dium. A 50-μl bacterial suspension in M9 medium (OD_{630}; 0.05 ± 0.01) was placed in a 96-well plate, and 50-μl aliquots of plasma samples were added. After 6 h of incubation at 37°C, the number of bacteria exposed to various plasma samples were determined by measuring the absorbance 630 nm using a microplate reader (model 450; Bio-Rad Laboratories, Hercules, CA).

Surgery for Splenectomy. For splenectomy, the rats were anesthetized using pentobarbital and, after shaving, an incision was made in the skin at the left flank. The peritoneal membrane was opened, and the entire spleen was removed intact after ligating the splenic vein and artery at the hilum. The peritoneal membrane and skin were separately sutured (Ishida et al., 2006). One week after surgery for splenectomy, the rats were used in experiments.

Measurement of Plasma IgG, IgM, and CH50. The plasma samples obtained 4 and 7 days after resuscitation by the administration of HbV and wRBC solutions were stored at −80°C before analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan). CH50 was measured in hemolytic assays as described previously (Mayer, 1961).

Measurement of Phagocyte Activity. Phagocyte activity was determined by the carbon clearance method, as described previously (Taguchi et al., 2011b). Hemorrhagic shock was induced in the rats, which were then resuscitated with HbVs and wRBCs. In a typical experiment, rats were anesthetized with pentobarbital. Polyethylene catheters (PE 50 tubing) containing saline and heparin were then introduced into the left femoral vein for the infusion of a carbon particle solution and blood collection. The carbon particle solution (Fount India Ink; Pelikan Co., Hannover, Germany) was infused at 10 ml/kg within 1 min. At 4, 10, 20, 30, 45, and 60 min later, approximately 100 μl of blood was withdrawn, and exactly a 50-μl aliquot was diluted with 5 ml of a 0.1% sodium bicarbonate solution. The absorption was measured at 675 nm by means of a spectrophotometer (U-2900; Hitachi, Tokyo, Japan). The phagocyte index (K) was calculated using the equation: $K = 1/(t_2 - t_1) \times \ln(C_1/C_2)$, where C_1 and C_2 are the concentrations (absorbance) at time t_1 and t_2 (min), respectively.

Preparation of Cecum Ligation and Puncture Model and the Measurement of Viable Bacterial Counts in Blood. Bacterial infection was induced by cecum ligation and puncture, following a previously reported method with minor modification (Hubbard et al., 2005). Hemorrhagic shock was induced in the rats, which were then resuscitated with HbVs and wRBCs. Four or 7 days after resuscitation with HbVs and wRBCs, the cecum was ligated without preventing passage of gastric contents. Ten punctures in cecum ligation were then performed using a 21-gauge needle. Survival rate was determined daily for 7 days after puncture. The blood samples from surviving rats (0.3 ml) were collected from tail vein 24 h after the puncture. After collecting blood samples, the aliquot blood (0.1 ml) was immediately placed on Luria-Bertani agar plates (9.9 ml) and the plates were incubated at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as colony-forming units/ml.

Data Analysis. Data are shown as the means ± S.D. for the indicated number of animals. Significant differences among each group were determined using the two-tail unpaired Student's t test. The Spearman test was used for correlation analyses. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test. A probability value of $p < 0.05$ was considered to indicate statistical significance.

Results

Survival Rate and Blood Gasses Analysis. All hemorrhagic shock rats resuscitated by an infusion of either isovolemic HbVs or wRBCs (1400 mg Hb/kg, 22.4 ml/kg) survived during experiments and were well tolerated with no apparent change in behavior. The PaO_2 before induction of hemorrhagic shock by bleeding was 88.8 ± 5.7 Torr and increased significantly to 129.3 ± 17.4 Torr after induction of hemorrhagic shock. After resuscitation with HbVs or wRBCs, PaO_2 recovered similar to PaO_2 measured at baseline (76.3 ± 7.8 and 93.5 ± 16.5 Torr for 4 and 7 days after HbV resuscitation, respectively; 86.8 ± 14.8 and 87.8 ± 11.1 Torr for 4 and 7 days after wRBC resuscitation, respectively). In addition, pH before and after hemorrhage was 7.33 ± 0.07 and 7.28 ± 0.08, respectively, and it recovered by resuscitation with HbVs (7.37 ± 0.06 and 7.43 ± 0.05 for 4 and 7 days after resuscitation, respectively) or wRBCs (7.41 ± 0.03 and 7.44 ± 0.02 for 4 and 7 days after resuscitation, respectively). The data showed similar tendency as that reported previously by Sakai et al. (2009a).

The Evaluation of Antibacterial Activity. To evaluate antibacterial activity, the collected plasma samples were incubated with *E. coli* (ATCC strain) for 6 h. As shown in Fig. 1A, the only plasma sample in HbV_{4day} showed a dramatic level of antibacterial activity, whereas other plasma samples obtained from the hemorrhagic shock rats did not show any activity. Because it is possible that HbV directly suppresses *E. coli* growth, HbV alone was incubated with *E. coli* (0, 0.049, 0.098, 0.195, 0.391, and 0.781 mg Hb/ml). As a result, HbVs did not show any antibacterial activity during 6 h (data not shown). This result clearly excluded the possibility of HbV itself directly contributing to the antibacterial activity observed in plasma samples from the hemorrhagic shock rats that had been resuscitated by HbV. Consequently, other factors in plasma induced by resuscitation with HbV seem to be indirectly involved in this unique phenomenon.

The Relationship between CH50 and Antibacterial Activity. In a previous study, we reported that CH50 in plasma was decreased after resuscitation with HbV in the rat model of hemorrhagic shock (Taguchi et al., 2011a). Because it is well known that complement represents the first line of the host defense system and possesses bacteriolytic effects, we examined the relationship between the antibacterial activity described above and CH50 in plasma.

As shown in the previous results, the CH50 for HbV_{4day} was significantly smaller than that in normal rats, whereas the other samples remained unchanged ($p < 0.01$; Fig. 1B). As shown in Fig. 1C, CH50 was well correlated with antibacterial activity ($r = 0.47$, $p = 0.013$). These results strongly suggest that CH50 is associated with the observed antibacterial activity.

The Relationship between Plasma IgM and Antibacterial Activity. Because Ishida and Kiwada (2008) demonstrated that the IgM produced from spleen is related to the reduction of CH50 after the administration of liposomes, it is possible that the change in plasma IgM levels after HbV administration might affect antibacterial activity via the reduction of CH50.

As shown in Fig. 2A, the plasma IgM level in the HbV_{4day} was significantly larger than that in normal rats, but the other plasma samples were not significantly changed ($p < 0.01$; Fig. 3A). Similar to CH50 (Fig. 1B), plasma IgM levels were well correlated with the observed antibacterial activity ($r = 0.64$, $p = 0.0006$; Fig. 2B). To further demonstrate this relationship, we prepared splenectomized rats to suppress the production of IgM by the administration of HbV, and the same experiments were then performed. The level of plasma IgM and CH50 were maintained at the normal rat level by

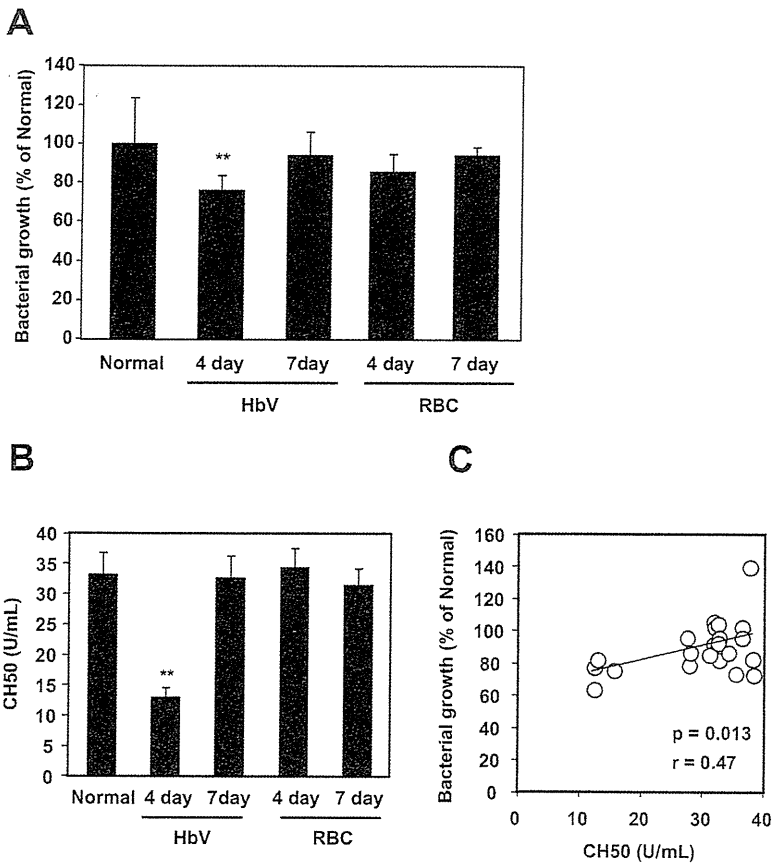

Fig. 1. A, bacterial (*E. coli* ATCC strains) growth rate after resuscitation with HbVs or wRBCs in a rat model of hemorrhagic shock. Blood was collected from the tail vein, and plasma was obtained. *E. coli* ATCC strains and each plasma sample were mixed (6 h, 37°C). Bacteria growth was determined by measuring the absorbance at 630 nm. Each bar represents the mean ± S.E.M. (n = 4). **, $p <$ 0.01 versus normal rats. B, the measurement of CH50in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation. Plasma samples were obtained at 4 and 7 days after resuscitation with HbV and wRBC. Each bar represents the mean ± S.D. (n = 4). **, $p < 0.01$ versus normal rats. C, relationship between CH50 and bacterial growth. The linear regression was calculated using the least-squares method ($y = 0.892x + 64.9$, $r = 0.47$, $p = 0.013$).

removing the spleen; even in 4 days after resuscitation with HbV (Fig. 3, A and B), no significant antibacterial activity was detected in any of the splenectomized rats (Fig. 3C). Consequently, no significant correlation was found between either plasma IgM and antibacterial activity or CH50 and antibacterial activity (r = 0.18 and 0.03, for plasma IgM and complement activity, respectively) (data not shown).

Evaluation of Antibacterial Activity in the Two-Hit Model of Hemorrhagic Shock and Sepsis Rat Model Induced by Cecum Ligation and Puncture. To examine whether the antibacterial activity caused by resuscitation with HbV were observed under conditions of bacterial infection in vivo, we prepared a double model of hemorrhagic shock and cecum ligation and puncture, as a model for sepsis, arguably one that closely replicates the nature and course of clinical sepsis in patients after trauma (Hubbard et al., 2005).

