We previously established a coculture system of human telomerase catalytic subunit-transfected bone marrow stromal cells and $CD34^+$ cells in vitro, by which the expansion of human hematopoietic stem/progenitor cells is visible. Using this in vitro expansion system, we found that the incubation of HbV with CB-derived $CD34^+$ cells up to 3 days had no adverse effect on the expansion of CB-derived hematopoietic stem/progenitor cells (data not shown) (Yamaguchi et al. 2009b). Taken together, the evidence shows that HbV are apparently biocompatible with human CB-derived hematopoietic stem/progenitor cells.

22.1.4 Effects of HbV on Complement Systems, Coagulation and the Kallikrein–Kinin Pathway in Human Plasma

Negatively charged liposomes activate complements via both classical and alternative pathways in rat and human models (Chonn et al. 1991; Cunningham et al. 1979; Devine et al. 1994). Consequently, the reticuloendothelial system rapidly removes opsonized liposomes from blood circulation. Furthermore, complement activation can engender cardiovascular and pulmonary adverse responses, called complement activation-related pseudoallergy (CARPA) (Szebeni 2005; Szebeni et al. 2000). Indeed, certain types of liposome-encapsulated hemoglobin cause CARPA in pigs (Szebeni et al. 1999).

A negatively charged surface also triggers intrinsic coagulation pathway and the kallikrein–kinin cascade by activating coagulation factor XII (Griep et al. 1985; Mitropoulos et al. 1989). PEGylation was regarded as effective for prevention of complement activation by liposomes (Bradley et al. 1998; Klibanov et al. 1990; Woodle and Lasic 1992).

We evaluated the interaction of HbV between human plasma using HbV of three types: PEGylated HbV having DHSG (DHSG-HbV), PEGylated HbV having DPPG (DPPG-HbV) and DPPG-HbV without PEGylation (DPPG-HbV (no PEG)) (Abe et al. 2007). Coatsome EL-A was used as a highly negative-charged liposome without PEGylation. The EL-A greatly reduced the complement titer, but DHSG-HbV had no effect (Table 22.4).

Table 22.4 Consumption of complement by HbV and liposome

Additive	CH50 (U/mL)	
	(additive: serum)	
	20:80	40:60
Saline	33.4 ± 2.8	21.4 ± 1.7
DHSG-HbV	33.5 ± 2.9	22.9 ± 2.4
EL-A	25.1 ± 2.7*	5.9 ± 0.7*

The complement titer (CH50) was measured using a 50 % hemolysis assay with a commercial kit. DHSG-HbV, saline or Coatsome EL-A (a negative-charged liposome) were mixed with serum at the indicated ratio (v/v) at 37 °C for 24 h. The lipid composition (mol %) of coatsome EL-A was DPPC:CHOL:DPPG = 30:40:30. Data are presented as mean ± SD using sera from five individuals. The CH50 of 100 % serum was 38 ± 3.2 U/mL. *$p < 0.05$ versus saline (cited from reference Fujihara et al. (2008))

Table 22.5 Consumption of complement by various types of HbV

Additive	CH50 (U/mL)	
	(additive: serum)	
	20:80	40:60
Saline	36.4	27.9
DHSG-HbV	37.6	31.4
DPPG-HbV	35.9	28.4
DPPG-HbV (no PEG)	29.9	Under detection limit

Complement titer (CH50) was measured using a 50 % hemolysis assay using a commercial kit DHSG-HbV, DPPG-HbV, DPPG-HbV (no PEGylation) or saline was mixed with serum as indicated ratio (V/V) at 37 °C for 24 h. The CH50 of 100 % serum was 45.1 U/mL (cited from reference Fujihara et al. (2008))

Among the three types of HbV, DHSG-HbV and DPPG-HbV show no reduction of the complement titer, although DPPG-HbV (no PEG) showed drastic reduction (Table 22.5).

In terms of coagulation activity, DHSG-HbV had no effect on the prothrombin time (PT) or on the activated partial thromboplastin time (APTT), but DPPG-HbV and DPPG-HbV (no PEG) tended to shorten APTT (data not shown). Furthermore, DHSG-HbV did not cause activation of the kallikrein–kinin cascade even when DHSG-HbV was mixed with plasma at 60 %, whereas DPPG-HbV (no PEG) and DPPG-HbV caused activation of the kallikrein–kinin cascade, producing a digested product. Collectively, DHSG-HbV, which is PEGylated HbV of the most advanced type, is highly biocompatible with human plasma protein.

22.1.5 Effects of HbV on Complement and Anaphylactic Reactions in Rats

CARPA represents a novel subcategory of acute (type I) hypersensitivity reactions (HSR), which are mostly mild, transient and preventable using appropriate precautions (Szebeni et al. 2011). However, in an occasional patient, it can be severe or even lethal. Because a main manifestation of complement activation is cardiopulmonary distress, CARPA might be a safety issue primarily in cardiac patients. Although PEGylation is regarded as effective for prevention of complement activation by liposome, clinical experience shows that even PEGylated liposomal anti-cancer drug caused CAPRA, suggesting that PEGylation is insufficient to escape from the complement system in vivo (Laing et al. 1994; Laverman et al. 2001; Chanan-Khan et al. 2003). Therefore, we evaluated whether the infusion of HbV into rats affects the complement titer in vivo (Abe et al. 2007).

A transient decrease of the complement titer of the rat serum was apparent 3 days after the infusion of HbV or empty vesicle without hemoglobin (EV) (Fig. 22.1).

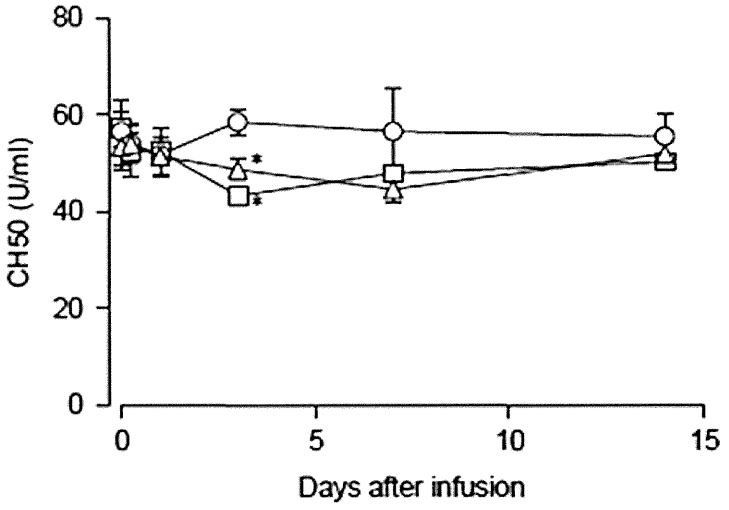

Fig. 22.1 Changes of the complement titer in the rat serum after HbV infusion. HbV (*triangles*), EV (*squares*) or saline (*circles*) was infused into the rats at top-load from the tail vein. The complement titer in the rat serum was measured. It is shown as CH50. $N = 3$–4, mean ± SD $^*p < 0.05$ (cited from reference Abe et al. (2007))

