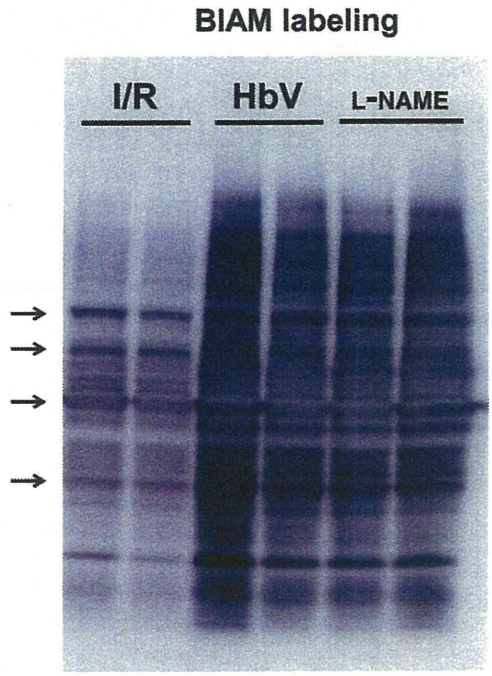

FIGURE 7. Results of BIAM labeling. Representative blotting is shown (n = 4; 2 pairs, 2 blotting). Proteins containing reduced reactive thiols were strongly stained. Arrows show nonspecific bands that were thought to be carboxylases/decarboxylases inherently containing biotin molecules.

polymerized placenta hemoglobin, one of the hemoglobin-based oxygen carriers, attenuated ischemia–reperfusion injury in isolated rat hearts. In their studies, polymerized placenta hemoglobin reduced inducible nitric oxide synthase–derived NO production and increased superoxide dismutase activity in cardiac tissues.

The BIAM labeling method has been developed to detect the H_2O_2-sensitive cysteine thiol of functional proteins.[13] This method is used to detect nitrosative modification of cysteine thiols of sarcoplasmic reticulum Ca^{2+}-ATPase[26] and to estimate the redox state of cellular proteins.[27] In this study, we found that the BIAM labeling of cardiac tissue proteins decreased in the I/R group after ischemia–reperfusion, and this decrease was suppressed in the L-NAME and HbV groups (Fig. 7). This means that oxidative stress increased under the condition of ischemia–reperfusion and oxidized the reactive thiol of the proteins in the I/R group, but this increase was inhibited by L-NAME and HbV.

The NO molecule has been shown to regulate the function of intracellular calcium handling proteins (L-type Ca^{2+} channel,[28] ryanodine receptor[29] and sarcoplasmic reticulum Ca^{2+}-ATPase[26]), with nitrosative modification of their thiol groups. We therefore speculated that both HbV and L-NAME suppressed oxidative stress and regulated the activity of these proteins, resulting in improved recovery of cardiac function after ischemia–reperfusion; however, this needs to be clarified.

In the present study, we considered the diametrically opposed actions of L-NAME and HbV on glucose metabolism in cardiac tissue to be an exceptional result (Fig. 4). HbV slightly increased pyruvate release into the perfusate during perfusion and did not affect lactate release (data not shown), and as a result, there was a nonsignificant decrease in the L/P ratio for the perfusate at 5 minutes after the start of reperfusion. In contrast, L-NAME tended to decrease the pyruvate release and hardly affect the lactate release, resulting in an increase in the L/P ratio. At 30 minutes, the L/P ratios for the perfusate were identical for I/R, L-NAME, and HbV groups. However, these small changes in anaerobic glucose metabolism during ischemia can hardly explain the marked improvement in cardiac function during reperfusion, so further studies on the effects of L-NAME and HbV on cardiac anaerobic metabolism during ischemia–reperfusion may be required.

In clinical usage, L-NAME is not approved for any of the shock state because of its vasoconstriction effects, difficulty of dose settings, or too short half-life. We considered that L-NAME inhibited the enzymes; however, HbV scavenged reactive nitrogen species. HbV has very mild effects on blood pressure compared with hemoglobin itself.[30] These properties of HbV may be more beneficial for clinical usage of hypotension than L-NAME or hemoglobin itself.

In conclusion, we found that HbV improves recovery of cardiac function after ischemia–reperfusion in isolated perfused rat hearts. HbV's inhibition of a decrease in BIAM labeling of the reactive thiol of functional proteins and an increase of cardiac tissue GSSG content after ischemia–reperfusion

TABLE 1. Activities of Glutathione Peroxidase, Glutathione Reductase, Cu/Zn-Superoxide Dismutase, Mn-Superoxide Dismutase, and Catalase in the Cardiac Tissues

	Control Group, n = 5	C + HbV Group, n = 5	I/R Group, n = 7	I/R + HbV Group, n = 6
Glutathione peroxidase (NADPH, nmol·min^{-1}·mg protein^{-1})*	223 ± 10	230 ± 15	248 ± 16	224 ± 30
Glutathione reductase (NADPH, nmol·min^{-1}·mg protein^{-1})*	11.2 ± 0.8	12.3 ± 0.7	14.6 ± 1.1	13.9 ± 0.7
Cu/Zn-superoxide dismutase (U/mg protein)	0.92 ± 0.07	0.86 ± 0.17	0.95 ± 0.12	0.91 ± 0.17
Mn-superoxide dismutase (U/mg protein)	0.44 ± 0.06	0.59 ± 0.08	0.60 ± 0.12	0.59 ± 0.11
Catalase (FA, nmol·min^{-1}·mg protein^{-1})†	5.57 ± 0.40	5.65 ± 0.62	6.12 ± 0.49	6.22 ± 0.48

All numerical data are shown as mean ± standard error.
*Enzyme activity is expressed as the rate of oxidation of NADPH per minute per milligram of protein extracted.
†Enzyme activity is expressed as the rate of generation of FA per minute per milligram of protein extracted.
FA, formaldehyde.

suggest that HbV improves recovery of cardiac function by suppressing the excessive increase in oxidative stress in cardiac tissue after ischemia–reperfusion (see **Figure, Supplemental Digital Content**, http://links.lww.com/JCVP/A52).

