic processing and biliary secretion of head-group and acyl chains of liposomal phosphatidylcholines. *Biochem J* 275: 139-144.
- Yamazaki M, Aeba R, Yozu R, Kobayashi K (2006) Use of hemoglobin vesicles during cardiopulmonary bypass priming prevents neurocognitive decline in rats. *Circulation* 114: I220-I225.

35. Plock JA, Rafatmehr N, Sinovic D, Schnider J, Sakai H, et al. (2009) Hemoglobin vesicles improve wound healing and tissue survival in critically ischemic skin in mice. *Am J Physiol Heart Circ Physiol* 297: H905-H910.
36. Komatsu H, Furuya T, Sato N, Ohta K, Matsuura A, et al. (2007) Effect of hemoglobin vesicle, a cellular-type artificial oxygen carrier, on middle cerebral artery occlusion- and arachidonic acid-induced stroke models in rats. *Neurosci Lett* 421: 121-125.
37. Yamamoto M, Izumi Y, Horinouchi H, Teramura Y, Sakai H, et al. (2009) Systemic administration of hemoglobin vesicle elevates tumor tissue oxygen tension and modifies tumor response to irradiation. *J Surg Res* 151: 48-54.
38. Sou K, Klipper R, Goins B, Tsuchida E, Phillips WT (2005) Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. *J Pharmacol Exp Ther* 312: 702-709.
39. Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, et al. (2009) Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J Control Release* 136: 232-239.
40. Taguchi K, Miyasato M, Watanabe H, Sakai H, Tsuchida E, et al. (2011) Alteration in the pharmacokinetics of hemoglobin-vesicles in a rat model of chronic liver cirrhosis is associated with Kupffer cell phagocyte activity. *J Pharm Sci* 100: 775-783.

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Swine hemoglobin as a potential source of artificial oxygen carriers, hemoglobin-vesicles

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Abstract

Hemoglobin-based oxygen carriers (HBOCs) have been developed as a transfusion alternative and oxygen therapy. The Hb source is usually outdated donated human blood or cow blood obtained from cattle industries because of its abundance. This study examined the feasibility of using swine Hb (ζ Hb) for preparation of cellular-type HBOCs, hemoglobin-vesicles (HbV). Purification of ζ Hb from fresh swine whole blood was conducted with processes including carbonylation (ζ HbO₂ → ζ HbCO), pasteurization (60 °C, 15 hours) and tangential flow ultrafiltration, with yield of 90%. Actually, differential scanning calorimetric analysis showed a denaturation temperature of ζ HbCO at 83 °C and assures its stability during pasteurization. Concentrated ζ HbCO together with pyridoxal 5'-phosphate (PLP) as an allosteric effector was encapsulated in phospholipid vesicles to prepare ζ HbV. After decarbonylation (ζ HbCO → ζ HbO₂), the oxygen affinity (P_{50}) of ζ Hb changes mainly by PLP, and the influence of Cl⁻ was small, in a manner similar to that of human Hb (η Hb). However, after encapsulation, vesicles of ζ HbV showed much lower oxygen affinity (higher P_{50}) than η HbV did. Autoxidation of ζ HbV was slightly faster than η HbV. Although some differences are apparent in oxygen affinity and autoxidation rates, results clarified that ζ Hb is useful as a starting material for HbV production.

Keywords: blood substitutes, encapsulation, hemoglobin, liposome

Introduction

Hemoglobin-based oxygen carriers (HBOCs) have been developed for use as a transfusion alternative (Chang 2006) because transfusion presents problems of possible infection, blood-type mismatching, and a short storage period during refrigeration. It is expected that HBOCs can resolve these problems. Cell-free HBOCs are now under clinical

trials. Some examples are intramolecularly crosslinked hemoglobin (Hb), polymerized Hb, and polyethylene glycol conjugated Hb (Natanson et al. 2008). The major remaining hurdle before clinical approval of this earliest generation of cell-free HBOCs is vasoconstriction and resulting hypertension, which are presumably attributable to the high reactivity of Hb with endothelium-derived nitric oxide (NO) (Olson et al. 2004, Yu et al. 2010). However, cellular Hb-vesicles (HbVs) that encapsulate concentrated Hb solution in phospholipid vesicles (Sakai et al. 2008, Tsuchida et al. 2009) induce neither vasoconstriction nor hypertension (Sakai et al. 2000).

Most HBOCs are made of human-derived Hb (η Hb) that has been purified from outdated donated blood or placenta blood. Because of the limited sources of outdated donated blood, some groups use bovine Hb (β Hb), which is purified from blood abundantly obtained from cattle industries. However, the concern of prion contamination remains. In Japan, the usage of biological raw materials derived from ruminant animals including cows is strictly regulated. Moreover, the possibility exists in some countries that religious reasons would hinder the usage of β Hb. Another possibility would be use of swine Hb (ζ Hb) (though it would also relate to religious restriction), because swine is not categorized as a ruminant animal. Structural similarity of ζ Hb and human Hb (η Hb) were confirmed from X-ray crystallography (Katz et al. 1994). In fact, usage of ζ Hb was tested to prepare chemically modified Hb solutions (Lee et al. 1992, Chang et al. 2004, Barnikol et al. 2002, Zhang et al. 2011).

In this study, we tested whether ζ Hb is applicable for the preparation of swine Hb-vesicles (ζ HbV) in terms of purification methods including pasteurization, encapsulation, and the fundamental chemical characteristics of the resulting swine HbV (ζ HbV) when compared with human Hb-vesicles (η HbV).