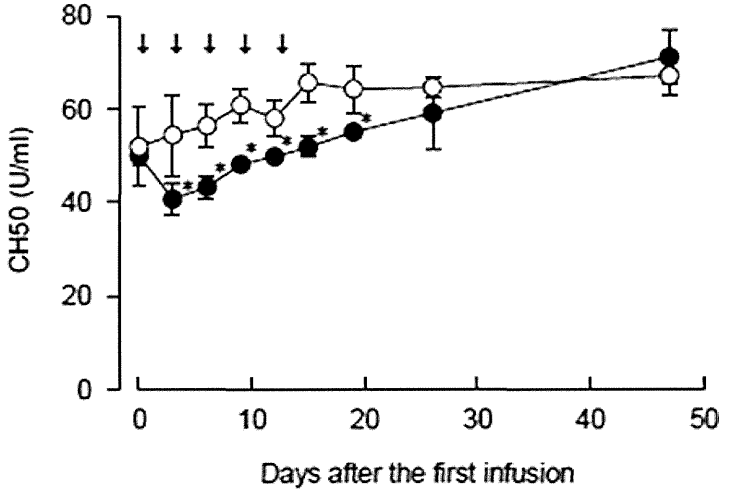

Fig. 22.2 Effects of repeated infusion of HbV on the rat serum complement. HbV (*closed circles*) or saline (*open circles*) was infused into rats at top-load from the tail vein at the time points indicated by arrows. $N = 3$–5, mean ± SD $^*p < 0.05$ (cited from reference Abe et al. (2007))

Neither HbV nor EV caused the consumption of the complement in rat serum in vitro (data not shown). It is particularly interesting that a repeated-infusion study showed that only first infusion of HbV reduced the complement titer. Despite additional infusions of HbV, gradual recovery of the complement titer occurred, suggesting that additional infusions of HbV did not cause the complement consumption (Fig. 22.2).

Furthermore, multiple administration of EV caused no anaphylactic shock, although ovalbumin-sensitized rats died with symptoms of respiratory distress after the second ovalbumin administration (data not shown). Regarding the evidence collectively, the administration of HbV is apparently safe, without allergic or anaphylactic reactions.

Intravenous injection of liposomes into pigs reportedly induces anaphylactoid reactions at small doses, resulting in circulatory disorder. Therefore, the pig model is regarded as useful for the safety evaluation of liposome drugs. HbV did not cause a significant anaphylactoid reaction in pigs, thereby reconfirming the high biocompatibility of HbV (Sakai H et al. 2012).

22.1.6 *Effect of HbV on Immune Response of Rat Splenocytes*

Large amounts of HbV must be transfused to substitute for allogeneic red blood cell transfusion in a clinical setting. Therefore, a considerable number of liposome particles must accumulate in the MPS after HbV infusion, mainly in the spleen and liver (Torchilin 2005). Consequently, it is possible that the immune response fluctuates because of phagocytic cells, which phagocytize HbV, because those cells can become not only positive regulators of immune response as an antigen presenting cells but also negative regulators designated as suppressor macrophages. Reportedly, the production of nitric oxide was involved in its suppressive effect (al-Ramadi et al. 1991; Dasgupta et al. 1999; Schleifer and Mansfield 1993). However, the latter effect has been of little concern, possibly because the amount of liposome used as a drug vehicle is so small that it has no notable negative effect on the immune system in an experimental animal model. This possibility has been addressed recently by our colleagues with the infusion of large numbers of liposomal particles (HbV) (Takahashi et al. 2011). Normal rat splenocytes proliferate well in response to Concanavalin A (Con A) stimulation. However, when the rat splenocytes were taken out 24 h after infusion of HbV (20 % of total blood volume), they failed to proliferate in response to Con A stimulation. When the splenocytes were taken at 7 days after HbV injection, this immune suppression was no longer observed (Fig. 22.3). These results show a transient effect of HbV infusion on immune response. The time course of the suppression appeared to be correlated with that of accumulation and disappearance of HbV from the spleen evaluated based on a histochemical analysis (Sakai et al. 2001; Sou et al. 2005).

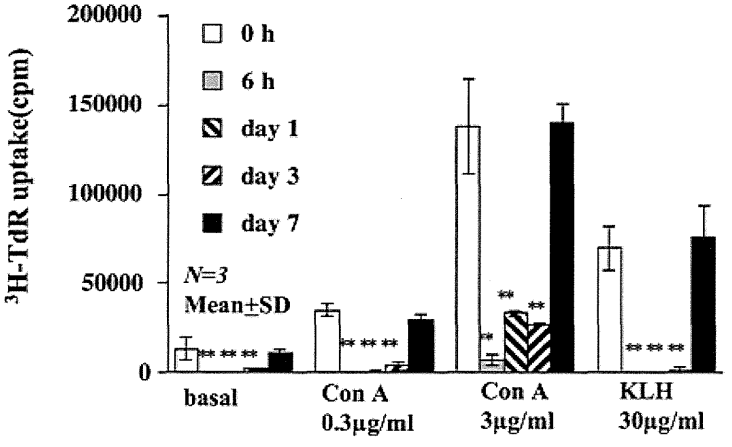

Fig. 22.3 Effect of the HbV and empty vesicles on proliferation of Con A-stimulated rat splenic T cells. Rats were immunized with KLH. After 7 days, they were injected with HbV. Spleens were excised at 6 h, 1, 3 and 7 days later. Bulk splenocytes were stimulated with Con A or KLH. The proliferative response of splenic T cells to Con A and KLH was inhibited from 6 h to 3 days after injection of HbV compared to control (**: $p < 0.01$). No suppression was observed after 7 days. Data are representative of at least three independent experiments and are expressed as the mean ± SD (cited from reference Takahashi et al. (2011))