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

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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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[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

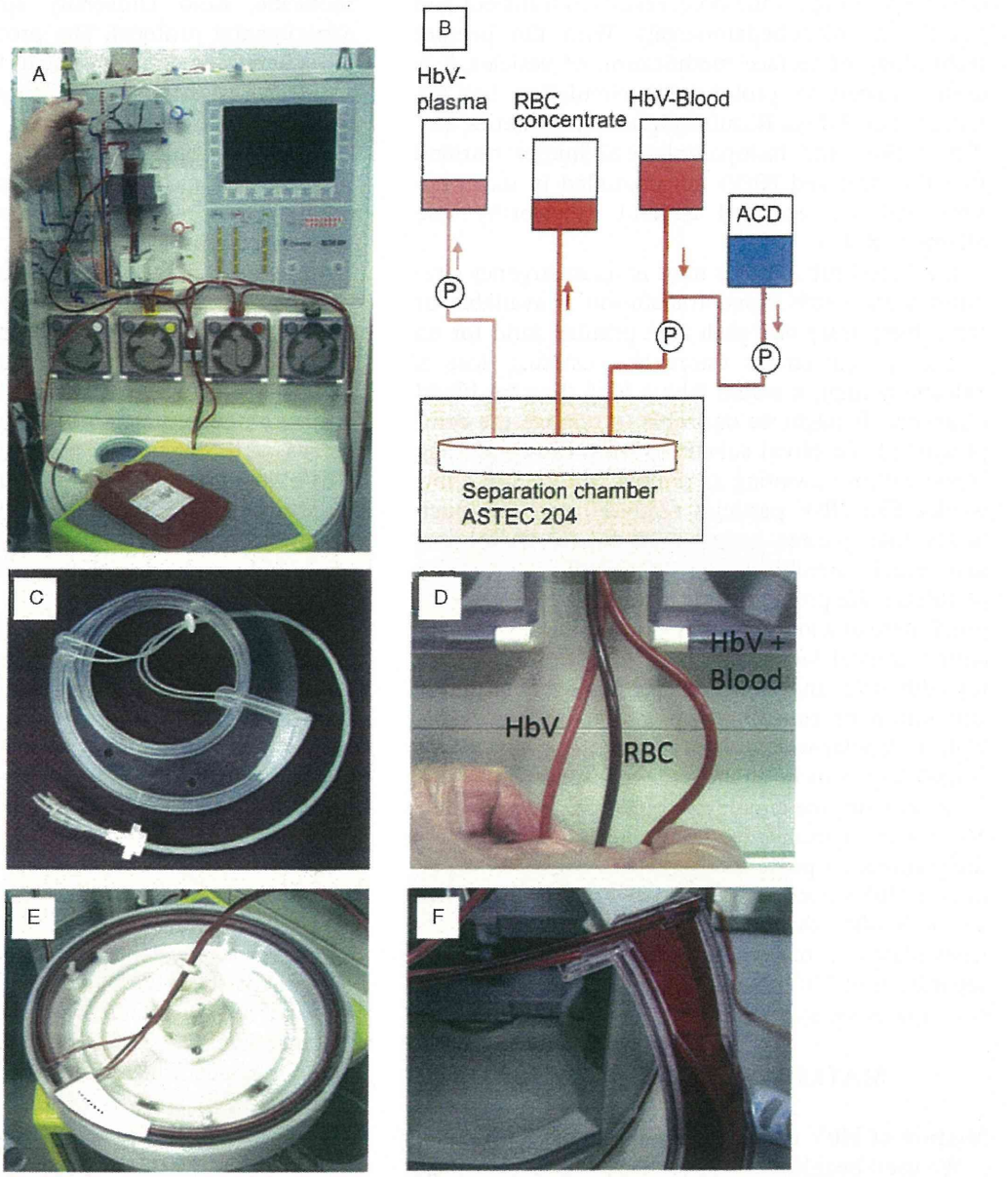

FIG. 1. Fresenius Cell Separator (ASTEC 204; Fresenius Kabi AG) used for the separation of HbV from beagle blood components: (A) experimental overview of the instrument and (B) schematic representation of the inlet and outlet fluids. The HbV–blood mixture was poured into the system with a small addition 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.).