Figure 4A shows the survival rates after the induction of cecum ligation and puncture. Ninety percent of the normal rats died within 36 h after the induction of cecum ligation and puncture. However, the survival times for HbV_{7day}, RBC_{4day}, and RBC_{7day} rats were prolonged, but 90% of these died within 120 h. It is noteworthy that a significant number of animals at HbV_{4day} survived compared with normal rats, and 40% of the rats in this group survived until 168 h after the induction of cecum ligation and puncture. We also measured the amount of bacteria in blood at 24 h after the induction of cecum ligation and puncture. As shown in Fig. 4B, the amount of bacteria in blood decreased in the HbV_{4day} and HbV_{7day} rats compared with the normal and RBC resuscitation groups. To investigate whether the amount of bacteria in blood had an influence on survival rate, we compared the numbers of bacteria in blood between the rats that survived for more than 72 h and the rats that died within 72 h. As shown in Fig. 4C, the amount of bacteria in the blood of the rats that survived more than 72 h was less than that in the rats that died within 72 h. These results indicate that the elevated complement activity after HbV administration contributed to the removal of bacteria, even under conditions of severe sepsis, and the clearance of bacteria at an earlier time was an important factor in the improved survival rates.

Phagocyte Activity. Phagocyte activity, especially in Kupffer cells, is strongly related to the removal of bacteria in vivo. Thus, to investigate the effect of resuscitation by HbV and wRBC solutions on phagocyte activity in Kupffer cells, we estimated the in vivo carbon clearance, an indicator of phagocyte activity in Kupffer cells [Kupffer cells engulfed more than 90% of the injected carbon particles (Zweifach and Benacerraf, 1958)].

As shown in Table 1, phagocyte activity in HbV_{4day} and HbV_{7day} rats was approximately 1.5 and 1.9 times higher than that in normal rats. In contrast, phagocyte activity for both RBC_{4day} and RBC_{7day} rats was comparable with that in normal rats. These data indicate that phagocyte activity was increased after resuscitation with HbV in the rat model of hemorrhagic shock, and the enhanced phagocyte activity played a role in the clearance of bacteria in vivo.

Discussion

The "two-hit" theory proposes that a host primed by an initial stress such as a massive hemorrhage is likely to show

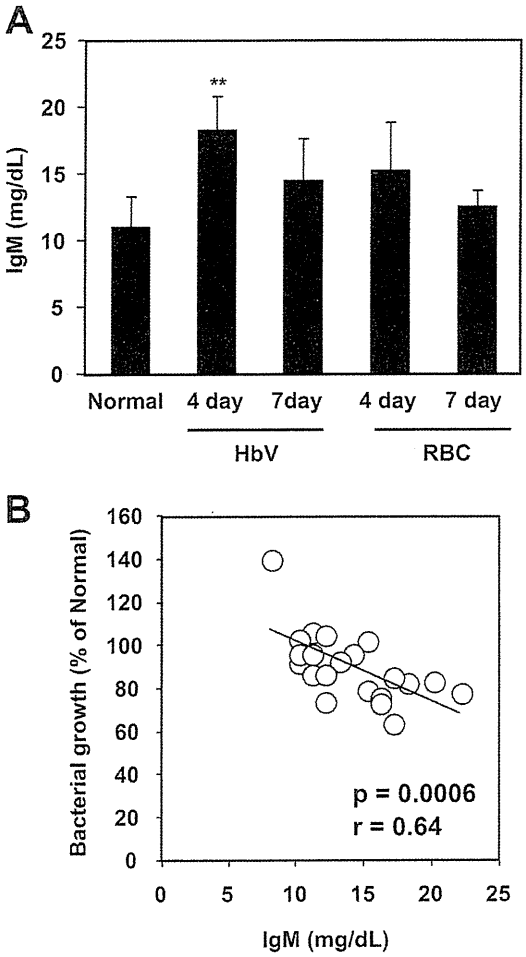

Fig. 2. A, the measurement of plasma IgM concentrations in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation. Plasma samples were obtained at 4 and 7 days after resuscitation with HbV and wRBC solution. The plasma samples were then ultracentrifuged to remove intact HbV (50,000*g*, 30 min). Each bar represents the mean ± S.D. (n = 4). **, $p < 0.01$ versus normal rats. B, relationship between plasma IgM concentration and bacterial growth. The linear regression was calculated using the least-squares method ($y = -2.77x + 129.8$, $r = 0.64$, $p = 0.0006$).

an abnormal response to a second stress such as an infection (Price et al., 1999). It has been shown that bacterial translocation from the gut to mesenteric lymph nodes induced by hemorrhagic shock, in combination with the immunosuppressive effects of hemorrhage, can amplify the second hit (Turnbull et al., 1995). In fact, Rachoin et al. (2009) reported that the approximately 10% of transfused patients developed nosocomial infections, and the number of patients infected with *E. coli* was similar to that of methicillin-resistant *Staphylococcus areus*. *E. coli* is classed as a gram-negative bacteria, in which the outer membrane contains a lipopolysaccharide as endotoxin, which causes endotoxin shock (sepsis) and multiple organ failure (Yang and Lee, 2008). Therefore, in terms of decreasing mortality and morbidity among transfused patients with massive hemorrhage, the removal of Gram-negative bacteria, especially *E. coli*, at an early phase is an important issue.

The findings reported herein showed that plasma of rats resuscitated with HbVs from hemorrhagic shock showed a suppressed *E. coli* growth correlated to reduction of CH50 (Fig. 1). Four possible mechanisms for the reduction of CH50 after HbV resuscitation can be proposed: 1) The liver injury decreases the production of complement; however, it was reported that HbVs did not directly injure the liver and other organ. Therefore, it would affect the production of complement. 2) The direct effect of HbV, which makes contact with complement, subsequently induces the complement activation, because it was previously reported that liposomes containing cholesterol induce complement activation (Alving et al., 1977; Cunningham et al., 1979). However, in the case of HbV, the level of CH50 was equivalent to that observed for saline treatment in both in vitro studies using human serum (Abe et al., 2006) and in vivo using healthy rats (Sou and Tsuchida, 2008). In addition, *E. coli* incubated with HbVs did not show antibacterial activity (data not shown). Therefore, it does not seem the HbV itself directly affects the reduction of CH50. 3) Plasma IgG, which activates complement by binding to an antigen, recognizes microbial polysaccharides, and by the lipid A component of lipopolysaccharide. However, plasma IgG was hardly induced by HbV administration, and no correlation with the antibacterial activity was found (r = 0.09, data not shown). 4) Plasma IgM, which activates complement via the same pathway as plasma IgG, as mentioned above. In this study, the plasma IgM produced in spleen was well correlated with *E. coli* growth (Fig. 2). Furthermore, it was observed that both CH50 and antibacterial activity disappeared by suppression of IgM production using splenoctomized rats (Fig. 3). Based on these results, it seems that complement is activated by binding IgM to *E. coli*, and this complement activity would result in suppressed *E. coli* growth.

The complement system contributes to host defense against infections by two different mechanisms: C3-mediated lysis, which leads to the formation of a membrane attack complex (Frank et al., 1987), and opsonic activity, which leads to phagocytosis by macrophages and Kupffer cells (Esser, 1994). Therefore, C3 is a key factor for antibacterial activation. In fact, a clinical C3 deficiency involves the loss of major complement opsonin and failure to activate the membrane attack complex pathway (Walport, 2001). It was previously reported that when liposomes are repeatedly injected into the same animal IgM is induced by the first injected liposomes and the binding of IgM to the second injected liposomes occurs, followed by C3 activation by IgM (Ishida and Kiwada, 2008; Taguchi et al., 2009c). Therefore, C3 could also be activated by the binding of IgM to *E. coli* in the present study.

In addition, we used a double-hit model of hemorrhagic shock and sepsis, which closely replicates the nature and course of clinical sepsis in patients after trauma (Hubbard et al., 2005). Under these conditions, only 10% of the normal and RBC resuscitation rats survived 168 h after the induction of cecum ligation and puncture, whereas 40% of the HbV_{4day} animals survived (Fig. 4A). Previously, Turnbull et al. (2004) reported that antimicrobial therapy with the broad-spectrum antibiotic imipenem at a dose of 25 mg/kg resulted in a significant improvement in survival rate (46%) compared with nontreatment (26%) in cecum ligation and puncture model mice. Furthermore, a imipenem-cilastatin treatment, administered 500 μg intraperitoneally every 12 h, prolonged the survival time in cecum ligation and puncture model mice, but did not prevent mortality (100% mortality;

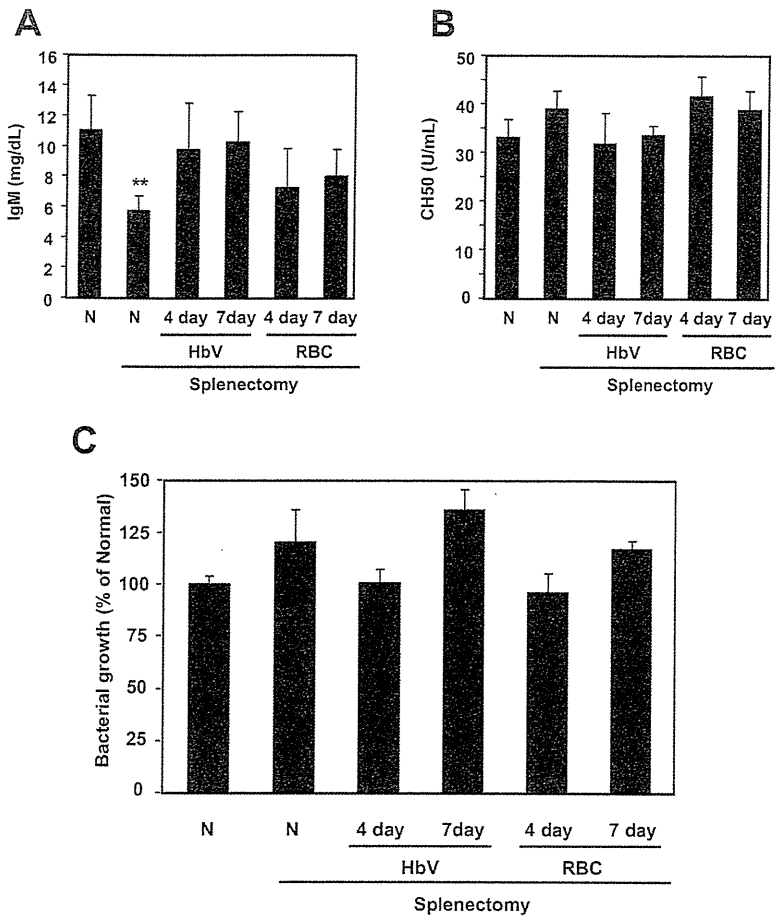

Fig. 3. A and B, the measurement of plasma IgM concentration (A) and CH50 (B) in normal healthy rats and hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation in splenectomy rats. Plasma samples from splenectomy rats were obtained 4 and 7 days after resuscitation with HbV and wRBC solutions. The plasma samples were then ultracentrifuged to remove intact HbV (50,000*g*, 30 min). Each bar represents the mean ± S.D. (n = 4). C, the antibacterial (*E. coli* ATCC strains) activity of plasma obtained from splenectomy rats. *E. coli* ATCC strains and each plasma sample obtained from splenoctomized rats were mixed (6 h, 37°C). Bacteria growth was determined by measuring the absorbance at 630 nm. There was no significant difference among groups. Each bar represents the mean ± S.E.M. (n = 4).

84 and 120 h, for cecum ligation and puncture alone and imipenem-cilastatin treatment groups, respectively) (Doerschug et al., 2004). Therefore, it might be expected that complement activation by IgM after HbV resuscitation would achieve a full therapeutic effect for antibacterial activity, similar to antibiotic therapy.