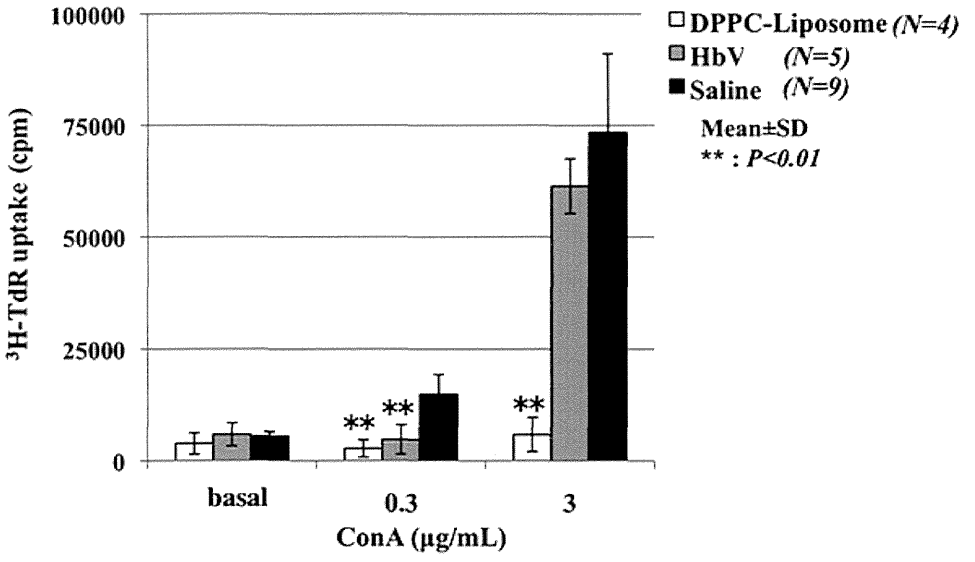

Fig. 22.4 Effect of DPPC-liposomes on immune suppression. HbV, EV, DPPC-liposome or saline was injected intravenously. The spleen was excised 18 h later. DPPC-liposome induced immune suppression. Data from 2–3 independent experiments are collected and expressed as the mean ± SD (cited from reference Takahashi et al (2011))

The suppressive effect can also be induced by injection of empty liposome particles composed of DPPC only (Fig. 22.4), indicating that transient immune suppression is unavoidable as long as the current liposome particle is used as a vehicle for Hb molecules.

Extensive analyses were performed to elucidate the mechanism underlying this phenomenon. Results obtained so far are the following: (1) T cells were activated and express IL2 receptor (CD25) but were unable to proliferate. (2) T cell proliferation specific to keyhole limpet hemocyanin (KLH) was also inhibited from 6 h to 3 days after the injection of liposomes (Fig. 22.3). (3) Direct cell-to-cell contact was necessary for the suppression. (4) Both iNOS and arginase inhibitors restored T cell proliferation to some degree (Fig. 22.5). (5) Cells that trapped vesicles were responsible for suppression. (6) Most of them expressed CD11b/c, but lacked class II molecules. To summarize these results, the phagocytosis of a large load of liposomal particles by rat CD11b/c$^+$, class II- immature monocytes temporarily renders them highly immunosuppressive. In addition, nitric oxide, possibly produced from cells that phagocytized HbV, is involved in immune suppression. It is noteworthy that the results from an additional experiment showed that HbV infusion did not interfere in the in vivo production of KLH-specific antibody (Fujihara et al.), suggesting that the observed immune suppression is restricted in spleen and not systemic phenomenon.

These data and the effects observed on other blood components revealed the excellent and satisfactory bioavailability of HbV and are expected to guarantee the application of HbV to human as a blood substitute in the near future. Finally, from a different perspective, the observed immunosuppressive effect induced by liposomes might open new fields for the clinical application of liposomes themselves.

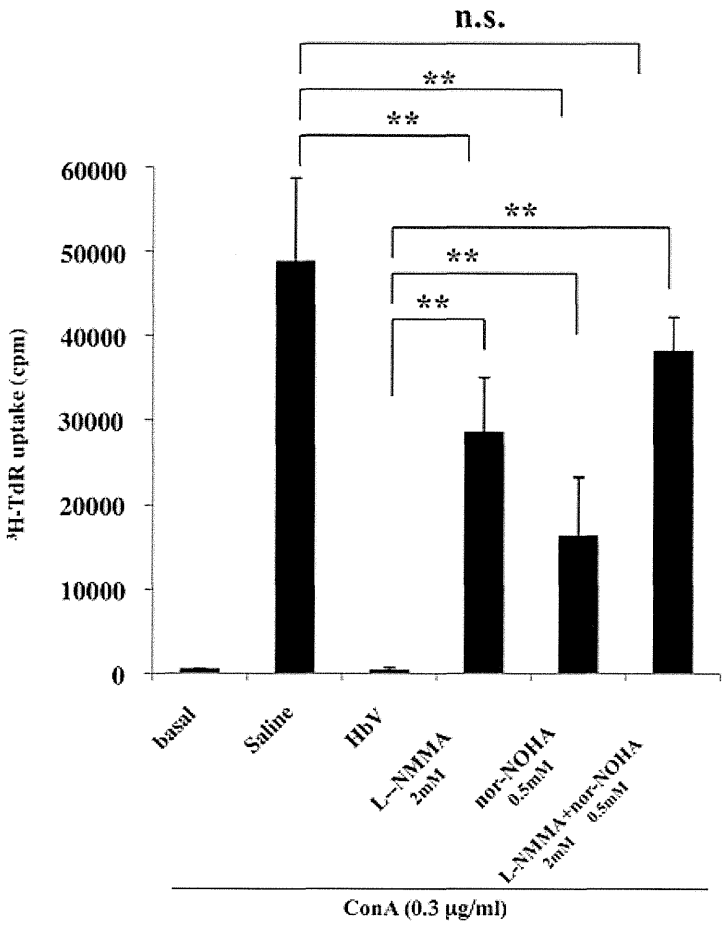

Fig. 22.5 Effect of L-NMMA and nor-NOHA on suppression of T cell proliferation. Each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) in the presence or absence of iNOS inhibitor (L-NMMA, 2 mM) or arginase inhibitor (nor-NOHA, 0.5 mM) or both. T cell proliferation was restored in the presence of each inhibitor to a certain degree. Significant inhibition disappeared in the presence of both inhibitors, suggesting that both iNOS and arginase were involved in the suppression. Data from two independent experiments were collected experiments were collected and expressed as the mean ± SD ($N = 5$) (cited from reference Takahashi et al (2011)). **: $p < 0.01$ ns: not significant

22.2 Conclusion

In this chapter, we presented the excellent biocompatibility of HbV with human blood cells, human plasma proteins in vitro and rat immune systems in vivo. Along with several lines of evidence, our data demonstrate that HbV are promising candidates for use as artificial oxygen carriers.

Acknowledgments The work presented here in was supported in part by Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan).