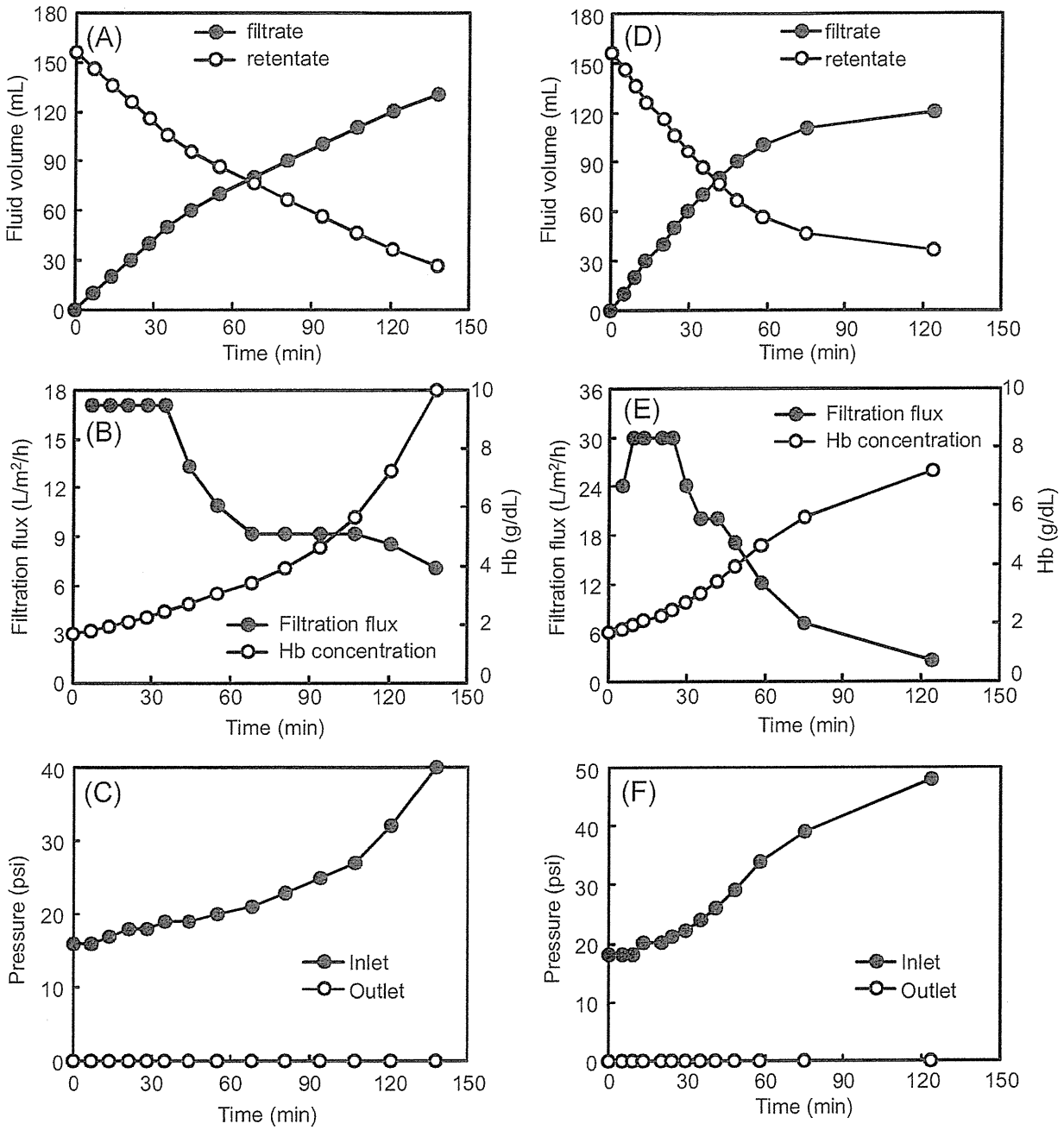

FIG. 3. Permeation profiles of the plasma layer separated from the mixture of HbV–plasma by Millipore ultrafilters using Labscale (Millipore Corp.) at an inlet flow rate 30 mL/min: (A,B,C) Durpore, (D,E,F) Biomax, (A,D) volume change of the retentate and filtrate solutions, (B,E) changes of permeation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

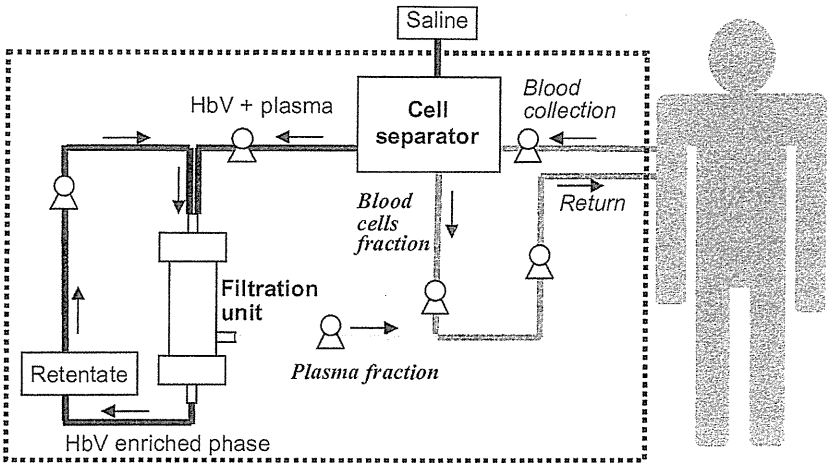

FIG. 4. Schematic representation of a proposed clinical device system connecting centrifugation and ultrafiltration to remove HbV from the circulating blood. Collected blood from a patient is centrifuged using a cell separator and separated to RBC fraction and HbV–plasma fraction. The HbV–plasma fraction 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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Fluid Resuscitation of Hemorrhagic Shock with Hemoglobin Vesicles in Beagle Dogs: Pilot Study

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Abstract: Resuscitation of hemorrhagic shock requires volume replacement and restoration of oxygen metabolism. Artificial oxygen carriers that can both expand blood volume and deliver oxygen have been developed as resuscitation fluids. We employed hemoglobin vesicles (HbV), a cellular-type artificial oxygen carrier, in a Beagle dog hemorrhagic shock model to prove the efficacy of HbV. Hemorrhagic shock was introduced in splenectomized Beagle dogs by withdrawing 50% of circulating blood from the femoral artery. Shock was maintained for 60 minutes before isovolemic resuscitation with HbV dispersed in 5% albumin in saline (HbV), lactated Ringer's solution (LR), 5% human serum albumin in saline (HSA), or autologous shed blood (ASB). One animal in the LR group died 150 min after resuscitation. All other animals survived 4 h of the experiment. The mean arterial pressure remained significantly lower in the LR group than in the HbV group but did not differ significantly among the HbV, Alb, and ASB groups. Immediately after resuscitation, the HbV group showed a significantly higher mean pulmonary arterial pressure, which decreased within 10 minutes to the baseline level. The cardiac output was significantly higher in the Alb group than in the others, indicating compensation for low oxygen delivery per unit blood. The post-resuscitation hematocrit was 36% in the ASB group and decreased in the other groups (20–22%). Serum chemistry data from the HbV group were unremarkable. HbV contributed 32% of the post-resuscitation oxygen delivery. Collectively, HbV is comparable to ASB and HSA as a resuscitation fluid and is an effective oxygen carrier.

Keywords: Artificial oxygen carrier; Trauma; Hemorrhage; Oxygen metabolism; Hypoxia

INTRODUCTION

Massive hemorrhage due to trauma requires large-volume fluid resuscitation as well as blood transfusion to maintain organ circulation and oxygen metabolism [1–3]. Blood type determination and cross-match testing must be performed before blood transfusion. Blood cannot always be immediately accessed in the emergency setting, and it may take hours to reach the trauma center or another facility that can provide definitive therapy. Artificial oxygen carriers may be beneficial in such situations.

Hemoglobin Vesicles (HbV) have been developed by Tsuchida's group [4–6], and we have previously reported their potential utility in small animals [7–9]. Rodent models preclude evaluation of the right heart and pulmonary circulation. To evaluate these circulatory parameters and the response to HbV in larger animals, we developed a 50% hemorrhagic shock model in Beagle dogs and conducted a pilot study to clarify the safety, efficacy, and influence on right heart function of HbV as an artificial oxygen carrier.