In addition to the enhancement in survival rate, the amount of bacteria in blood of the HbV resuscitation groups was less than those of the other groups (Fig. 4B). In ex vivo conditions, although the HbV_{7day} group did not show antibacterial activity, the amount of bacteria in blood was suppressed. Because the mononuclear phagocyte system, especially Kupffer cells, plays a major role in the clearance of microorganisms (Bilzer et al., 2006), the suppression of bacterial count in the HbV_{7day} group seems to be the result of increased phagocyte activity. Actually, phagocyte activity in the HbV_{7day} rats was approximately 1.9 times higher than that in normal rats (Table 1).

It might be a concern that the elevated complement activity induced by IgM after HbV resuscitation damaged some host organisms. However, when the plasma samples were added to human umbilical vein endothelial cells, which are commonly used as a model of vascular endothelial cells, to examine cellular injury using WST-8, no significant difference in human umbilical vein endothelial cell viability was found among the groups at 6 or 24 h (data not shown). Therefore, it is unlikely that the observed complement activation damages host organisms. In fact, it was reported that histological changes and an enhancement in organ injury markers were not observed in the hemorrhagic shock rat model after resuscitation by HbV (Sakai et al., 2009a). Moreover, complement activation is a subject of great concern as a pseudoallergic reaction (Laverman et al., 2001). In this study, no allergic-like reactions, such as anaphylactic reactions, were observed, and previous reports demonstrated that healthy rats, which were repeatedly injected a massive dose of HbV (10 ml/kg/day for 14 days), all survived and showed no toxicity (Sakai et al., 2004a). Therefore, an enhancement in complement activity after HbV injection would not be expected to induce allergic reactions or have an effect on host organisms. However, it is possible that the HbV administration might produce detrimental outcome in conditions in which complement activation increases the risk of survival, because HbV enhanced the complement activation. In the case of using HbV clinically, resuscitation should be the first priority as in hemorrhagic shock that decreased the survival rate. Having said that, the effect of complement activation via HbV administration in conditions in which complement activation increases the risk of survival deserves further investigation.

Although the complement activation induced by IgM after HbV administration showed antibacterial activity, our model has several limitations with respect to providing a complete explanation for antibacterial activity. It is well known that reactive oxygen species (ROS) and cytokines, such as interleukin-6, tumor necrosis factor-α, and C-reactive protein, are

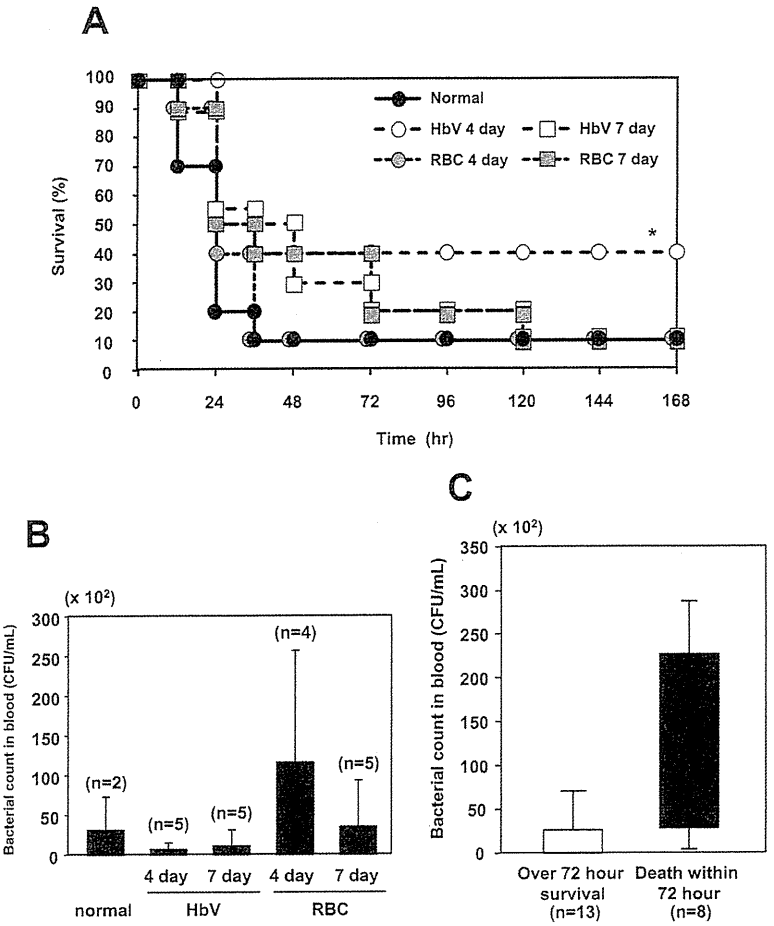

Fig. 4. A, survival rate of the two-hit model of hemorrhagic shock and sepsis. Sepsis was induced by cecum ligation and puncture. The cecum was ligated, and then 10 punctures in the cecum ligation were performed using a 21-gauge needle. The survival rate was compared using Kaplan-Meier survival curves and the log-rank test. *, $p < 0.05$ versus normal rats ($n = 10$). B, amount of bacteria in blood at 24 h after cecum ligation and puncture. C, the comparison of the amount of bacteria in blood at 24 h after cecum ligation and puncture between rats that survived over 72 h and rats that died within 72 h after the induction of sepsis. Blood samples from survival rats (0.3 ml) were collected from the tail vein 24 h after puncture. After collecting the blood samples, aliquots (0.1 ml) were immediately placed on Luria-Bertani agar plates (9.9 ml) followed by incubation at 37°C for 24 h. The numbers of bacterial colonies were then counted and expressed as colony-forming units (CFU)/ml. Each bar represents the mean ± S.D.

TABLE 1

Phagocyte activity in hemorrhagic shock rats at 4 or 7 days after HbV or wRBC resuscitation

Carbon clearance was estimated, and K was calculated from the clearance of carbon particles. Results represent the mean ± S.D. ($n = 5$).

	Percentage of Normal	
	4 Days	7 Days
HbV	163 ± 14*	189 ± 38**
wRBC	83 ± 19	96 ± 19

* $P < 0.05$, ** $P < 0.01$ vs. normal rats.

also related to antibacteria action. In this study, the effect of ROS was estimated by determining the oxidative albumin ratio, which serves as a nonspecific marker of ROS (Shimoishi et al., 2007). As a result, we found that the relationship between ROS and antibacterial activity was significantly correlated ($r = 0.42, p < 0.05$; data not shown). Therefore, it is possible that ROS and cytokines are also involved in the antibacteria action observed here. However, further study will be needed to clarify the contribution of these factors. Moreover, in clinics, patients frequently become infected with various species of bacteria including Gram-negative and -positive bacteria, but we used only Gram-negative bacteria, *E. coli*. In general, Gram-positive bacteria are less sensitive to lysis by complement than Gram-negative bacteria (Mold, 1999). However, because Gram-positive bacteria affected the opsonization by complement, antibacterial activity of Gram-positive bacteria under in vivo conditions would not be unexpected. Further study will be needed to demonstrate this fact.

Based on the present findings, resuscitation with HbV in a rat model of hemorrhagic shock suppressed the growth of *E. coli* via complement activation induced by IgM, compared with wRBC resuscitation. However, this possible therapeutic efficacy was limited to 4 days after resuscitation with HbV. Fortunately, in clinical settings, the timing of infections from the first transfusion is typically approximately 4 to 5 days, which is consistent with the present results (Rachoin et al., 2009). Given the above findings and ongoing progress in antibacterial therapy, HbV resuscitation may contribute to a reduction of infections in patients with a massive hemorrhage, especially in cases of inadequately resourced health service areas.

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Authorship Contributions

Participated in research design: Taguchi, Watanabe, Maruyama, and Otagiri.

Conducted experiments: Taguchi and Ogaki.

Contributed new reagents or analytic tools: Sakai, Kobayashi, and Horinouchi.

Performed data analysis: Taguchi and Kadowaki.

Wrote or contributed to the writing of the manuscript: Taguchi, Sakai, Maruyama, and Otagiri.

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Address correspondence to: Dr. Masaki Otagiri, Department of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Kumamoto University, 5-1 Oe-honmachi, Kumamoto 862-0973, Japan. E-mail: otagirim@gpo.kumamoto-u.ac.jp

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Intravenous infusion of Hb-vesicles (artificial oxygen carriers) after repetitive blood exchange with a series of plasma expanders (water-soluble biopolymers) in a rat model†

Hiromi Sakai[a,b,*], Norihito Miyagawa[c], Hirohisa Horinouchi[d], Shinji Takeoka[b,c], Masuhiko Takaori[e], Eishun Tsuchida[a,§] and Koichi Kobayashi[d]

Hemoglobin-vesicles (HbV) are artificial oxygen carriers developed for use as a transfusion alternative. The extremely high concentration of the HbV suspension (solutes, *ca.* 16 g/dl; volume fraction, *ca.* 40 vol%) provides a sufficient oxygen carrying capacity to maintain oxygen metabolism. A suspension of HbV has no colloid osmotic pressure (COP). Consequently, a combination of a plasma expander is necessary for a massive dose of HbV. Clinically available plasma expanders include hydroxyethyl starch (HES), modified gelatin (MFG), or recombinant human serum albumin (rHSA). Our previous studies confirmed that these water-soluble biopolymers interact with HbV to induce flocculation of HbV reversibly by depletion interaction, especially with MFG and high molecular weight HES. It remains unknown whether such flocculate formation in blood might affect animal's hemodynamics. Using a rat model, we tested infusion of a series of plasma expander to maintain the blood volume (level of blood exchange led to 60%) at repeated hemorrhages and the subsequent infusion of HbV (20 ml/kg, 36% of blood volume). All rats survived for 4 hr after the infusion of HbV; hemodynamic and respiratory functions were preserved, indicating that the flocculation does not induce capillary embolism. Blood exchange with rHSA and subsequent infusion of HbV showed more stable systemic parameters because of the longer retention of rHSA in blood than other plasma substitutes, indicating that rHSA is suitable for combination with HbV in this experimental model. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: blood substitutes; artificial red cells; biomacromolecules; hemoglobin; liposome

INTRODUCTION

Hemoglobin-vesicles (HbV) are artificial oxygen carriers that encapsulate a concentrated Hb solution in phospholipid vesicles (280 nm particle diameter). HbV has been developed by the Oxygen Infusion Project of Waseda University, directed by Prof. Eishun Tsuchida since 1990s from the view point of molecular assembly and macromolecular metal complexes.[1–3] The oxygen carrying capacity and safety of HbV as a transfusion alternative have been evaluated energetically in animal tests aimed at clinical applications. In contrast to conventional liposomal products, the concentration of the recent HbV suspension is extremely high (Hb, 10 g/dl). One infusion as a transfusion alternative causes the substitution of a large volume of blood: about 40% of the circulating blood volume.[4] Accordingly, it is important to evaluate its safety, not only in terms of the