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Chapter 28
Cellular-Type Hemoglobin-Based Oxygen Carrier as a Resuscitative Fluid for Hemorrhagic Shock: Acute and Long-Term Safety Evaluation Using Beagle Dogs

Tatsuhiko Ikeda, Hirohisa Horinouchi, Yoraro Izumi, Hiromi Sakai and Koichi Kobayashi

28.1 Introduction

Blood transfusion is one of the most important measures in clinical medicine. However, some unresolved issues threaten the achievement of safe transfusion: the possibility of contamination of pathogens; mismatching of blood types; and numerous immunological difficulties. Guideline for safer blood transfusion has been revised repeatedly, such as the reduction of a transfusion trigger, the critical hemoglobin (Hb) level, to 6 g/dL to minimize unnecessary transfusion strictly or to avoid allogeneic transfusion as long as possible to prevent such side effects (American Society of Anesthesiologists Task Force 2006). In this respect, Hb-based oxygen carriers (HBOCs) are considered superior to allogeneic transfusion because they are free of blood-type antigens, and microbial pathogens. The stability of HBOCs for a long-term storage over years is also advantageous when

Supported by Health and Labour Sciences Research Grants, Ministry of Health, Labour and Welfare, Japan

H. Horinouchi (✉)
Department of General Thoracic Surgery, Saitama-city Hospital,
Mimuro 2460, Midori-ku, Saitama-city, Saitama 336-8522, Japan
e-mail: twoeseight@gmail.com

T. Ikeda · H. Horinouchi · Y. Izumi · K. Kobayashi
Department of General Thoracic Surgery, School of Medicine,
Keio University, Tokyo 160-8582, Japan

H. Sakai
Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan
e-mail: hirosakai@naramed-u.ac.jp

H. W. Kim and A. G. Greenburg (eds.), *Hemoglobin-Based Oxygen Carriers as Red Cell Substitutes and Oxygen Therapeutics*, DOI: 10.1007/978-3-642-40717-8_28,

compared with RBC transfusion (Sakai et al. 2008a). Considerably shorter half-life ($t_{1/2}$) of the HBOCs in the blood stream (2–3 days) limit their use (Lee et al. 1995), but they are applicable for shorter periods of use as: (1) a resuscitative fluid for hemorrhagic shock during an emergency situation temporarily or for bridging until RBCs are available (Johnson et al. 2001); (2) a fluid for preoperative hemodilution or perioperative O_2 supply fluid for a hemorrhage during elective surgery to avoid or delay allogeneic transfusion (Standl et al. 1998); (3) a priming solution for the circuit of an extracorporeal membrane oxygenator during cardiac surgery (Yamazaki et al. 2006); (4) an alternative use for other potential indications, for example, so-called O_2 therapeutics to oxygenate ischemic tissues (Contaldo et al. 2005; Nozue et al. 1996; Horinouchi et al. 2008).

A phospholipid vesicle or liposome-encapsulating concentrated human Hb (Hb-vesicle, HbV) is an HBOC (Djordjevich et al. 1987; Awasthi et al. 2004). HbV (particle diameter, approx. 250 nm) has characteristics that resemble those of natural RBCs because both have lipid bilayer membranes that prevent the direct contact of Hb with blood components and the endothelial lining, thus shielding all side effects of molecular Hb (D'Agnillo and Alayash 2001; Sakai et al. 2000a). Once in circulation, HbV particles are captured by the phagocytes in the reticuloendothelial system (RES) and are metabolized in the physiologically normal pathway after topload infusions (Sakai et al. 2001, 2004a, b; Sou et al. 2005). It was reported that the efficacy of HbV suspended in recombinant human serum albumin (rHSA) in extreme normovolemic hemodilution (80–90 % blood exchange) and resuscitation from hemorrhagic shock was proven (Izumi et al. 1997; Cabrales et al. 2005; Yoshizu et al. 2004; Sakai et al. 1997, 2004c). However, those experiments were mainly conducted with small animals. We couldn't evaluate the influence of HbV on pulmonary circulation in small animals. Therefore we conducted hemorrhage-resuscitation study using Beagle dog and reported the results (Yamamoto et al. 2012). In the previous report, observation was limited to four hours. Transient increase of pulmonary arterial pressure was observed in HbV group compare to the other groups that SAB(Shed Autologous Blood), rHSA(recombinant Human Serum Albumin), and LR (lactate Ringer solution) was used as resuscitative fluid. Splenectomy was performed in the previous study so that we could maintain uniform hemorrhagic shock state. In the present study, long term influence and safety as well as acute phase safety of HbV was studied. This time we preserved spleen to investigate the role of spleen in shock-resuscitation.

In the present study, we observed the animals for a long period (1 year) after resuscitation. Hemorrhagic shock was induced by 50 % bleeding (acute phase study) or 40 % bleeding (long term study). We analyzed systemic hemodynamics and O_2-transporting capacity within 4 h, and hematological, plasma biochemical, and histopathological examination within 1 year to clarify the impact on organ functions.

28.2 Materials and Methods

28.2.1 Preparation of HbVs Suspended in rHSA

HbVs were prepared under sterile conditions, as reported in previous studies (Sou et al. 2003; Sakai et al. 2000b). The Hb was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 14.7 mmol per L pyridoxal 5′-phosphate (PLP) (Sigma-Aldrich Co., St. Louis, MO) as an allosteric effector at a molar ratio of PLP/Hb of 2.5. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd, Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan), at a molar composition of 5/5/1/0.033. The lipopolysaccharide content, measured with a modified *Limulus* amebocyte lysate test, was less than 0.1 EU per mL (Sakai et al. 2004d). The physicochemical parameters are P_{50}, 27 Torr; 251 ± 81 nm particle diameter; and less than 3 percent MetHb content. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of rHSA (25 g/dL, 1.4 mL; Nipro Corp. Osaka, Japan) to regulate the rHSA concentration in the suspending medium to 5 g per dL. Consequently, the Hb concentration became 8.6 g/dL. Under these conditions, the colloid osmotic pressure and the viscosity at 300 s^{-1}, 37 °C) of the HbV/rHSA were 20 mmHg and 2.9 cP, respectively.

28.2.2 Animal Preparation

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 carried out using 26 male beagle dogs (7.07 ± 0.33 kg b.w., NARC Corp., Chiba, Japan). The dogs were fasted 18 h before the experiment, but had free access to water up to 2 h before the anesthesia. The animals were bred in the cages individually. We used 10 beagle dogs for the acute phase study, and 16 beagle dogs for the chronic phase study. The dogs for the acute phase study were divided to HbV/rHSA group (n = 4), shed autologous blood (SAB) group (n = 3), and rHSA group (n = 3). The dogs for the chronic phase study were divided to HbV/rHSA group (n = 9) and SAB group (n = 7). The 9 dogs of the HbV/rHSA group were randomly divided into three and sacrificed at 28 days (n = 3), 168 days (n = 3), or 365 days (n = 3) after resuscitation experiment. Among 7 dogs of the SAB group, two dogs were randomly selected and sacrificed at 28 days, two dogs at 168 days, and the remained three dogs at 365 days after resuscitation.

28.2.3 Animal Preparation and Instrumentation

The animals, pre-medicated with atropine sulfate (0.07 mg/kg i.m.). Anesthesia was induced by intramuscular injection of ketamine hydrochloride (5 mg/kg i.m.). Animals were orally intubated, inhalation anesthesia was maintained with 2.0–2.5 %–sevoflurane mixed air supplied by an anesthesia apparatus (SN–487, Shinano Seisakusho Co., Tokyo, Japan). The concentration of sevoflurane (2.0–2.5 %) was adjusted as necessary to maintain the animal at a stable plane of anesthesia. Visual monitoring of spontaneous respiration was performed.