This study was partially supported by Health Science Research Grants (Research on Regulatory Science) from the Ministry of Health, Labor, and Welfare, Japan. This paper was read at the XI[th] International Symposium on Blood Substitutes held in Beijing, 2007. We are grateful to Professor Eishun Tsuchida for his instructions and deep insight. He was a member of this study and passed away in April 2010.

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MATERIALS AND METHODS

Animals

The study was conducted on 13 healthy young male Beagle dogs (6.84 ± 0.41 kg body weight, Sankyo Animal, Shinagawa, Japan) after approval by the Institutional Animal Care and Use Committee of Keio University and in compliance with the Guide for the Care of Laboratory Animals (National Institute of Health publication 86–23, revised 1985). The animals were maintained in cages with a 12-hour light-dark cycle, dog chow was provided twice daily, and water could be accessed freely. The animals were fasted overnight before the experiments but had free access to water up to 2 hour prior to anesthesia.

Animal Preparation and Instrumentation

Anesthesia was induced with ketamine (10 mg/kg intramuscularly) and atropine (0.02 mg/kg intramuscularly). After orotracheal intubation, animals were mechanically ventilated with an animal ventilator (Shinano SN 480–4, Tokyo, Japan) using a tidal volume (VT) of 20 mL/kg and respiratory rate of 15 breaths/min. Sevoflurane was administered via a vaporizer throughout the experiment. The concentration of sevoflurane (2.0–2.5%) was adjusted as necessary to maintain the animal at a stable plane of anesthesia. Prior to catheterization, a small pararectal laparotomy was made and splenectomy performed, a tissue oxygen tension (PtO_2) needle electrode (POE-10N; Eiko Kagaku Co., Tokyo, Japan) was placed at the cortical area of the left kidney and connected to the tissue oxygen tension monitor (PO_2–100; Intermedical. Co., Tokyo, Japan), and the peritoneal cavity was closed. In dogs, hemorrhage induces contracture of the spleen, which supplies additional blood from the spleen parenchyma to the systemic circulation. This mechanism alleviates the severity of hemorrhagic shock. To avoid this compensation, we surgically removed the spleen prior to the experiment. The resected spleen was placed in a tin basin and allowed to contract at room temperature for 30 minutes. The blood that was expressed from the spleen was collected and measured, and this volume was added to the experimental blood loss.

Electrocardiogram (EKG) electrodes were attached to the feet. A 5.5-F Thermo-dilution catheter (631Hf55; Edwards Lifescience, Irvine, CA, USA) was placed in the pulmonary artery via the right femoral vein. 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, UT, 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).

Preparation of Resuscitation Fluid

The Hb vesicles were manufactured according to the method developed by Waseda's group and provided by Oxygenic (Oxygenic Co., Ltd., Japan), and 25% human recombinant albumin solution was provided by Nipro (Nipro Co., Osaka, Japan) [4–6]. For the HbV group, Hb vesicles were re-suspended in a 5% albumin solution. Hb concentration of the supplied HbV suspension fluid was adjusted to 10 g/dL in saline. The HbV suspension fluid was then mixed with 25% albumin solution at a ratio of 8.6 to 1.4 to be used as a colloidal resuscitation fluid containing 5% albumin. This mixture was filtered through a 0.45-μm filter unit (DISMIC®–25cs; Toyo Roshi Kaisha, Ltd., Japan) to dissociate any large aggregates. The final Hb vesicle solution contained 8.6 g/dL of hemoglobin.

For the ASB group, the exsanguinated blood was drawn into a 50-mL syringe containing 7 mL of citrate-phosphate-dextrose (CPD) solution (Karmi C; Kawasumi, Tokyo, Japan). This blood was preserved at room temperature until use.

For the Alb group, 5% albumin solution was prepared by diluting 25% albumin solution with saline. Lactated Ringer's solution (Lactec; Otsuka Pharmaceutical Co., Tokushima, Japan) was used for the LR group.

Study Protocol

After establishment of stable anesthesia, animals were assigned randomly to four experimental groups, the Autologous shed blood (ASB) (n = 4), HbV (n = 3), Albumin (HSA) (n = 3), and Lactated Ringer's solution (LR) (n = 3) groups. The mean arterial pressure (MAP), pulmonary arterial pressure (PAP), central venous oxygen saturation (SvO_2), EKG, heart rate (HR), and regional saturation of oxygen (rSO_2) of the brain and muscle and tissue oxygen tension (PtO_2) of the renal cortex were continuously monitored throughout the experiment. Pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), arterial blood gas analysis (BGA), mixed venous BGA, and cardiac output were measured and complete blood count and serum chemistry panels obtained every 30 minutes after resuscitation for the first 2 hours and every 60 minutes thereafter until 4 hours post-resuscitation.

Fifty percent of the estimated circulating blood volume was withdrawn from the right femoral artery catheter at a rate of 20 mL/min. The estimated blood volume was calculated from the following formula: Estimated blood volume (mL) = 86 × body weight (kg).

Shock was maintained for 60 minutes after exsanguination. After the shock period, fluid resuscitation with the designated resuscitation fluid (either autologous shed blood (ΛSB), HbV suspension fluid (HbV), Albumin (5% albumin in saline), or Lactated Ringer's solution) was performed. Λ volume of resuscitation fluid equal to that of the withdrawn blood was administered via the right femoral vein at a rate of 20 $mL \cdot kg^{-1} \cdot min^{-1}$. After resuscitation, no additional intravenous fluid was allowed except for the cold 5% glucose required to measure cardiac output.

The arterial and pulmonary arterial pressures and the PtO_2 of the renal cortex were monitored continuously and cardiac output and arterial blood gases measured every 30 minutes for the first 2 hours after resuscitation and every 60 minutes thereafter until 4 hours after resuscitation.