* *Correspondence to: H. Sakai, Principal Investigator and Associate Professor, Waseda Bioscience Research Institute in Singapore, 11 Biopolis Way, #05-01/02 Helios, Singapore 138667, Singapore.*
E-mail: hirosakai@aoni.waseda.jp

a *H. Sakai, E. Tsuchida*
Oxygen-Infusion Project, Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan

b *H. Sakai, S. Takeoka*
Waseda Bioscience Research Institute in Singapore (WABIOS), Singapore 138667, Republic of Singapore

c *N. Miyagawa, S. Takeoka*
Graduate School of Advanced Sciences and Engineering, Waseda University, Tokyo 162-8480, Japan

d *H. Horinouchi, K. Kobayashi*
Department of Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan

e *M. Takaori*
Higashitakarazuka Sato Hospital, Takarazuka 665-0873, Japan

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† *Dedicated to late Emeritus Professor Eishun Tsuchida.*

§ *Emeritus Professor Eishun Tsuchida passed away during preparation of this manuscript. The co-authors acknowledge his contribution to the research of blood substitutes.*

biocompatibility of the HbV particles themselves, but also in terms of the rheological property of the HbV suspension, the infusion fluid, compared to the blood hemorheology.[5,6]

Albumin, dissolved in a blood plasma at *ca.* 5 g/dl, provides sufficient colloid osmotic pressure (COP, *ca.* 20 Torr) to play an important role in equilibrating COP between blood and interstitial fluid, thereby maintaining the overall blood volume. This COP is one requisite for a transfusion alternative to sustain blood circulation for transporting oxygen and metabolites. However, an HbV suspension shows no COP in an aqueous solution because about 30 000 Hb molecules are compartmentalized in one HbV particle. Accordingly, HbV must be suspended in, or co-injected with, a plasma expander (plasma substitute solution). This requirement is identical to that for emulsified perfluorocarbon, which does not possess COP,[7] and it contrasts to characteristics of other Hb-based oxygen carriers (HBOCs), intramolecular crosslinked Hbs, polymerized Hbs, and polymer conjugated Hbs, which all possess very high COP as protein solutions.[8]

In animal experiments, HbV suspended in plasma-derived human serum albumin (HSA) or recombinant HSA (rHSA) showed oxygen transporting capacity that was comparable to that of blood.[4,9,10] We reported previously that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian and no aggregation was detected microscopically.[9,11] In Japan, rHSA was approved for clinical use quite recently (but is not commercially available yet).[12] Various plasma substitutes are used worldwide, such as hydroxyethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG).[13,14] The selection among these plasma substitutes should be determined not only by safety and efficacy, but also by price, experience of clinicians, and customs of respective countries.

Water-soluble polymers generally interact with particles including polystyrene beads, silica, red blood cells (RBCs), and liposomes to induce aggregation or flocculation.[15–17] Actually, we confirmed in previous studies that HbV interacts with plasma substitutes through depletion interaction and induce flocculate formation, depending on the species of a plasma substitute and its molecular weight.[18,19] Even though the flocculate formation changes Newtonian to non-Newtonian characteristics with a shear-thinning profile and the flocculation is completely reversible, it is important to determine the compatibility of HbV with these plasma substitutes in blood circulation. Using a rat model, we tested infusion of a series of plasma substitutes at repeated hemorrhages to maintain the blood volume and the subsequent infusion of HbV and observed the respiratory and hemodynamic parameters.

EXPERIMENTAL

Preparation of HbV

The HbV used for this study was prepared under sterile conditions, as reported previously.[20–22] The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP; Sigma) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. The lipid bilayer comprised a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemical Co. Ltd., Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly (ethylene glycol) (NOF Corp., Tokyo, Japan, 0.3 mol% of the total lipid). The particle diameter was 279 ± 95 nm. The HbVs were suspended in a physiologic saline solution at [Hb] = 10 g/dl ([lipids] = *ca.* 6 g/dl). Then they were deoxygenated with N_2 bubbling and sealed in vials for storage.[23]

Plasma expanders (water-soluble biopolymers)

The plasma substitutes used for this study are presented in Table 1. rHSA (M_w 67 kDa, 25 wt%) was a gift from Nipro Corp. (Osaka, Japan). Before use, it was diluted to 5-wt% using saline solution (Otsuka Pharmaceutical Co. Ltd., Osaka, Japan). An HES_{70} solution (Saline-HES™, M_w 70 kDa, 6 wt% in a physiological saline solution) was purchased from Kyorin Pharmaceutical Co. Ltd. (Osaka, Japan). An HES_{130} solution (Voluven™, M_w 130 kDa, 6 wt% in a physiological saline solution) was a gift from Fresenius Kabi AG (Homburg v.d.H., Germany). An HES_{670} solution (Hextend™, M_w 670 kDa, 6 wt% in a physiological Ringer lactate solution) was obtained from Hospira Inc. (Lake Forest, IL, USA). An MFG solution (Gelofusin™, M_w 30 kDa, 4 wt% in a physiological saline solution) was a gift from B. Braun Melsungen AG (Melsungen, Germany). The COP was measured using a colloid osmometer (Model 4420; Wescor Inc., Logan, Utah, USA, Cut-off M_w = 10 000). We did not

Table 1. Plasma substitute solutions and their physicochemical properties. Viscosities at 10 and 1000 sec^{-1} are almost identical, indicating that these polymer solutions are Newtonian fluids

Plasma substitute solutions	M_w (kDa)	M_n (kDa)	M_w/M_n	Conc. (g/dl) in saline	Degree of substitution	COP (Torr)	Viscosity (cP) at 25°C	
							at 10 sec^{-1}	at 1000 sec^{-1}
MFG	30[a]	23[a]	1.3	4[a]	—	44	2.2	2.3
HES_{670}	670[a]	194[b]	3.5	6[a]	0.75	27	4.5	4.4
HES_{130}	130[a]	50[a]	2.6	6[a]	0.38–0.45	35	2.3	2.3
HES_{70}	68[a]	17[a]	4.0	6[a]	0.5–0.55	34	2.0	2.0
rHSA	67[a]	67[a]	1.0	5	—	19	1.3	1.2

HES, hydroxyethyl starch; MFG, modified fluid gelatin; rHSA, recombinant human serum albumin; COP, colloid osmotic pressure.
[a] Data provided by the manufacturer.
[b] Calculated from the concentration dependence of COP.[19]

use DEX in this experiment because it induces anaphylactic reactions in rats.[24]

Animal experiment of repeated hemorrhage and infusion of plasma expander and HbV

The Laboratory Animal Care and Use Committee of the School of Medicine, Keio University, approved the entire experimental protocol. The protocol complies with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council—National Academy of Sciences (Washington, DC: National Academy Press, 1996).

Experiments were conducted using 63 male Wistar rats (240–310 g b.w.). The rats were anesthetized using 1.5%-sevoflurane-mixed air inhalation (Maruishi Pharmaceutical Co. Ltd., Osaka) with a vaporizer (Model TK-4 Biomachinery; Kimura Medical Instrument Co. Ltd., Tokyo) throughout the experiment (fraction of inspired O_2:$FiO_2 = 21\%$) while spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing, OD 0.8 mm, ID 0.5 mm; Natsume Seisakusho Co. Ltd., Tokyo) filled with a saline solution containing 40 IU/ml heparin were introduced through the right jugular vein into the right atrium and into the right common carotid artery. The arterial catheter was connected to a strain-gauge transducer and arterial blood pressure was displayed on polygraph (LEG-1000; Nihon Kohden Corp., Tokyo).

The systemic blood volume was estimated as 56 ml/kg body weight. After withdrawing 10% of the blood volume as a hemorrhage (5.6 ml/kg, 1 ml/min) from the carotid artery, a plasma substitute solution (rHSA, HES_{70}, HES_{130}, HES_{670}, or MFG) was injected isovolemically into the right jugular vein. This procedure was repeated six times to attain 60% blood exchange. At this point, the hematocrit (Hct) decreased to about 16.8% from 42%. After the blood exchange with a plasma expander, HbV suspension or saline (20 ml/kg) was infused at a rate of 1 ml/min. The numbers of animals of 10 experimental groups were rHSA + HbV ($n = 5$), rHSA + saline ($n = 6$), HES_{70} + HbV ($n = 5$), HES_{70} + saline ($n = 7$), HES_{130} + HbV ($n = 6$), HES_{130} + saline ($n = 7$), HES_{670} + HbV ($n = 6$), HES_{670} + saline ($n = 5$), MFG + HbV HbV ($n = 5$), and MFG + saline ($n = 6$). For comparison, one group received saline for blood exchange and subsequent saline infusion (saline + saline group, $n = 5$).

Measurements

Changes in systemic hemodynamics and blood gases were observed before blood exchange with a plasma substitute (baseline), after 60% blood exchange, immediately after infusion of 20 ml/kg HbV or saline infusion, and 1, 2, and 4 hr after infusion (Fig. 1). Blood samples were collected in 70-IU/ml heparinized microtubes (125 μl, Clinitubes™; Radiometer A/S, Copenhagen, Denmark) for blood gas analyses, and in glass capillaries (Terumo Corp., Tokyo) for Hct measurements. A pH/blood gas analyzer (model ABL 700; Radiometer A/S) was used for analyzing arterial blood O_2 partial pressure (PaO_2), CO_2 partial pressure ($PaCO_2$), pH, lactate, and base excess (BE). A recording system (Polygraph System 1000; Nihon Kohden Corp., Tokyo) was used for continuous monitoring of the mean arterial blood pressure (MAP) and heart rate (HR).

After a final measurement at 4 hr, about 2 ml of blood was collected into a heparinized syringe for viscosity measurement. Then the rats were euthanized by increased concentration of sevoflurane for anesthetic inhalation. Steady-shear viscosity measurements were taken using a rheometer (Physica MCR 301; Anton Paar GmbH, Graz, Austria).[18] The cone diameter was 50 mm; the gap angle between the cone and plate was 1°. About 650 μl of the sample was sandwiched between the cone and plate. The excess solution was wiped away. All measurements were performed at 25°C.

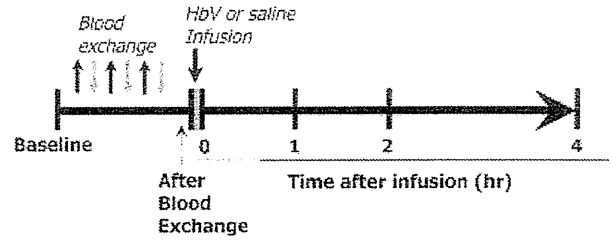

Figure 1. Time points for the measurements before and after 60% blood exchange with a plasma expander, and after infusion of HbV or saline.

All data are presented as mean ± standard deviation of the indicated number of animals. Data were analyzed using software (StatView ver. 5.0; Abacus Concepts Inc., Berkeley, CA). Time-related differences compared to the baseline within each group were assessed using paired *t*-tests. Differences were inferred as significant when $p < 0.01$.

RESULTS AND DISCUSSION

All rats that received a plasma expander at the repeated hemorrhages and the subsequent infusion of HbV or saline survived for 4 hr of the experimental period. The saline + saline group, receiving saline at the repeated hemorrhages and the subsequent infusion of saline, showed three deaths among five rats.