Electrocardiogram (EKG) electrodes were attached to the feet. A 5.5-F Thermodilution catheter (631Hf55; Edwards Lifescience, Irvine, CA, USA) was placed in the pulmonary artery via the right femoral vein for measurements of the mean pulmonary arterial pressure (MPAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), and cardiac output. The left femoral artery was cannulated to monitor arterial pressure as well as for blood sampling. The pressure line was connected to transducers (5100TW; Edwards Lifescience, Irvine, CA, USA), and these transducers and the EKG line were connected to a polygraph system (LEG-1000, Nihon Kohden Co., Tokyo, Japan). The right femoral artery was cannulated with a 16G I.V. cathter (Angiocath; Becton–Dickinson, Sandy, Utah, USA) to control the bleeding. rSO_2 (regional saturation of oxygen) was monitored using the rSO_2 monitor INVOS 4100 (Somanetics Inc., Troy, MI) at the forehead (brain rSO_2) and abdomen (rectus abdominis muscle rSO_2).

28.2.4 Experimental Protocol

After establishment of stable anesthesia, animals were randomly assigned to three experimental groups in acute study, i.e. shed autologous blood (SAB) group ($n = 3$), 5 g/L recombinant human serum albumin in Saline (rHSA) group ($n = 3$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 4$). In chronic study, dogs were assigned to two groups, i.e. shed autologous blood (SAB) group ($n = 7$), and HbV suspended in 5 % rHSA/saline solution (HbV/rHSA) group ($n = 9$).

The systemic blood volume was estimated to be 86 mL per kg of the total body weight. In acute phase study, a 50 % volume of the circulation blood was withdrawn from the right femoral artery cathter at a rate of 20 ml/min. In the chronic phase study, a 40 % volume was withdrawn. Withdrawn blood was preserved in a several 50 ml syringe containing 7 ml of CPD solution (Karmi C, Kawasumi Laborataories Inc. Tokyo, Japan) in SAB group.

The hemorrhagic shock state was maintained for 1 h. Thereafter, designated isovolemic resuscitative fluid was injected intravenously. In all experiment, infusion rate of resuscitative fluid were maintained at 20 mL/kg/min. After resuscitation, no additional intravenous fluid was allowed except for the cold 5 % glucose required to measure cardiac output.

28.2.5 Measurements

In the acute phase study, 0.5 mL of arterial blood and 2.0 mL of mixed–venous blood were collected from the femoral and pulmonary arteries at the following ten time–points: before hemorrhage, immediately after hemorrhage, 1 h after the shock, immediately after resuscitation, and 0.5, 1, 1.5, 2, 3 and 4 h after resuscitation. In the chronic phase study, 10 mL of venous blood was collected from cepharic vein at the following ten time–points: before the bleeding, and 1, 3, 7, 14, 28, 56, 84, 168 and 365 days after resuscitation.

MAP was monitored through the right femoral artery, and MPAP, PCWP and CVP were through the flow directed pulmonary artery catheter connected to a transducer (5100TW, Edwards Lifesciences, Irvine, CA., USA). These transducer and EKG line were connected to a polygraph system (PEG-1000, Nihon Kohden, Tokyo, Japan). MAP, MPAP and HR were continuously monitored, and cardiac output was assessed by a thermodilution procedure with the rapid injection of cold saline (5 mL, 4 °C) in duplicate using a cardiac output measurement apparatus (Vigilance system, Edwards Critical-Care Division Irvine, CA, USA). The systemic vascular resistance (SVR) and pulmonary vascular resistance (PVR) were calculated as SVR = 79.92 × (MAP − CVP)/cardiac output, and PVR = 79.92 × (MPAP − PCWP)/cardiac output, respectively. MAP, MPAP, PCWP, CVP, cardiac output and $PtO_2(R)$ were measured at the same points stated above.

Withdrawn blood specimen (approximately 1 mL) was rapidly applied to a blood gas system (ABL555, Radio Meter Trading, Copenhagen, Denmark) to measure the pH, O_2 pressure (PaO_2) and CO_2 pressure ($PaCO_2$) of the arterial blood, and the O_2 pressure (PvO_2) and lactic acid level of the venous blood. Arterial O_2-saturation (SaO_2) and mixed venous O_2-saturation (SvO_2) were calculated by PaO_2 and PvO_2, respectively, using an O_2-equilibrium curve of the canine RBC, which was measured by a Hemox Analyzer (TCS medical products, Philadelphia, USA).

The hematocrit (Hct) was measured by using the glass capillary and centrifugation (4,500 rpm, 5 min). The Hb concentration of the arterial blood was obtained using a multi-system automatic blood cell counter (KX-21, Sysmex, Kobe, Japan). The presence of HbV in the blood interferes in the measurement of Hb concentration, thereafter the Hb concentration of the HbV/rHSA group was measured with a modified cyanomet-hemoglobin method.

The plasma Hb concentration derived from HbV was calculated as follows. Blood samples from the venous line after the HbV infusion were centrifuged at 4 °C (3,500 rpm, 10 min). The HbV molecule in the supernatant was converted to the cyanomet form using a Hemoglobin Test Wako (Wako Pure Chemical Industries, Ltd., Tokyo), and its concentration was determined by the absorption spectral measurement using a UV–vis absorption spectrophotometer (V-570, JASCO, Tokyo, Japan). The percentage of metHb within the vesicles was periodically calculated by the ratio of absorbance at 405 nm (metHb) and 430 nm

(deoxyHb) in the Soret band using a UV–vis spectrometer without destruction of the HbV (Atoji et al. 2006).