Materials and methods

Purification of ζ Hb from swine whole blood

Purification procedures with carbonylation and tangential flow ultrafiltration were almost identical to those presented in our previous report describing preparation of β Hb (Sakai et al. 2002). Swine fresh whole blood containing EDTA as an anticoagulant was obtained from Innovative Research Inc. (Michigan, USA). A total of 1004 g ($[\zeta\text{Hb}] = 10.78$ g/dL) of swine blood was used for centrifugation. The swine blood was separated in equal volumes into four 250 ml centrifuge tubes. Each was spun down at 3000 rpm for 20 min. Supernatants from all four tubes were discarded. The sedimented red blood cells (RBCs) were re-suspended with saline solution. The whole process was repeated for four centrifugation cycles.

The RBC concentrate was transferred into a 2 L Pyrex bottle, and was topped up to 1.5 L with pure water. A tangential flow ultrafiltration system of a Biomax V-screen (cutoff Mw.:1000 kDa; Millipore Corp.) was set up for the hemolysis and removal of stroma of swine RBCs. Hemolysis and removal of stroma were done simultaneously by adding pure water to the retentate RBC concentrate in the Pyrex bottle while maintaining a total volume of 1.5 L in the bottle. The ultrafiltrated ζ Hb solution was collected.

The ζ Hb solution as oxyhemoglobin (ζHbO_2) was converted to carbonylhemoglobin (ζHbCO) by bubbling CO gas into the ζ Hb solution for approximately 4 hours. ζHbCO solution was heated overnight (15 hours) at 60 °C to inactivate viruses and to denature other proteins. After heating overnight, the solution was cooled to around 15 °C using ice/water. Then it was centrifuged at 3000 rpm for 30 minutes for two centrifugation cycles. The supernatant was collected into a new 2 L Pyrex bottle.

Dialysis and concentration of the ζHbCO was performed using an ultrafiltration system (Biomax V-screen, Cut off Mw., 8 kDa; Millipore). After confirming that the sodium concentration (as NaCl) decreased below 0.01%, the ζHbCO was concentrated to 42 g/dL.

Preparation of ζ Hb-vesicles (ζHbV)

With slight modifications, ζHbVs were prepared using a method reported previously (Sakai et al. 2009). Pyridoxal 5'-phosphate (PLP; Sigma Chemical Co., St Louis, MO) was added to the ζHbCO solution as an allosteric effector at a molar ratio of PLP/ ζHb tetramer = 0, 0.5, 1.0, and 2.5. After filtration of the ζHbCO solution through a sterilizable filter (pore size 0.45 μm ; Advantec Co.), it was mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG; Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG5000 (DSPE-PEG; NOF Corp., Tokyo, Japan) at the molar composition of 5/4/0.9/0.03. The particle diameter was regulated using a rapid mixing method. The encapsulated ζHbCO was converted to ζHbO_2 by exposing the liquid membranes of HbVs to visible light under an aerobic atmosphere. The ζHb concentration was measured using

a modified cyanomethemoglobin (metHb-CN) method (Alfresa Pharma Corp., Tokyo). The phospholipid (DPPC) concentration was measured using a choline oxidase-DAOS method (Wako Pure Chemical Industries Ltd., Osaka, Japan). Finally, the Hb concentration of the suspension was adjusted to 10 g/dL. The particle diameter distribution was measured using a dynamic light-scattering method (Nanoparticle analyzer SZ-100; Horiba Ltd., Tokyo).

Analysis of thermal stability of ζ Hb

Thermograms (30–120 °C) of ζHb solutions in oxy, deoxy, and carbonyl states were obtained using a differential scanning calorimeter (DSC8500; Perkin Elmer Inc., MA, USA). The solutions (10 g/dL, 60 μl) were sealed with stainless steel capsules (Large volume capsules, LVC; Perkin Elmer Inc.). The LVCs eliminate the interfering effects of heat of vaporization by suppressing the vaporization of water even at 120 °C. The scanning rate was 1.0 °C/min.

Measurements of oxygen equilibrium curves of ζHb and ζHbV

The ζHbCO solution was adjusted to about 2.5 g/dL in saline. It was converted to ζHbO_2 by photoreaction as described above. A Hemox analyzer (TCS Scientific Corp., PA, USA) was used to obtain the oxygen equilibrium curves. Either phosphate-buffered saline (PBS, pH 7.4; Gibco) or 30 mM phosphate buffer (PB, pH 7.4, without Cl^-) was used to dilute the solutions. ζHbVs with different PLP contents were diluted with a Hemox solution. Oxygen equilibrium curves were obtained in the same manner.

Measurements of autoxidation rates of ζHb and ζHbV

A ζHbO_2 solution or a ζHbV containing ζHbO_2 was suspended in PBS (pH 7.4) and incubated at 37 °C with gentle shaking. The metHb level of ζHb solution was measured periodically using the method of Evelyn–Malloy using CN^- (Matsubara 1987). For ζHbV suspension, 3 ml of PBS was added to the Thunberg cuvette together with 10 μL of sample. The solution was subjected to bubbling of N_2 gas for 4 minutes, resulting in the presence of deoxyHb and metHb. Absorbance reading was then measured at 405 nm (metHb) and 430 nm (deoxyHb) using a spectrophotometer (V-650; Jasco Corp., Tokyo) and recorded. Calculations of two absorptions were then done to ascertain the level of metHb (Hamada et al. 1995).

For all the analyses described above, μHb was used for comparison.

Results

Purification of ζHb and preparation of ζHbV

Within 2 weeks after the withdrawal of swine blood, we were able to start the ζHb purification. The yield of Hb from the first rinsing procedure with saline was 98% because of the lesser amount of hemolysis in the fresh blood. Tangential flow ultrafiltration (cut-off Mw. 1000 KDa) effectively removed stroma components in the turbid retentate fraction, and