We took serum samples before exsanguination and after measuring other parameters 240 min post-resuscitation. Blood samples were ultracentrifuged to collect serum as ordinary centrifugation could not separate HbV. Standard laboratory testing was performed for TP, Alb, A/G ratio, AST, ALT, LDH, ALP, GGTP, LAP, ChE, Tbil, Dbil, Cr, BUN, UA, Amylase, Lipase, CPK, Tcho, Free Cholesterol, beta Lipase, HDL-C, TG, Total lipid, FFA, Phospholipid, K, Cl, Ca, IP, Mg, Fe, and Cu.

Statistical Analysis

Because of the small sample size, the Tukey Kramer test was used to evaluate the differences between groups. $P < 0.05$ was considered statistically significant. Student's t-test was used for comparisons within the same group.

RESULTS

Survival

We observed one death in the LR group 150 min after resuscitation. All other animals survived the study.

Heart Rate

After exsanguination, the HR increased slightly in all groups and remained stably high until the beginning of resuscitation (Figure 1a). After resuscitation, the HR first decreased slightly to its initial level, then increased again in all groups at 30 minutes post-resuscitation. In the LR group, HR decreased after 120 minutes, and one animal subsequently died before the end of the experiment. The severity of shock in this model was thought to be such that isovolemic LR resuscitation could not compensate for the hemorrhagic shock. EKG showed no changes in the ST elevation or heart rhythm throughout the experiment.

Mean Arterial Pressure

After exsanguination, the mean arterial pressure (MAP) measurements of each group were 18 ± 7.0 mmHg in the HSA group, 23.7 ± 1.15 mmHg in the ASB group, 21 ± 3 mmHg in the HbV group, and 22.3 ± 8.5 mmHg in the LR group, indicating that hemorrhagic shock was uniformly established (Figure 1b). Immediately after resuscitation, the MAP was 62.7 ± 6.8 mmHg in the HSA group,

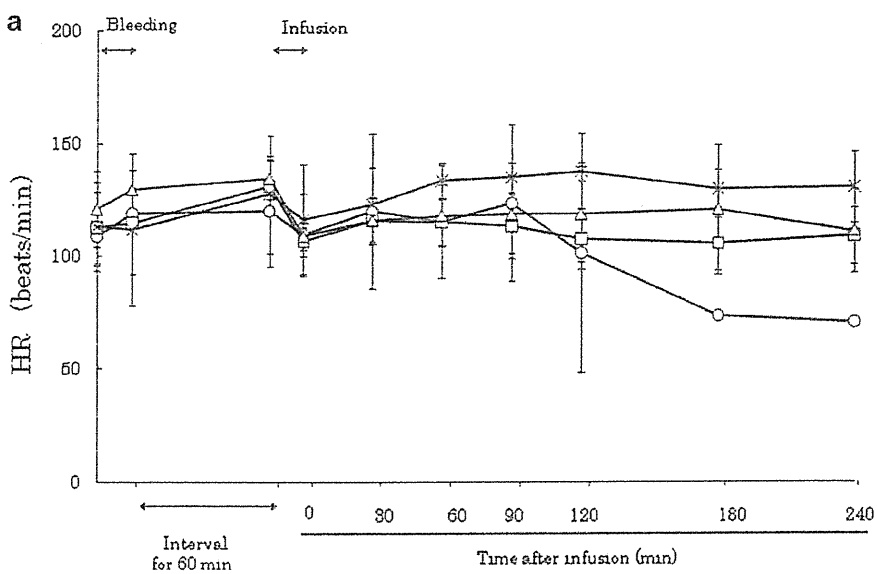

Figure 1a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on heart rate (HR) in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group, ×: ASB group; △: HbV group).

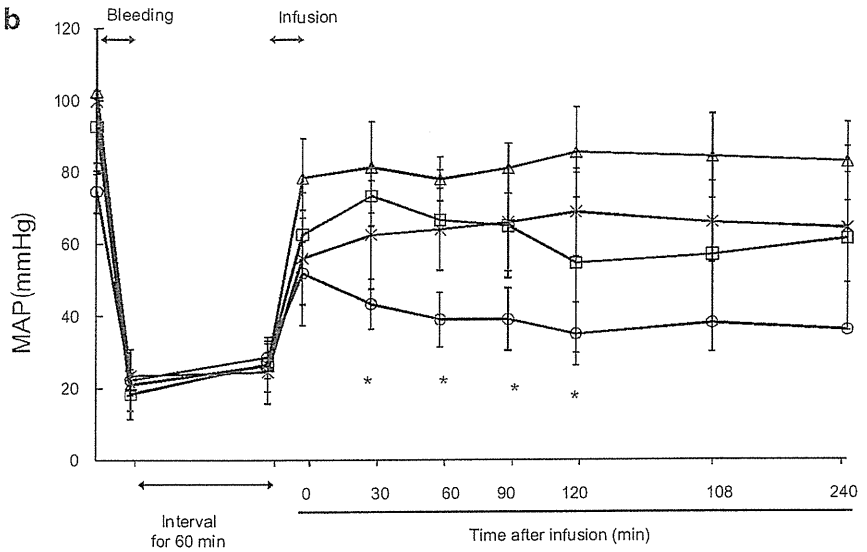

Figure 1b. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on MAP (mean arterial pressure) in anesthetized dogs subjected to 50% exsanguination. There were significant differences between the LR and HbV groups 30 minutes after resuscitation. (*) (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

51 ± 19 mmHg in the ASB group, 78 ± 11.0 mmHg in the HbV group, and 52 ± 8.7 mmHg in the LR group. There was no significant difference in pre- or immediate post-resuscitation MAP among the groups.

After resuscitation, MAP was low in the LR group and high in the HbV group. There were significant differences between the HbV and LR groups at 30 ($p = 0.01$), 60 ($p = 0.02$), 90 ($p = 0.03$), and 120 ($p = 0.048$) minutes after resuscitation. There were no significant differences between the HSA and ASB groups, the ASB and HbV groups, or the HSA and HbV groups at any timepoint.

Mean Pulmonary Arterial Pressure (MPAP)

After exsanguination, MPAP dropped below 5 mmHg in all groups (Figure 1c). Immediately after resuscitation, MPAP recovery was observed in all groups. MPAP was 20.0 ± 3.5 mmHg in the HbV group, significantly higher than in the ASB, HSA, or LR group. This elevation quickly returned to the normal MPAP range, and 30 minutes post-resuscitation, there were no significant differences between HbV and the other groups.