The arterial O_2-content (CaO_2 (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen delivery (DO_2 (RBC)) of the beagle dog's RBC was calculated as the product of Qt and CaO_2 (RBC).

$$CaO_2(\mathrm{RBC}) = [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SaO_2 \times 10^{-2}\right\}$$

$$DO_2(\mathrm{RBC}) = CaO_2(\mathrm{RBC}) \times 10 \times \mathrm{Qt}$$

The arterial O_2-content (CaO_2 (HbV)) of HbV was estimated by the following equation. The oxygen delivery (DO_2 (HbV)) of HbV was calculated as the product of the cardiac output (Qt) and CaO_2 (HbV).

$$\begin{aligned} CaO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SaO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ DO_2(\mathrm{HbV}) &= CaO_2(\mathrm{HbV}) \times 10 \times \mathrm{Qt} \end{aligned}$$

The arterial O_2-content (CaO_2 (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen delivery (DO_2 (DO)) of the dissolved oxygen was calculated as the product of Qt and CaO_2 (DO).

$$CaO_2(\mathrm{DO}) = 0.003 \times PaO_2$$

$$DO_2(\mathrm{DO}) = CaO_2(\mathrm{DO}) \times 10 \times \mathrm{Qt}$$

The total oxygen delivery (DO_2) was calculated by the following equation.

$$DO_2 = DO_2(\mathrm{RBC}) + DO_2(\mathrm{HbV}) + DO_2(\mathrm{DO})$$

The mixed venous O_2-content (CvO_2 (RBC)) of the beagle dog's red blood cell (RBC) was estimated by the following equation. The oxygen consumption (VO_2 (RBC)) of the beagle dog's RBC was calculated as the product of Qt and the difference between CaO_2 (RBC) and CvO_2 (RBC).

$$\begin{aligned} CvO_2(\mathrm{RBC}) &= [\mathrm{Hb}]_{\mathrm{RBC}} \times 1.34 \times \left\{SvO_2(\mathrm{RBC}) \times 10^{-2}\right\} \\ VO_2(\mathrm{RBC}) &= \left\{CaO_2(\mathrm{RBC}) - CvO_2(\mathrm{RBC})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous O_2-content (CvO_2 (HbV)) of HbV was estimated by the following equation. The oxygen consumption (VO_2 (HbV)) of HbV was calculated as the product of Qt and the difference between CaO_2 (HbV) and CvO_2 (HbV).

$$\begin{aligned} CvO_2(\mathrm{HbV}) &= [\mathrm{Hb}] \times 1.34 \times \left\{1 - \text{metHb percentage} \times 10^{-2}\right\} \\ &\quad \times \left\{SvO_2(\mathrm{HbV}) \times 10^{-2}\right\} \\ VO_2(\mathrm{HbV}) &= \left\{CaO_2(\mathrm{HbV}) - CvO_2(\mathrm{HbV})\right\} \times 10 \times \mathrm{Qt} \end{aligned}$$

The mixed venous O_2-content (CVO_2 (DO)) of the dissolved oxygen was estimated by the following equation. The oxygen consumption (VO_2 (DO)) of the dissolved oxygen was calculated as the product of Qt and the difference between Cao_2 (DO) and CVO_2 (DO).

$$CvO_2(DO) = 0.003 \times PvO_2$$
$$VO_2(DO) = \{CaO_2(DO) - CvO_2(DO)\} \times 10 \times Qt$$

The total oxygen consumption (VO_2) was calculated by the following equation.

$$VO_2 = VO_2(RBC) + VO_2(HbV) + VO_2(DO)$$

In the chronic phase study, the collected venous blood was used for blood cell counts with an automatic blood cell counter. The rest of the blood was centrifuged (5,000 rpm, 10 min) to separate the plasma which was then ultracentrifuged (50,000 rpm, 20 min) to sediment the HbV particles from the plasma at 1, 3 and 7 days after the resuscitation with HbV/rHSA to avoid their interference by HbV particles in the plasma biochemical assays (Sakai et al. 2003). The obtained transparent serum specimens were stored at –80 °C until biochemical tests (Biken, Kyoto, Japan). The selected analyses were aspartate aminotransferase phosphatase (ALP), γ-glutamylteansferase (γ-GTP), cholinesterase (ChE), total protein (TP), albumin (ALB), creatine phosphokinase (CPK), amylase (AMY), lipase, leucine aminopeptidase (LAP), urea nitrogen (BUN), creatinine (Cre), uric acid (UA), total cholesterol (T-chol), free cholesterol (F-chol), high density lipoprotein cholesterol (HDL-chol), triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, total bilirubin (T-Bil), Fe, Cu, K, Ca, inorganic phosphate (IP), and Mg.

28.2.6 Histopahological Examination

The animals were finally euthanized with large dose of pentobarbital and exsanguination. Then autopsy was performed to get the specimen of organs (esophagus, small intestine, large intestine, liver, pancreas, spleen, thymus, lung, trachea, heart, kidney, testis, and adrenal) were obtained for a histopahological study. They were fixed in a 10 % formalin neutral buffer solution (Wako Pure Chemicals, Osaka, Japan) immediately after removal, and the paraffin sections were stained with hematoxylin & eosin (Mitsubishi Chemical Safety Institute, Kumamoto, Japan).

28.2.7 Statistical Analyses

Data are reported as mean ± standard deviation (SD) for all measurements. Data were analyzed using Stat View (SAS Institute, Inc., Cary, N.C., USA). Differences compared with the control (baseline) group were analyzed with paired *t* test, and

differences between the groups were analyzed with Mann–Whitney U test. The changes were considered significant if the p value was less than 0.05 in the acute phase study, and 0.01 in the chronic phase study.

28.3 Results

28.3.1 Acute Phase Study

Beagle dogs of all groups tolerated well the 50 % bleeding inducing hemorrhagic shock and resuscitation. They survived for 4 h after the resuscitation without any change in their appearance.

Circulation

MAP before hemorrhage was 102 ± 19 mmHg on the average; it decreased significantly to 19 ± 5 mmHg immediately after hemorrhage (Fig. 28.1). After resuscitation, both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group showed significantly lower MAP than the HbV/rHSA group at 1, 3, and 4 h after infusion.

All groups showed significantly higher MPAP immediately after infusion than the baseline values. However, there were not significant differences between groups. After that, all groups showed almost stable values. The HbV/rHSA group showed slightly lower PCWP after resuscitation than the other groups. The SAB group showed slightly higher CVP after infusion than the other groups.

There was no significant change in the time course of the HR during the experiment. CO before hemorrhage was 1.4 ± 0.3 L/min on the average; it decreased significantly to 0.2 ± 0.1 L/min immediately after hemorrhage. After resuscitation, all groups showed immediate recovery, and rHSA group showed significantly higher values than the baseline. The SAB group showed significantly lower values than the HbV/rHSA group at 0.5, 1, 1.5, and 3 h after resuscitation.

All groups showed significantly lower SVR at 0, 0.5, 1, 1.5, and 2 h after resuscitation than the baseline values. The rHSA group showed lower SVR than the HbV/rHSA group at 0, 0.5, 1, 1.5, 2 h after infusion. The 50 % hemorrhage increased the PVR, however, after the hemorrhage, all groups showed stable values for 4 h of the observation period.

Blood gas analysis (Fig. 28.2).