Figure 2 shows changes of Hct of the rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups as representatives, and saline + saline group as the negative control group. Hct data of other groups are shown only at 4 hr. Groups receiving rHSA and HES_{670} showed stable Hct for 4 hr (18–19%). However, the groups receiving MFG, HES_{70}, and HES_{130} showed a gradual increase of Hct (20–23%), indicating the reduction of circulating blood volume (hemoconcentration). For example, a 5%-Hct increase indicates the reduction of 20% blood volume. The circulation persistence of HES depends on its molecular weight and degree of substitution (DS).[25] The DS is the ratio of hydroxyethyl group conjugation to OH groups on the starch backbone. As Table 1 shows, HES_{130} shows the smallest DS and is more subjective to be decomposed by amylase in blood. Therefore, the rats receiving HES_{130} and low molecular weight HES_{70} showed similar Hct at 4 hr. On the other hand, HES_{670} has the highest molecular weight and highest DS; it was expected to have a longer retention time of COP and circulating blood volume and the results showed as expected. The saline + saline group showed the significant increase of Hct (24.0 ± 3.2% at 2 hr) because of the fast extravasation of saline fluid to interstitium tissue and hemoconcentration.

Mean arterial pressure (MAP) of the saline + saline group showed significant decrease to 44.8 ± 5.4 mmHg after blood exchange and remained lowest throughout the experiment

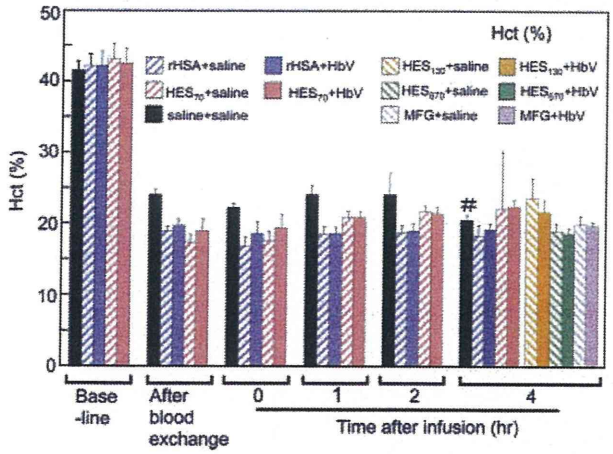

Figure 2. Changes in hematocrit (Hct) after blood exchange with rHSA and HES_{70} and for 4 hr after the subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other groups are shown only at 4 hr. $^{*}p < 0.01$ versus baseline; $^{\#}n = 2$. This figure is available in color online at wileyonlinelibrary.com/journal/pat

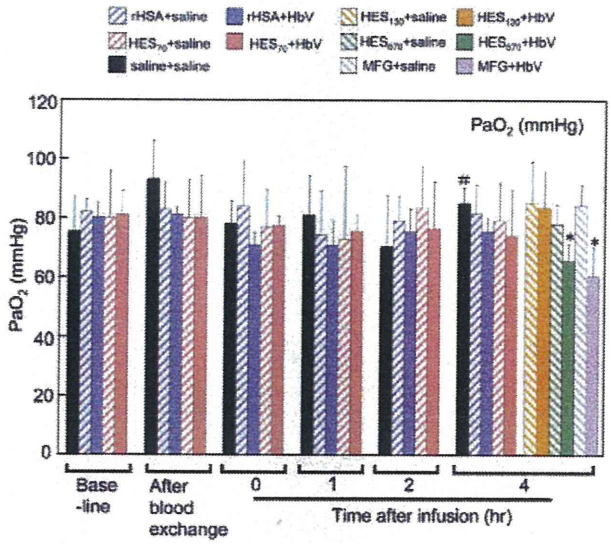

Figure 4. Changes in arterial blood oxygen partial pressure (PaO_2) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other groups are shown only at 4 hr. $^{*}p < 0.01$ versus baseline; $^{\#}n = 2$. This figure is available in color online at wileyonlinelibrary.com/journal/pat

(baseline value, 98.7 ± 9.6 mmHg) (Fig. 3). The hypovolemic and anemic conditions, together with the worsened blood gas parameters shown below, caused death in three rats among five. In contrast, other groups receiving plasma expanders showed higher MAP values (80–90 mmHg). Especially those receiving rHSA showed stable values. The groups of +HbV tended to show higher values in MAP compared with the groups receiving saline alone.

Arterial blood oxygen partial pressure (PaO_2) (Fig. 4) and arterial blood carbon dioxide partial pressure ($PaCO_2$), pH, and

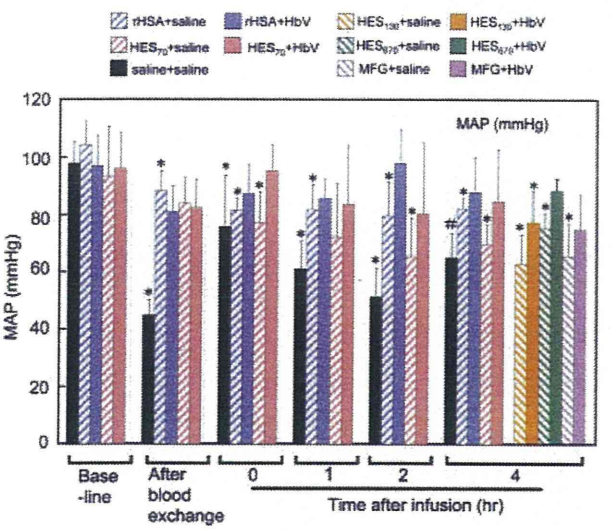

Figure 3. Changes in mean arterial pressure (MAP) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other groups are shown only at 4 hr. $^{*}p < 0.01$ versus baseline; $^{\#}n = 2$. This figure is available in color online at wileyonlinelibrary.com/journal/pat

lactate were stable in all groups receiving plasma expanders. Table 2 presents parameters at 4 hr for rats receiving plasma expanders, and the saline + saline group at 2 hr. The saline + saline group showed a significant increase in PaO_2 and a decrease in $PaCO_2$, indicating hyperventilation because of tissue hypoxia and metabolic acidosis, as evident from the increased lactate level. Three rats died during the 4 hr observation. Venous blood oxygen partial pressure, PvO_2, of the saline + saline group decreased remarkably to around 20 mmHg after blood exchange; the low level continued throughout the experiment because of hypovolemia and decreased oxygen delivery to tissues (Fig. 5). Results show that oxygen extraction (arterio-venous difference of oxygen saturation) increased compensating reduced oxygen delivery to tissues. Other groups receiving plasma expanders showed higher PvO_2 values, indicating that oxygen delivery to tissues was less reduced after blood exchanges. Especially, those receiving rHSA showed higher PvO_2 values than those receiving other plasma expanders.

Blood viscosity was measured 4 hr after the infusion of HbV or saline (Table 2). Fundamentally, the presence of RBCs is the reason for high viscosity of normal blood. Because of the dilution of blood by the blood exchange with a plasma expander and infusion of HbV or saline, the blood viscosity of all groups was lower than the baseline level. In our previous reports of *in vitro* rheological observation, some plasma expanders interact with HbV and induce flocculation of HbV and the consequent increased viscosity.[18,19] However, in the present *in vivo* experiment, all the components (blood, plasma expander, and HbV) were mixed mutually and diluted after the blood exchange and the infusion. The resulting blood viscosity was lower than the baseline level. Perhaps for this reason, we observed no deteriorative effect of the co-infusion of HbV and a plasma

Table 2. Summary of systemic parameters. The data at 4 hr observation period was summarized for the groups receiving plasma expanders. For the saline + saline group, the data at 2 hr were shown because three rats died among five by 4 hr

Fluid for 60% blood exchange	rHSA		HES_{70}		HES_{130}		HES_{670}		MFG		Saline	
Fluid for last injection (20 ml/kg b.w.)	HbV	saline	HbV	saline	HbV	saline	HbV	saline	HbV	saline	Saline	Baseline (w/o blood exchange)
Hct (%)	19.2 ± 0.8^a	18.3 ± 1.5^a	22.4 ± 0.9^a	22.0 ± 1.1^a	21.5 ± 1.7^a	23.6 ± 2.7^a	18.5 ± 0.7^a	18.8 ± 1.1^a	19.6 ± 0.5^a	19.8 ± 1.2^a	24 ± 3.2	41.8 ± 1.7
PaO_2 (mmHg)	75.7 ± 4.4	81.5 ± 9.7	74.2 ± 15.6	79.3 ± 12.9	81.5 ± 9.7	84.9 ± 13.8	65.2 ± 5.6^a	77.8 ± 6.6	60.1 ± 9.7^a	83.9 ± 6.9	70.3 ± 17.3	80.5 ± 8.0
$PaCO_2$ (mmHg)	37.7 ± 5.2	42.9 ± 7.9	35.5 ± 4.2	34.1 ± 2.8	35.6 ± 2.5	36.3 ± 2.9	36.3 ± 1.5	35.8 ± 1.8	39.2 ± 3.4	37.1 ± 2.5	41.9 ± 14.2	39.5 ± 4.0
BE (mM)	2.8 ± 1.4	2.8 ± 1.1	1.5 ± 0.6^a	1.0 ± 1.0^a	1.6 ± 1.6	1.5 ± 1.0^a	3.0 ± 1.0	3.3 ± 1.1	3.0 ± 1.3	0.0 ± 5.1	-3.6 ± 4.5	3.4 ± 0.9
pH	7.46 ± 0.03	7.42 ± 0.05	7.44 ± 0.03	7.45 ± 0.03	7.42 ± 0.05	7.45 ± 0.03	7.47 ± 0.01	7.48 ± 0.03	7.45 ± 0.03	7.42 ± 0.08	7.34 ± 0.15	7.45 ± 0.03
Lactate (mM)	1.1 ± 0.4	1.0 ± 0.4	0.8 ± 0.2	0.8 ± 0.2	1.2 ± 0.7	0.8 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	0.9 ± 0.5	0.9 ± 0.2	3.6 ± 2.5	1.0 ± 0.3
PvO_2 (mmHg)	34.9 ± 5.5	40.7 ± 13.0	22.9 ± 8.8^a	30.4 ± 4.1^a	26.2 ± 10.0^a	34.2 ± 4.6^a	29.6 ± 4.8^a	35.9 ± 3.0	23.9 ± 4.9^a	27.0 ± 9.8	20.9 ± 10.0	44.6 ± 7.8
$PvCO_2$ (mmHg)	41.6 ± 5.0	45.2 ± 8.7	41.3 ± 6.9	37.2 ± 3.5	42.7 ± 4.6	40.3 ± 2.3	41.0 ± 2.2	37.9 ± 1.6	42.6 ± 4.2	43.0 ± 7.6	53.0 ± 19.5	42.9 ± 5.6
MAP (mmHg)	87.8 ± 12.0	82.0 ± 3.8^a	84.6 ± 18.1	69.4 ± 7.1^a	77.5 ± 10.9^a	62.9 ± 10.3^a	88.4 ± 4.4	75.4 ± 5.3^a	75.0 ± 12.1	65.5 ± 11.4^a	51.3 ± 10.0	98.7 ± 9.6
HR (beat/min)	393 ± 22	395 ± 32	354 ± 23	364 ± 60	345 ± 22	369 ± 36	366 ± 23	393 ± 36	354 ± 42	338 ± 53	420 ± 21	403 ± 34
Blood viscosity (cP) at $10\ sec^{-1}$	4.1 ± 0.7	6.8 ± 4.3	4.6 ± 0.72	6.7 ± 4.7	10.5 ± 9.0	8.5 ± 6.1	6.5 ± 2.5	8.8 ± 9.0	5.3 ± 1.5	4.8 ± 2.2	—	12.7 ± 2.5
Blood viscosity (cP) at $1000\ sec^{-1}$	2.7 ± 0.2	1.7 ± 0.2	2.9 ± 0.1	2.1 ± 0.2	2.8 ± 0.4	2.2 ± 0.4	3.0 ± 0.2	2.2 ± 0.4	3.0 ± 0.9	2.0 ± 0.2	—	3.6 ± 0.3

Mean ± SD.
[a] $p < 0.01$ versus baseline values.