Pulmonary Capillary Wedge Pressure (PCWP)

The change of PCWP was similar in all groups except the LR group (Figure 1d). Immediately after resuscitation, PCWP recovered to the baseline levels and remained stable in the ASB, LR, and HbV groups. However, the PCWP increased proportionally more in the HSA group than in the other groups and remained high throughout the experiment. This indicated that the HSA group required a higher end-diastolic left-ventricular pressure in order to maintain cardiac output.

Cardiac Output

The pre-exsanguination cardiac output (CO) was 1.4 ± 0.3 L/min in the LR group, 1.1 ± 0.1 L/min in the HSA group, 1.14 ± 0.26 L/min in the ASB group, and 1.57 ± 0.6 L/min in the HbV group (Figure 2a). There were no significant differences between the groups. Exsanguination decreased CO to 0.20.47 L/min. Post-exsanguination, CO was 0.40 ± 0.10 L/min in the LR group, 0.4 ± 0.1 L/min in the HS group, 0.25 ± 0.1 L/min in the ASB group, and 0.35 ± 0.13 L/min in the HbV group. After resuscitation, cardiac output increased in every group (2.03 ± 0.4 L/min in the LR group, 1.47 ± 0.21 L/min in the HSA group, 1.11 ± 0.34 L/min in the ASB group, and 1.25 ± 0.23 L/min in the HbV group). The increase was greater in the HSA group than in the other groups, and there was a significant difference between the HSA and HbV groups ($p = 0.04$) immediately after resuscitation. After resuscitation, CO was stable in and not significantly different between the HbV, ASB, and HSA groups. In the LR group, CO decreased with time and was significantly lower than in the HbV group 90 minutes post-resuscitation.

Vascular Resistance

After shock was established, the systemic vascular resistance (SVR) rose in every group (Figure 2a, 2b). Resuscitation restores the circulation volume, which in turn decreases the SVR. After resuscitation, the SVR

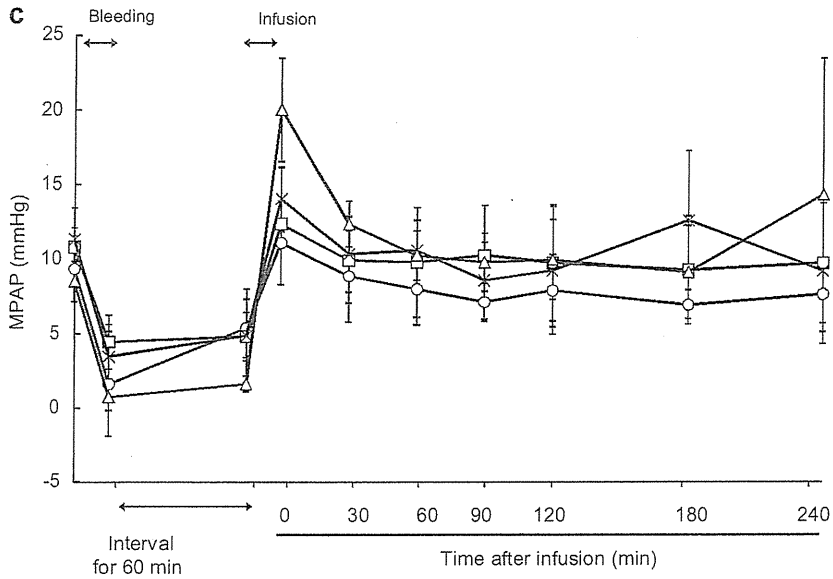

Figure 1c. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on mean pulmonary arterial pressure (MPAP) in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

decreased to 42.7–53.8% of its pre-exsanguination level in the HSA group and to 88.8–109.8% of its pre-exanguination level in the HbV group. In the LR group, SVR first decreased after resuscitation to 70.4% of initial pressure and then gradually increased to 115.0%. In the ASB group, SVR increased gradually after resuscitation from 55.1 ± 9.5% to 92.0 ± 8.4% of its pre-exanguination level. This gradual increase in SVR may have been due to the lack of maintenance fluid administration after resuscitation.

The effect of resuscitation fluid on pulmonary vascular resistance (PVR) seemed independent from that on SVR. The PVR was stable after resuscitation in all groups except for LR. In the LR group, PVR increased gradually after resuscitation, although there was no significant difference from the other groups.

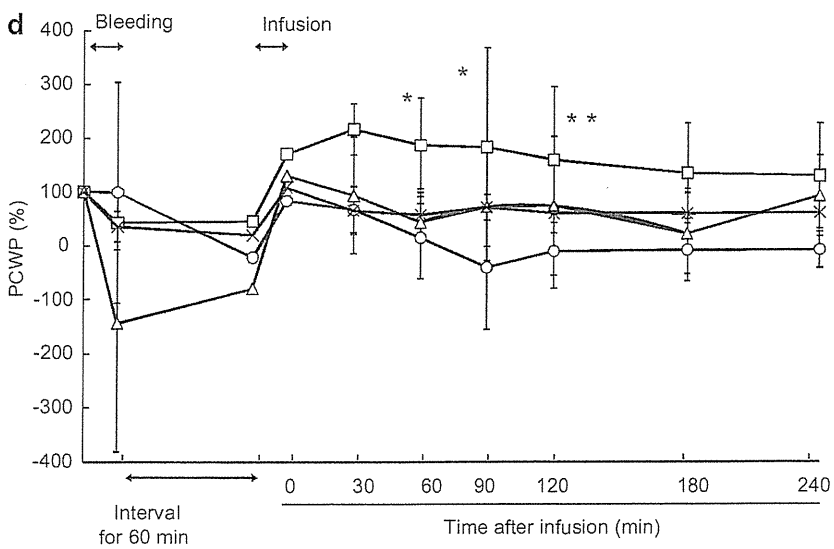

Figure 1d. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on pulmonary capillary wedge pressure (PCWP) in anesthetized dogs subjected to 50% exsanguination. Value is expressed as a ratio to that of the baseline. There were significant differences between the LR group and the HSA, ASB, and HbV groups (η) and between the HSA and HbV and ASB groups (*). (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