The pH value decreased to 7.13–7.22 after hemorrhage, but both the SAB and HbV/rHSA groups showed immediate recovery and stable values for the 4 h. The rHSA group tended to recover late. BE decreased to 11.5–15.6 mmol/L after hemorrhage, but all groups showed gradual recovery to the initial level after infusion. The lactic acid decreased to 3.07–5.07 mmol/L after hemorrhage, but all groups showed gradual recovery like BE. As a result of the hyperventilation, the slight elevation of PaO_2 and the decline of $PaCO_2$ were seen after hemorrhage. However, all groups showed recovery and similar tendency. PvO_2 before

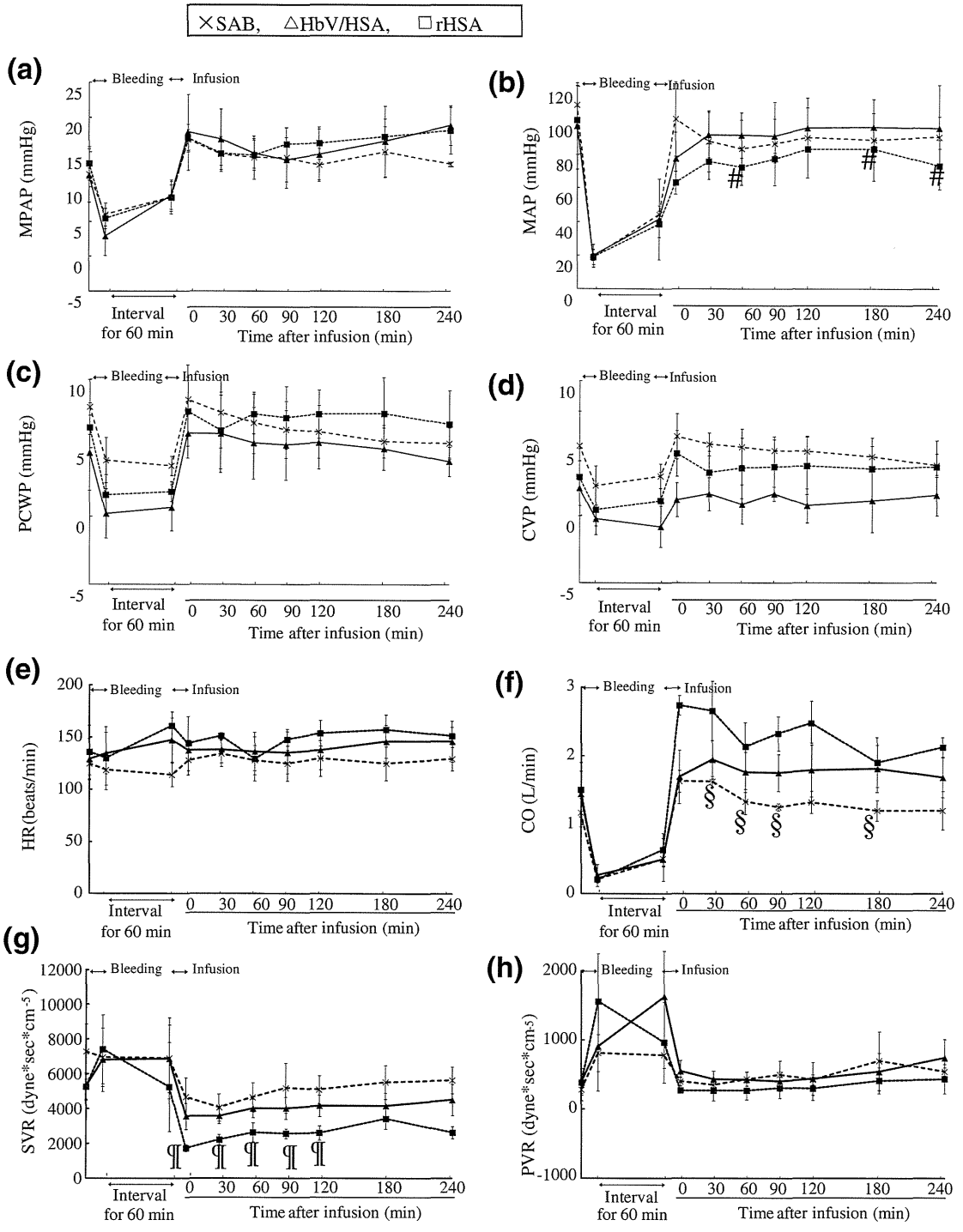

Fig. 28.1 Changes in mean pulmonary arterial pressure (MPAP), mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), heart rate (HR), cardiac output (CO), systemic vascular resistance (SVR), and pulmonary vascular resistance (PVR) during hemorrhagic shock and resuscitation with infusion of rHSA alone, shed SAB and HbV/rHSA. The values are mean ± SD. #,§: significantly different between HbV/rHSA group ($p < 0.05$)