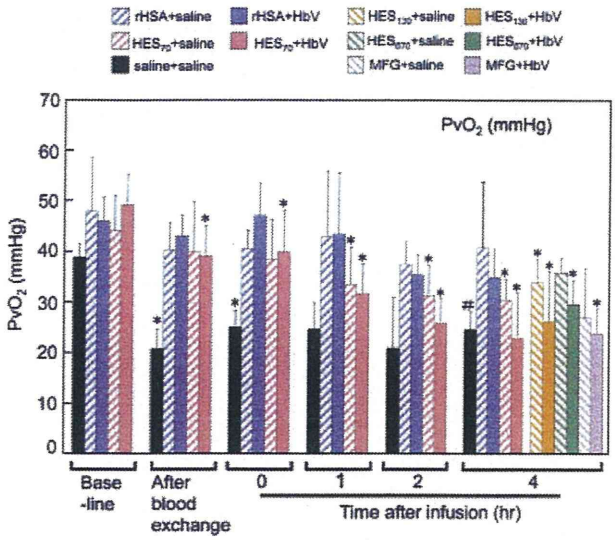

Figure 5. Changes in venous blood oxygen partial pressure (PvO_2) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (the rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other groups are shown only at 4 hr. $^{*}p < 0.01$ versus baseline; $^{\#}n = 2$. This figure is available in color online at wileyonlinelibrary.com/journal/pat

expander. We were unable to test DEX because rats reportedly show anaphylactic reaction toward DEX.[24] Even though DEX is becoming less used in Japan because of some clinical incidents of allergic reaction, the combination of DEX and HbV should be tested using other animal species if necessary.

The hemodynamic and respiratory parameters were maintained; no deteriorative sign was observed even after 60% blood exchange with the plasma expanders in this rat model with only 4 hr of observation. Significant differences might have been observed between +HbV and +saline group if the level of blood exchange was increased, such as to 80–90% blood exchange[9] or if the observation period was prolonged. However, the purpose of this experiment was to clarify the safety, not efficacy, of the combination of HbV and plasma expanders, expecting clinical practice. It is expected in a clinical setting that a crystalloid or colloid will be injected primarily at hemorrhage to maintain the blood volume. Subsequently, transfusion of packed RBCs (or artificial oxygen carrier) will be required when the blood Hb concentration decreases to a critical condition, such as less than 6 g/dl.[26] Infusion of HbV (Hb, 10 g/dl) is expected to increase blood Hb concentration to a safer level. In our clinically relevant repeated hemorrhage animal model, we clarified that no notable side effect of the combination of HbV and plasma expanders.

Another important safety issue of HBOC is hypertension resulting from vasoconstriction, induced by chemically modified Hb solutions such as intramolecular or intermolecular crosslinked Hbs that interact with endothelium-derived NO as a vasorelaxation factor.[27] In our experiment, we observed no hypertension caused by the corpuscular structure of HbV, which retards the reaction of encapsulated Hb and NO.[28,29] We confirmed that HbV is vasoinactive.

CONCLUSION

In this study, we tested intravenous infusion of HbV after blood exchange with a series of plasma expanders in a rat repeated hemorrhage model to clarify whether the combination of a plasma expander and HbV might affect hemodynamics because we previously observed flocculate formation of HbV when dispersed in some biopolymer aqueous solutions. The rats showed stable hemodynamic and respiratory parameters. No serious symptom was observed in this animal experiment of 60% blood exchange. Among the plasma expanders, rHSA showed the most stable parameters. Rats receiving HES showed some reduction of blood volume and blood pressure, which might be attributable to HES's own characteristics of susceptibility to enzymatic degradation and the resulting shorter circulation persistence, and which is expected to be unrelated to the presence of HbV. Additional infusion of a plasma substitute would re-adjust blood volume and the resulting hemodynamic and respiratory parameters.

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Removal of Cellular-Type Hemoglobin-Based Oxygen Carrier (Hemoglobin-Vesicles) From Blood Using Centrifugation and Ultrafiltration

*†Hiromi Sakai, †‡Keitaro Sou, §Hirohisa Horinouchi, †[1]Eishun Tsuchida, and §Koichi Kobayashi

**Waseda Bioscience Research Institute in Singapore, Singapore; †Research Institute for Science and Engineering; ‡Center for Advanced Biomedical Sciences, Waseda University; and §Department of Surgery, School of Medicine, Keio University, Tokyo, Japan*

Abstract: The hemoglobin-vesicle (HbV) is an artificial oxygen carrier encapsulating a concentrated hemoglobin solution in a phospholipid vesicle (liposome). During or after transporting oxygen, macrophages capture HbVs in the reticuloendothelial system (RES) with an approximate circulation half-life of 3 days. Animal studies show transient splenohepatomegaly after large doses, but HbVs were completely degraded, and the components were excreted in a few weeks. If a blood substitute is used for emergency use until red blood cell transfusion becomes available or for temporary use such as a priming fluid for an extracorporeal circuit, then one option would be to remove HbVs from the circulating blood without waiting a few weeks for removal by the RES. Using a mixture of beagle dog whole blood and HbV, we tested the separation of HbV using a centrifugal Fresenius cell separator and an ultrafiltration system. The cell separator system separated the layers of blood cell components from the HbV-containing plasma layer by centrifugal force, and then the HbV was removed from plasma phase by the ultrafiltration system. The HbVs (250–280 nm) are larger than plasma proteins (<22 nm diameter) but smaller than blood cell components (>3 μm). The size of HbVs is advantageous to be separated from the original blood components, and the separated blood components can be returned to circulation. **Key Words:** Blood purification—Cardiopulmonary bypass—Separation—Blood substitute—Hemoglobin-vesicles—Centrifugation—Oxygen carrier.

Hemoglobin-vesicles (HbVs) are artificial oxygen-carrying particles that encapsulate a concentrated hemoglobin (Hb) solution in phospholipid vesicles (liposomes) (1). The concentration of the HbV suspension is adjusted to 10 g/dL to attain a sufficient oxygen-carrying capacity that is comparable with that of blood. Although the most abundant protein in blood is Hb (12–15 g/dL), it becomes toxic when released from red blood cells (RBCs) (2). Each HbV has a cellular structure, with diameter of about 250–280 nm. The cellular structure is important to shield and eliminate the toxic effect of cell-free Hb. Moreover, that cellular structure enables coencapsulation of small functional molecules, such as allosteric effectors to regulate oxygen affinity or metHb reduction system to provide a longer functional half-life. Our safety and efficacy evaluations of HbV in animal experiments have clarified that HbVs transport oxygen for extreme hemodilution experiments with a level of blood exchange of 80–90%, for a fluid resuscitation from hemorrhagic shock (3), and for an oxygen carrier to improve the oxygenation of ischemic tissues such as brain and skin (4,5). Furthermore, HbVs are effective as a priming fluid for extracorporeal circulation systems (artificial lung) (6). During or after transporting oxygen to peripheral tissues, HbVs are captured by the reticuloendothelial system (RES) just as senescent RBCs are (7). However, their shorter circulation half-life, about 3

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Address correspondence and reprint requests to Dr. Hiromi Sakai, Waseda Bioscience Research Institute in Singapore, 11 Biopolis Way, #05-01/02 Helios, Singapore 138667, Republic of Singapore. E-mail: hirosakai@aoni.waseda.jp
[1]Emeritus Professor Eishun Tsuchida passed away during the preparation of this publication.

days depending on the dose, results in transient and significant splenohepatomegaly. With the present technology of surface modification of vesicles, it is quite difficult to prolong the circulation half-life longer than 3 days. Results of pharmacokinetics, biodistribution, and histopathological studies clarified that the captured HbVs are degraded in macrophages and are excreted without noteworthy side effects (2,8–12).

If a blood substitute is used in an emergency situation until a safe blood transfusion is available or for a temporary use, such as a priming fluid for an extracorporeal circuit where the resulting dose is extremely high, it would take a long time for blood clearance. It might be desirable to remove the component of the blood substitute from the circulating blood without awaiting its removal by RES in a few weeks. The HbV particles (250–280 nm) are much larger than plasma proteins (22 nm for fibrinogen) and much smaller than blood cells (3 μm for platelets). We previously reported that HbVs do not precipitate at a low centrifugal force, thereby complicating clinical laboratory tests of colorimetric and turbidimetric analyses (13,14). However, ultracentrifugation or centrifugation in the presence of a high-molecular-weight dextran can precipitate HbVs completely, which implies the possibility of HbV removal from the circulating blood cell components. Moreover, we usually use an ultrafiltration system for the production procedures of HbVs, such as separation of HbVs from unencapsulated Hb solution (15), which implies the possibility of removal of HbVs from plasma proteins. For this study, we tested the separation of HbVs from blood components using a cell separation system and an ultrafiltration system.

MATERIALS AND METHODS

Mixture of HbV and blood components

We used beagle dog blood obtained during an efficacy study of HbVs. The preparation method of the HbV (Table 1) is described elsewhere (2). We tested HbVs as resuscitative fluid for a hemorrhagic shock using male beagle dogs (16,17). The Laboratory Animal Care and Use Committee of the School of Medicine, Keio University approved the entire experimental protocol. The protocol complies with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council—National Academy of Sciences (Washington, DC: National Academy Press, 1996). The animals, premedicated with atropine sulfate (0.07 mg/kg, intramuscularly [i.m.]), were anesthetized by ketamine hydrochloride (5 mg/kg i.m.). Inhalation anesthesia was maintained with 2.0–2.5% sevoflurane-mixed air by the spontaneous respirations using an anesthesia apparatus (SN–487, 3 mL/min, Shinano Seisakusho Co., Tokyo, Japan). The level of blood exchange was approximately 50%. The details of the results of the resuscitative effects of HbVs were presented at the 11th International Symposium on Blood Substitutes (October 18–21, 2007, Beijing, China) (17). After the 4 h-observation experiment and immediately before the dogs were euthanized, arterial blood was withdrawn from a femoral artery using a heparinized syringe. The withdrawn blood (280 mL), containing about 40 mL of acid citrate dextrose (ACD) solution (ACD-A, Kawasumi Laboratories, Inc., Tokyo, Japan; sodium citrate, 2.2 g/dL; citric acid hydrated, 0.8 g/dL; glucose, 2.2 g/dL) was used for a separation study within 1 h. To prime the circulation volume of the cell separation system, a small amount of ACD solution (<100 mL) was used. The Hb concentration was measured using the cyanmethemoglobin method (Hemokit-N; Alfresa Pharma Corp., Osaka, Japan). For the HbVs, the destruction of the cellular structure was confirmed by the shape of the spectrum between 450 and 700 nm for determining the completion of the reaction. The yields of HbVs were calculated from the concentration and volume of each fraction.