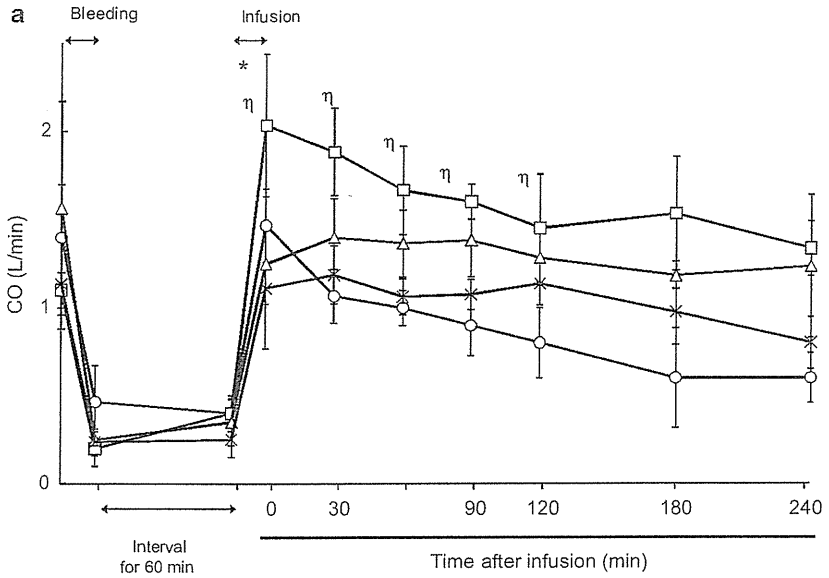

Figure 2a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on cardiac output (CO) in anesthetized dogs subjected to 50% exsanguination. There were significant differences between the LR and HSA groups (η) and between the HSA and HbV groups (*). (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

Arterial (PaO_2) and Mixed Venous (PvO_2) Oxygen Tension

Animals were intubated and ventilated mechanically; therefore, PaO_2 remained consistently above 80 mmHg at every time point (Figure 3a). In contrast, mixed venous PvO_2 decreased after exsanguination and recovered after resuscitation in every group (Figure 3b). However, in the LR group, the SvO_2 declined gradually after resuscitation and was significantly different from that of the HbV group.

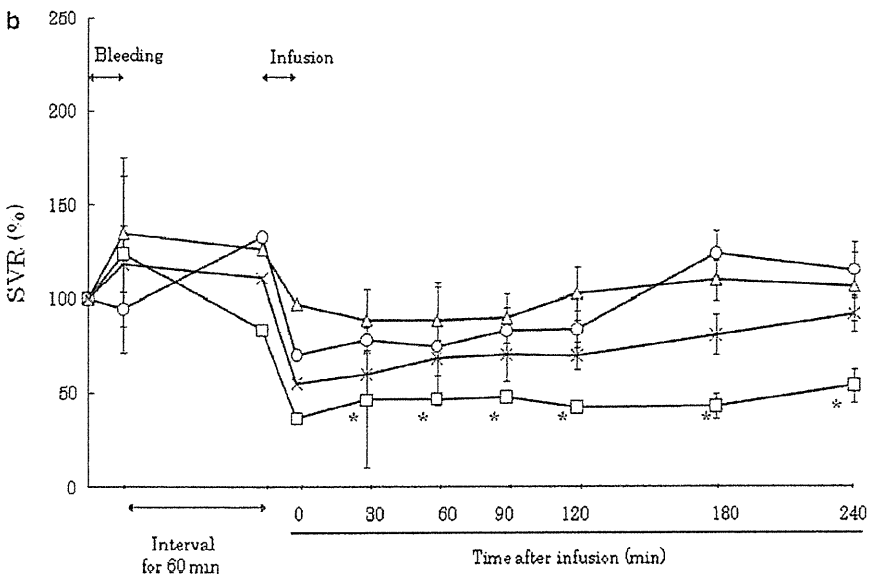

Figure 2b. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on systemic vascular resistance (SVR) ratio in anesthetized dogs subjected to 50% exsanguination. There were significant differences between the HSA and HbV groups after resuscitation. (○; LR group, □; HSA group, ×; ASB group, △; HbV group).

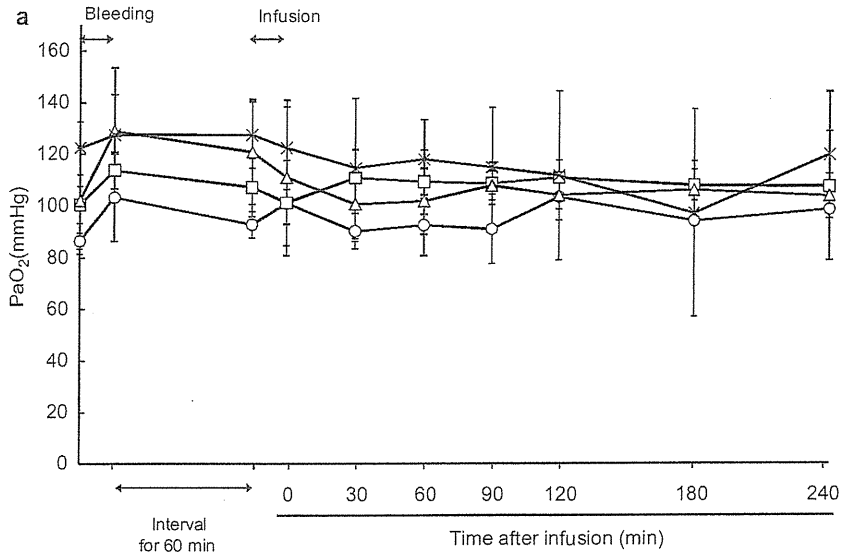

Figure 3a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on PaO_2 in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

Tissue Oxygen Tension of Renal Cortex and Regional Oxygen Saturation of Brain and Rectus Abdominis Muscle

The renal cortical oxygen tension decreased after exsanguination and recovered after resuscitation (Figure 4a). After resuscitation, the PtO_2 recovered to the baseline level in every group and there were no significant differences among the groups. The regional oxygen saturation was the mixed value of tissue blood flow and oxygen saturation (Figure 4b). During shock, brain rSO_2 decreased but recovered and was maintained at the baseline level after resuscitation in all groups.