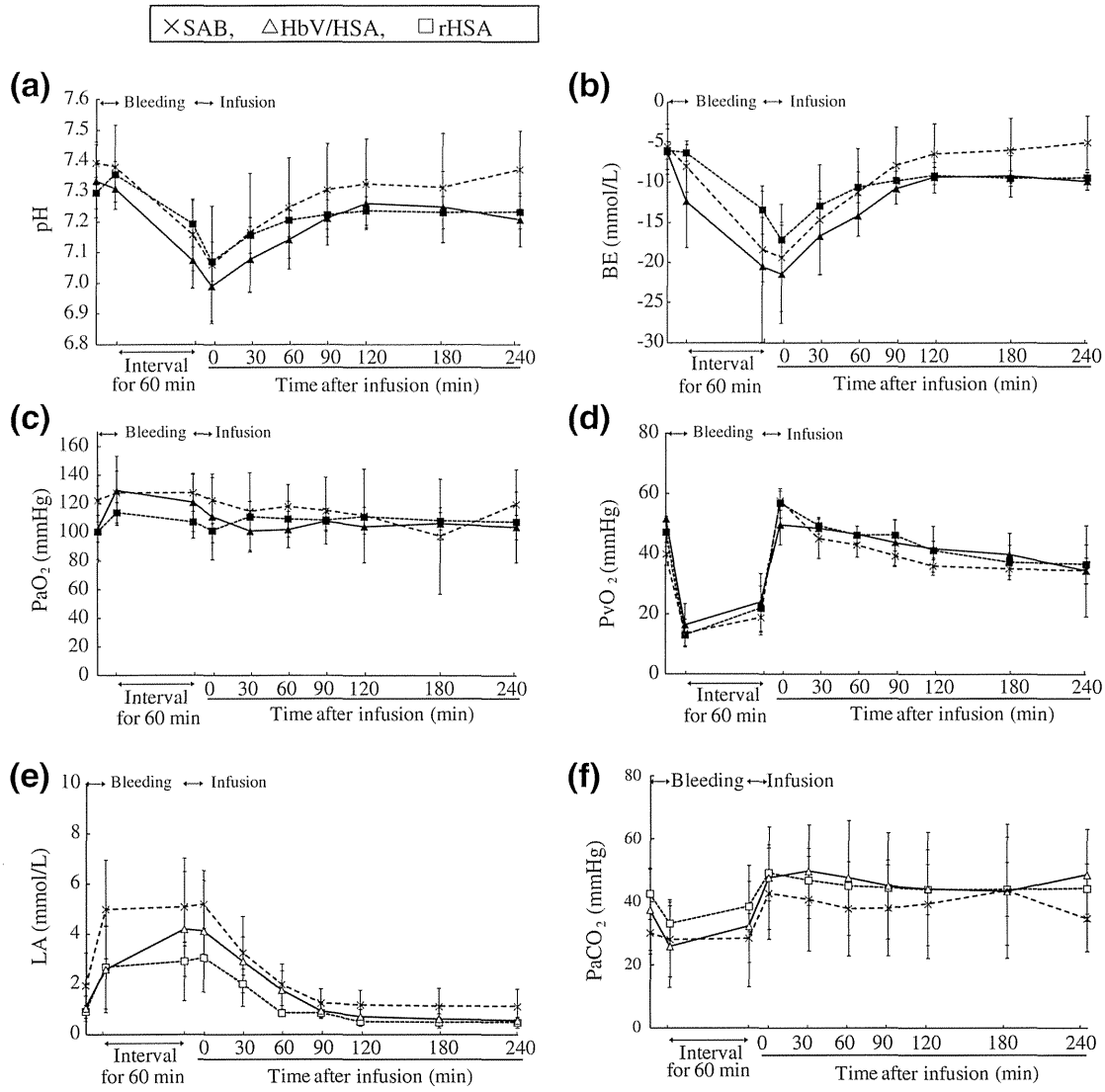

Fig. 28.2 Changes in pH, PaO_2, $PaCO_2$, base excess (BE), lactate, and PvO_2 during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB and HbV/rHSA. The values are mean ± SD. There was no significant difference between rHSA or SAB and HbV/rHSA group

hemorrhage was 48 ± 5 Torr on the average; it decreased significantly to 15 ± 6 Torr immediately after hemorrhage. After resuscitation, all groups showed immediate recovery and similar tendency.

Hematology (Fig. 28.3).

There was no significant change after bleeding in Hct for all groups. As a result of the dilution of blood, the rHSA and HbV/rHSA groups showed significantly lower values than the baseline values, while the SAB group showed higher values during the experiment (Fig. 28.3c). In the HbV/rHSA group the total Hb levels before hemorrhage was 10.4 ± 1.6 g/dL, and after resuscitation it was 13.8 ± 1.6 g/dL at 0 h, and 9.9 ± 1.2 g/dL at 4 h (Fig. 28.3b). The concentration of Hb derived from HbV was 4.2 ± 0.5 g/dL (37.5 ± 4.5 % of total Hb) at 0 h,

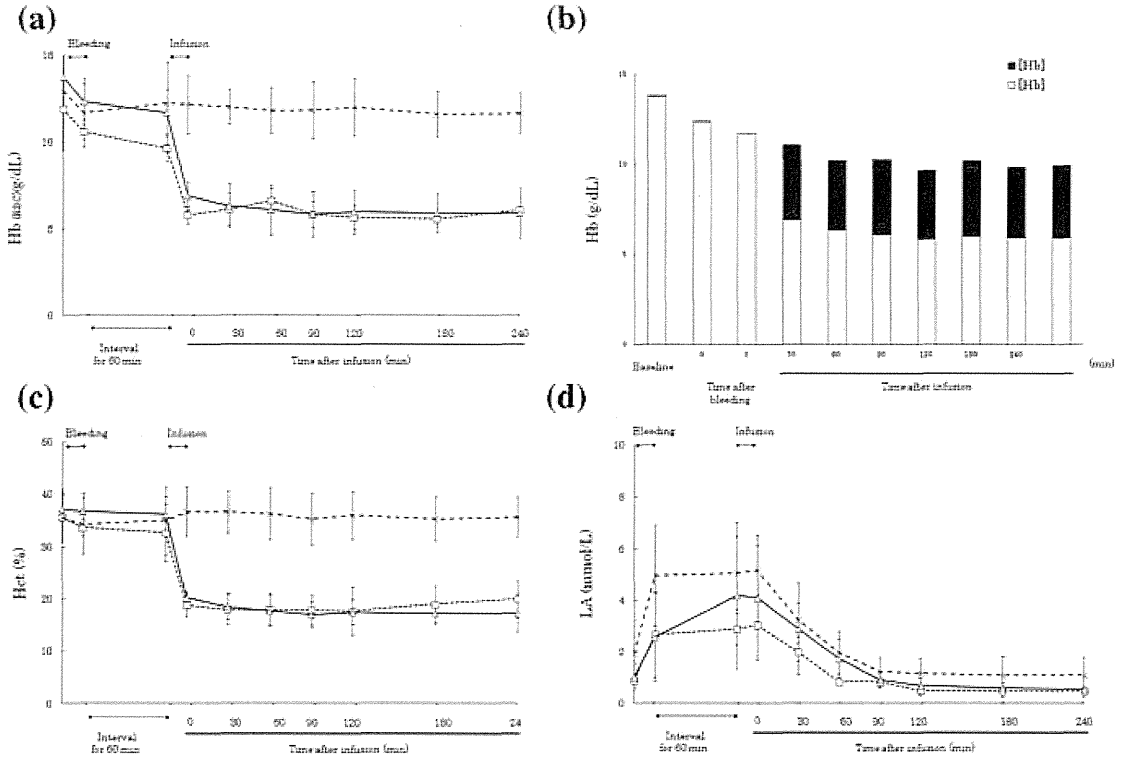

Fig. 28.3 Change in hemoglobin (Hb) concentration, hematocrit (Hct), and lactic acid (LA) level during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB, and HbV/rHSA. The values are mean ± SD. Composition of Hb concentration in the whole blood in HbV/rHSA group (B)

and 4.0 ± 0.8 g/dL (40.5 ± 8.1 % of total Hb) at 4 h. The level of metHbV increased to 9.1 ± 3.0 % at 4 h.

Oxygen delivery and consumption (Fig. 28.4).

As regards to the oxygen delivery and consumption, the rHSA group tended to show lower DO_2 than the other groups after the resuscitation, and showed significantly lower value than HbV/rHSA group 3 h after resuscitation (Fig. 28.4a). In the HbV/rHSA group, DO_2(HbV) was 34–38 % of the total DO_2. Oxygen consumption was not significantly different between HbV/rHSA group and SAB, or rHSA group. In HbV/rHSA group VO_2(HbV) showed 26–29 % of the total VO_2.

28.3.2 Long Term Study

Beagle dogs of all groups tolerated well the 40 % bleeding inducing hemorrhagic shock and resuscitation. They survived without any change in their appearance until their intentional sacrifice. The body weight of beagle dogs before resuscitation was 7.2 ± 0.3 kg, which increased monotonously to 12 ± 1.8 kg at one year after resuscitation in both groups (Fig. 28.5). The Hct before the resuscitation