For detailed analysis of the separation of HbVs from plasma with the tangential-flow ultrafiltration system, we used a mixture of beagle plasma and HbV. Beagle blood (260 mL) was withdrawn from a femoral artery using a heparinized syringe for a hemorrhagic shock experiment. The blood was centrifuged at 5000 rpm for 10 min. Then the supernatant plasma phase was collected (130 mL) and mixed with HbV (10 g/dL) at a volume ratio of 20 vol%.

TABLE 1. *Physicochemical characteristics of Hb-vesicles*

Parameter	Values
Diameter (nm)	250–280
[Hb] (g/dL)	10
Suspending medium	Saline (0.9% NaCl)
P_{50} (mm Hg)	27
Viscosity (cP)	3

Blood purification system using clinical devices

A blood cell separator (ASTEC 204; Fresenius Kabi AG, Bad Homburg, Germany) was used for the separation of HbVs from blood cell components. This instrument enables a continuous in-line blood supply, centrifugation, and separation into plasma and cell

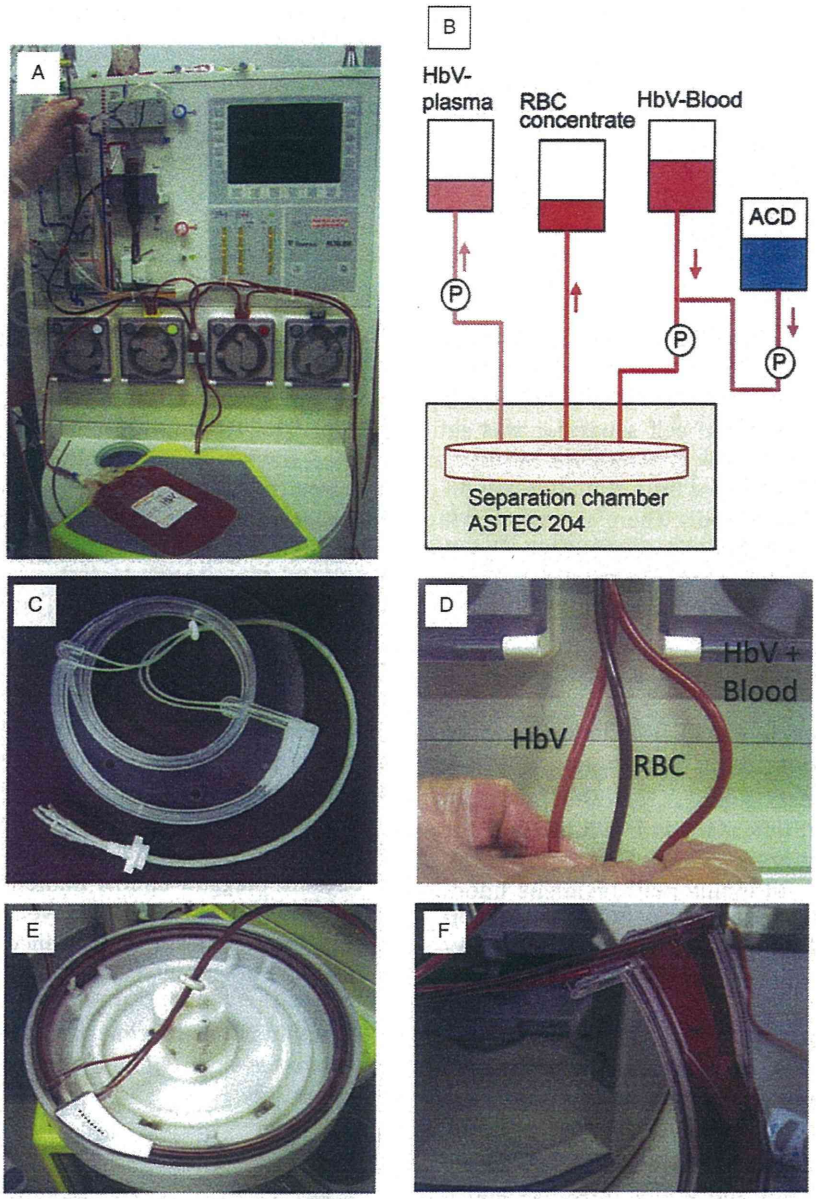

FIG. 1. Fresenius Cell Separator (ASTEC 204; Fresenius Kabi AG) used for the separation of HbV from beagle blood components: (A) experimental overview of the instrument and (B) schematic representation of the inlet and outlet fluids. The HbV–blood mixture was poured into the system with a small addition of ACD solution for priming. It was centrifuged using a hoop-shaped rotor and separated into HbV + plasma phase and RBC phase. (C) Hoop-shaped rotor connected with three tubes. (D) The three tubes showed different colors, indicating the separation of HbV–blood mixture into HbV–plasma and RBCs. (E) View of a hoop-shaped rotor when the operation was temporarily stopped. (F) Magnified view of the hoop-shaped rotor showing the interface between the HbV–plasma layer and the dense red RBC layer.

components (Fig. 1). The hoop-shaped centrifugal rotor was provided as sterilized. The system contains an optical system to detect the interface of blood cell layers and plasma phase by light absorption (transparency of a plasma phase). However, because of the marked turbidity of the HbV-containing plasma, we visually detected the interface and chose the timing for separation.

A hollow fiber module of a sterilized membrane-type plasma separator (Plasmaflo OP-02W; Asahi

TABLE 2. *Results of separation of HbV from beagle dog blood immediately after the cell separator treatment*

	Before separation HbV–blood	HbV–plasma + ACD	RBC concentrate
Volume (mL)	280	252	29
Hct (%)	17.4	0.7	85.5
[Hb] (g/dL)	8.5	2.9	25
$[Hb]_{HbV}$ (g/dL)	2.9	2.7	—
$[Hb]_{RBC}$ (g/dL)	5.6	0.2	25

Kasei Medical Co. Ltd, Tokyo, Japan, pore size, 0.3 μm, membrane 0.2 m^2) was used to separate HbVs from plasma components. The Hb-containing plasma obtained using the cell separator was subjected directly to the hollow fiber module. Because of the small volume of the test fluid, the retentate fluid was not circulated repeatedly. Then, we collected the filtrated plasma. The albumin concentration in the plasma phase before and after separation was measured by bromocresol green method (13).

Tangential-flow ultrafiltration for separation of HbV and plasma

An ultrafiltration unit (Labscale TFF System, Millipore Corp., Billerica, MA, USA), equipped with a pump and two pressure gauges, was used for detailed analysis of filtration efficiency with two different ultrafiltration unit membranes. One is Durpore (Millipore Corp.) made of hydrophilic polyvinylidene fluoride with pore size of 0.1 μm and 50 cm^2 filtration area. The other is Biomax (Millipore Corp.) made of polyether sulfone with a cutoff molecular weight (Mw) of 1000 kDa and 50 cm^2 filtration area. This filter can be sterilized by perfusion of 1-N NaOH solution, but we did not sterilize it because we tested the separation profile and did not intend to reinfuse the components into the animals in this experiment. The mixture of HbVs and plasma was input to the unit at a flow rate of 30 mL/min. The inlet and outlet pressures and the permeation flux (in L/m^2/h) were monitored.

RESULTS

Separation of HbVs from blood components using clinical devices

The HbV–blood mixture obtained from a beagle dog contained 2.9 g/dL of Hb derived from HbV, approximately 34% of the total Hb. The hematocrit (Hct) was 17.4% (Table 2). During centrifugal separation using the Fresenius cell separator (ASTEC 204; Fresenius Kabi AG), the outlet tubes clearly showed the components' respective colors: a dense red fluid as the RBC fraction and a pink, opaque fluid as a HbV–plasma fraction (Fig. 1d). The centrifugal ring unit clearly showed the interface between the two different layers: the RBC layer and HbV–plasma layer. The Hb concentrations and Hct of the separated fractions are presented in Table 2. The HbV–plasma fraction showed Hct of only 0.7%, and Hb concentration derived from RBC, $[Hb]_{RBC}$, was calculated as 0.2% (Table 2). Although some fluid remained in the system, the removal efficiency of HbVs was calculated as 84%. The RBC fraction was highly concentrated to Hct 85%; most importantly, it contained no HbVs.

The HbV–plasma fraction (252 mL) was input to the Plasmaflo hollow fiber module. Because of the small volume of the test fluid, the retentate fluid was not circulated repeatedly. Then, we collected the filtrated plasma. Because of the limited volume of the applied fluid and the dead-end filtration, the filter became plugged almost immediately. However, the filtrated plasma was pale yellow and transparent (Fig. 2). It showed no presence of Hb by the cyanomethemoglobin method, indicating the complete removal of HbVs and recovery of plasma proteins. Albumin concentration in the plasma was 1.7 g/dL, which was slightly lower (2.0 g/dL) than that before separation.

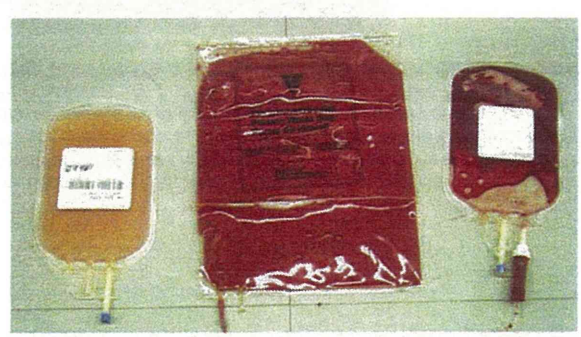

FIG. 2. Photograph of the plasma fraction, HbV fraction, and RBC fraction separated from the HbV–blood mixed beagle blood using the clinically available cell-separation system (Cell Separator ASTEC 204; Fresenius Kabi AG) and an ultrafiltration system (Plasmaflo OP-02W; Asahi Kasei Corp.).

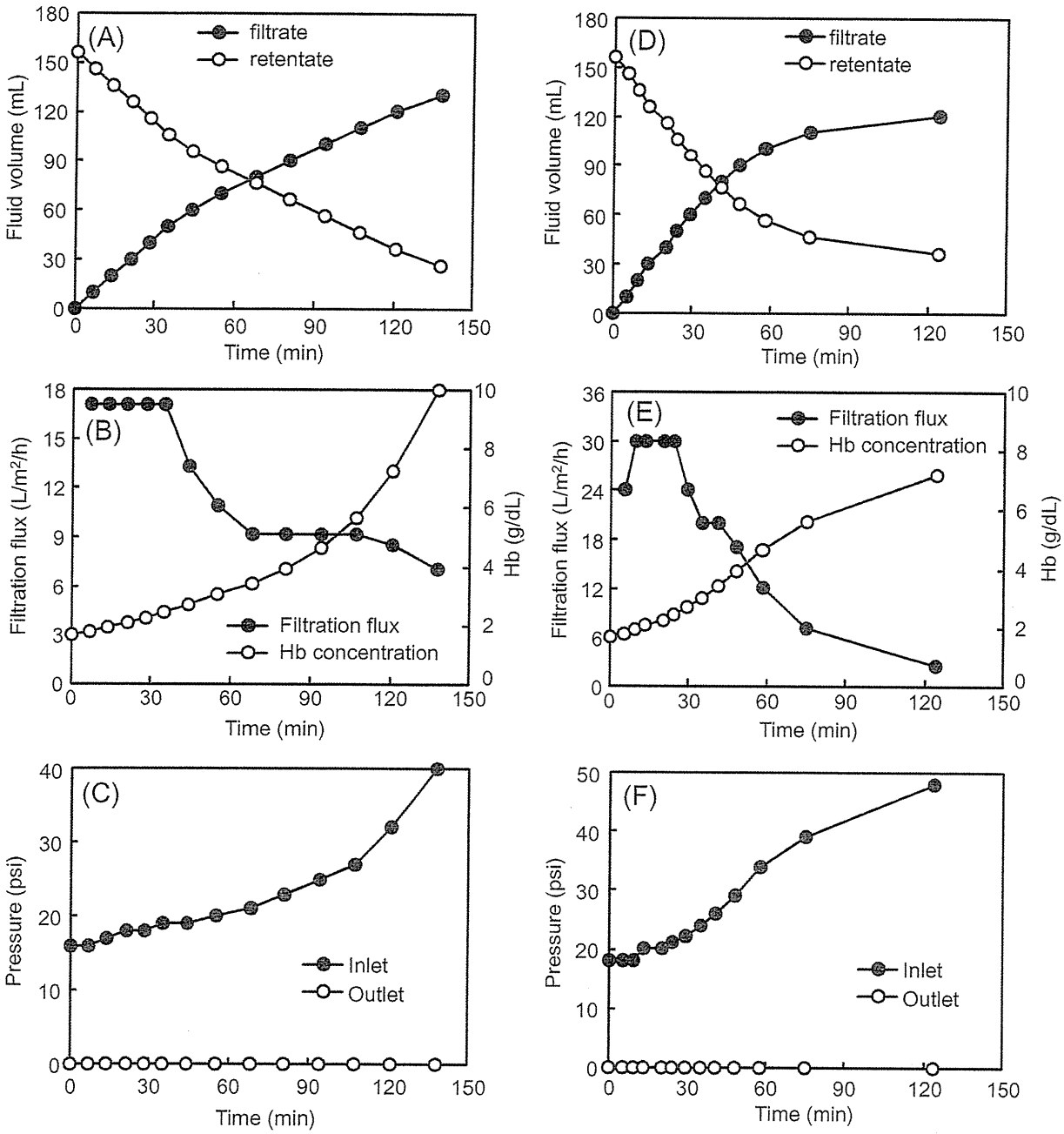

FIG. 3. Permeation profiles of the plasma layer separated from the mixture of HbV–plasma by Millipore ultrafilters using Labscale (Millipore Corp.) at an inlet flow rate 30 mL/min: (A,B,C) Durpore, (D,E,F) Biomax, (A,D) volume change of the retentate and filtrate solutions, (B,E) changes of permeation flux in L/m²/h and the retentate Hb concentration of HbV, (C,F) changes of inlet and outlet pressures during the ultrafiltration procedure.

Tangential-flow ultrafiltration for separation of HbV and plasma

The results of tangential-flow ultrafiltration are summarized in Fig. 3. The permeation fluxes of Durpore and Biomax at the beginning were, respectively, 17 and 30 L/m²/h (Fig. 3B,E). Biomax showed double the filtration efficiency of Durpore. Moreover, the filtrate for Durpore showed the presence of HbVs, indicating that a small amount of HbVs passed through the membrane despite its nominal

pore diameter of 0.1 μm. In contrast, Biomax showed no such leakage of HbVs; the filtrated solution was transparent. Both filters showed reduction of the permeation flux when the retentate HbV concentration became 2.5 g/dL. The inlet pressure increased gradually and eventually reached 40–50 psi (Fig. 3C,F). When the inlet pressure reached 40 psi, Durpore and Biomax, respectively, provided the filtrate solution volumes of 130 and 120 mL (initial volume 156 mL), and the yield was 83 and 77%. Both ultrafilters passed large quantities of the plasma phase.

DISCUSSION AND CONCLUSIONS

The dosage of a blood substitute is expected to be comparable with that of a conventional fluid therapy. Even though its particles are eventually captured by the RES and the components are excreted through urine and bile, and although the RES has a high capacity to manage the large dosage of a blood substitute, the shorter circulation half-life of a blood substitute would burden the RES during that short period, and might, therefore, influence the immunological function to some degree. Therefore, circumstances might arise that would necessitate that blood substitutes be removed from circulating blood without waiting for RES purification of the circulating blood. In this study, we tested the possibility of removing an injected blood substitute from the circulating blood using conventional clinical instruments.

The cell separator system we used is now used clinically for the collection and removal of blood cells such as platelets, CD34 positive cells, lymph cells, and platelets, and for plasma separation. This system enabled the separation of HbV-containing plasma fraction and RBC fraction. The intracellular Hb concentration of the HbV is about 35 g/dL, which is almost identical to that of RBC. However, the smaller particle diameter of HbVs (250–280 nm) increases their diffusivity, and the lipid components of the bilayer membrane of HbVs (Hb/lipid weight ratio is 1.6–1.9) make the density of HbVs lighter. Consequently, HbV particles remain in a plasma phase at a low centrifugal force. The problem of the system is that the plasma layer became turbid because of the presence of HbVs, and the system was unable to detect the interface between the RBC and plasma layers using an optical method. The detection system therefore requires some modification.

In our production procedure of HbVs, unencapsulated Hb is easily separated from HbVs using a tangential-flow ultrafiltration system with a cutoff Mw of 1000 kDa or larger (15). Therefore, it is readily inferred that HbVs can be removed from plasma protein similarly using an ultrafiltration system. Common and gigantic plasma proteins are fibrinogen (Mw = 340 kDa, hydrodynamic diameter 21.9 nm), immunoglobulin (Ig)A (162 kDa, 13 nm), IgG (150 kDa, 10.6 nm), IgM (950 kDa, 25.3 nm), and albumin (67 kDa, 6.2 nm) (18). These plasma proteins are much smaller than HbVs, and they pass through the ultrafiltration filter. It is speculated that a cell-free Hb-based oxygen carrier would be difficult to separate from the plasma protein because they are of nearly equal size. In this sense, the HbV size is advantageous and suitable for its separation from plasma proteins and blood cells, as we reported previously for the avoidance of interference effects of HbVs on plasma clinical chemistry based on colorimetric and turbidimetric analyses (13).

Because of the limited volume of the fluid and the dead-end filtration in our experimental setting, we were unable to evaluate the clinically used Plasmaflo appropriately. Further study is necessary to test Plasmaflo using a circulating system to determine the duration of passage through the filter. We tested the tangential-flow Millipore ultrafiltration system for separation of HbVs and plasma—even though it is not used for clinical use but for industrial use—because we are accustomed to using it and because the permeation flux is analyzed easily using the Lab-scale system with tangential-flow procedure. Results clarified that Biomax showed better permeation flux than Durpore; it also showed no permeation of HbVs. Judging from results of this study, Biomax, which is made of polyether sulfone with a cutoff Mw of 1000 kDa, is suitable for HbV separation. A drawback of the ultrafiltration for HbV separation is the decreased permeation flux with increased inlet pressure by increasing the concentration of HbVs in retentate fluid. This would be caused by the increased viscosity of the retentate fluid with an increase in the concentration of HbVs. The Biomax filtration efficiency can be enhanced further by adding a saline solution to the retentate to maintain the concentration of HbVs at a lower level though the concentration of the filtrate decreases gradually and another ultrafiltration is required to remove water. The filtration surface area can be enlarged easily by piling up the filter units of Biomax for permeating a larger volume of fluid in a shorter period with a lower applied pressure. Moreover, control of anticoagulation is thought to be important because it possibly affects interaction of blood (or plasma) with devices used for removal procedure and its removal efficiency.

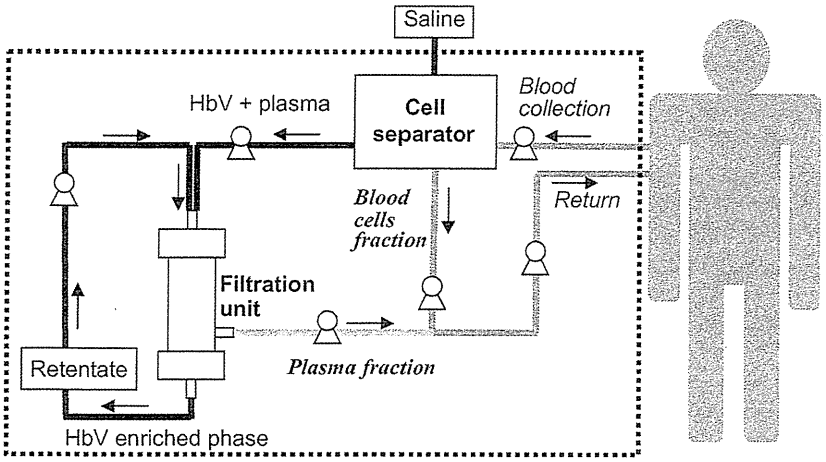

FIG. 4. Schematic representation of a proposed clinical device system connecting centrifugation and ultrafiltration to remove HbV from the circulating blood. Collected blood from a patient is centrifuged using a cell separator and separated to RBC fraction and HbV–plasma fraction. The HbV–plasma fraction is input to an ultrafiltration unit. Then the filtrated plasma fraction is mixed with the RBC fraction and returned to the patient.

Actually, HbVs were tested experimentally as a primer of the extracorporeal cardiopulmonary bypass in a rat model (6). For a subsequent long-term neurological safety study, all the blood–HbV mixture in the circuit was collected and centrifuged manually; then the precipitated RBCs were collected and returned intravenously. Results indicate the possibility of using the continuous HbV-removal system for the post-treatment of the cardiopulmonary bypass when the level of blood exchange is high, especially for infant and neonate surgery, and autologous RBCs should be returned to a patient. Figure 4 portrays a possible clinical device system including a connection of centrifugation and ultrafiltration.

Results of our recent study showed that a rat model of chronic hepatitis induced by tetrachloromethane tolerated the injection of HbVs; the RES managed the metabolism and excretion of a large amount of HbVs (19). Nevertheless, it would be desirable to establish a method to remove the injected HbVs from the circulating blood when blood transfusion is available and HbVs become unnecessary, for example, a situation of resuscitative injection at an uncontrolled hemorrhage and the level of blood exchange exceeds 80 or 90%, and then blood transfusion becomes available. Even though the critical dosage (level of blood exchange) is not identified yet, it might be better to reduce the burden on the RES and to avoid a hemoconcentration-like situation (a high HbV concentration with a high Hct). We admit that the present study might be premature, the system demands optimization, and further discussion is required about the necessity of this separation system at a clinical situation. However, it is the first reported attempt to remove the injected HbVs from circulating blood. Recent development of nanomedicine has brought the creation of small particles of various kinds used not only as blood substitutes, but also for use as drug delivery systems, contrast agents, etc. for intravenous injection. The combination of conventional centrifugation and ultrafiltration reported herein is applicable for the removal of such particles.

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Conflict of interest: Of the authors, HS, KS, and ET are the inventors holding patents related to the production and utilization of Hb-vesicles.

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