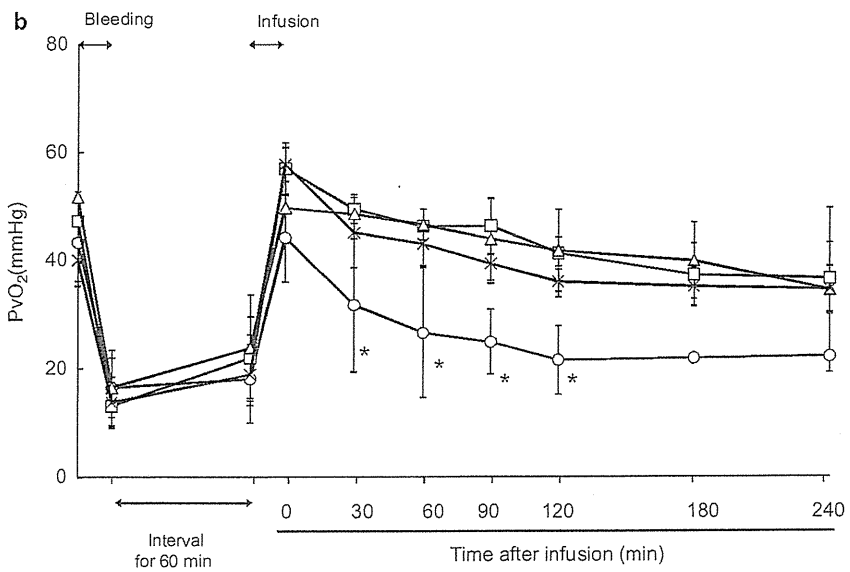

Figure 3b. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on PvO_2 in anesthetized dogs subjected to 50% exsanguination. There were significant differences between the LR group and the other groups 30 minutes after resuscitation (*). (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

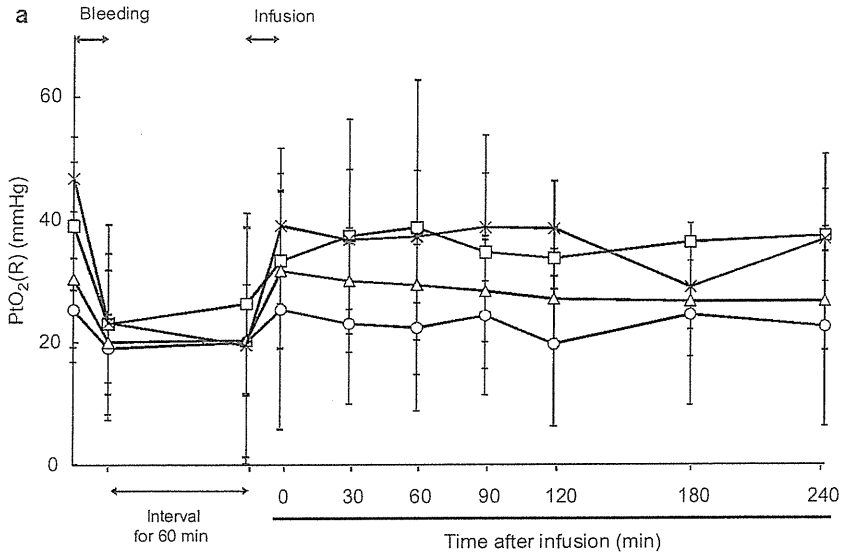

Figure 4a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on PtO_2 of the renal cortex in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

Ht and Hb Concentration

Ht decreased after resuscitation in all groups except ASB (LR: 21.7 ± 1.2, HSA: 20.0 ± 1.0, ASB: 36 ± 3.5, HbV: 22.0 ± 2.6). After resuscitation, Ht gradually increased in the LR group (21.7 ± 1.2% immediately after resuscitation to 25.5 ± 2.1% 180 minutes after resuscitation) (Figure 5a). In the HSA group, Ht did not change after resuscitation (20.0 ± 1.0% to 21.3 ± 2.3%). For accurate measurement of the Hb concentration in the HbV group,

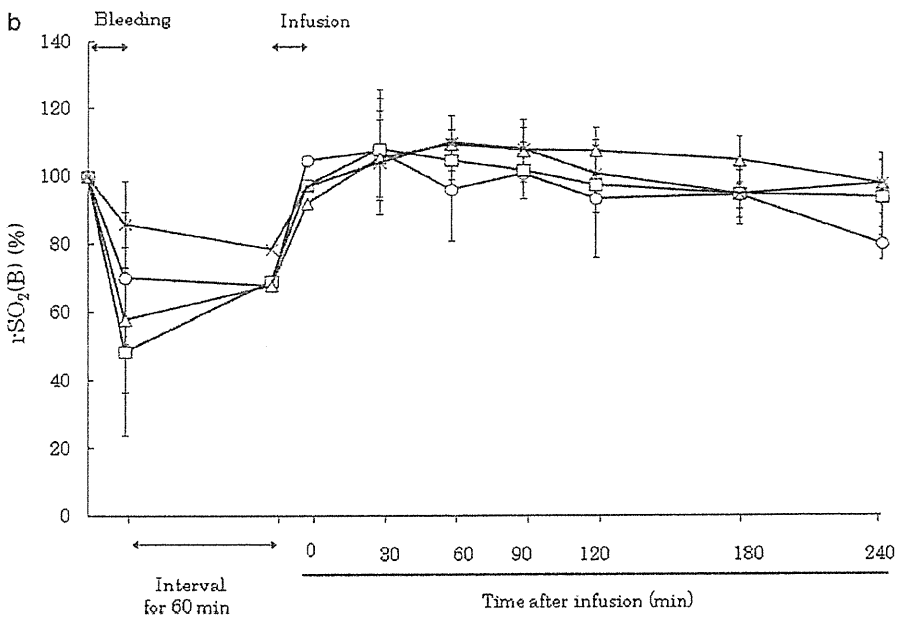

Figure 4b. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on regional oxygen saturation of the brain (rSO_2 (B)) in anesthetized dogs subjected to 50% exsanguination. The value was expressed as a ratio to the baseline. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

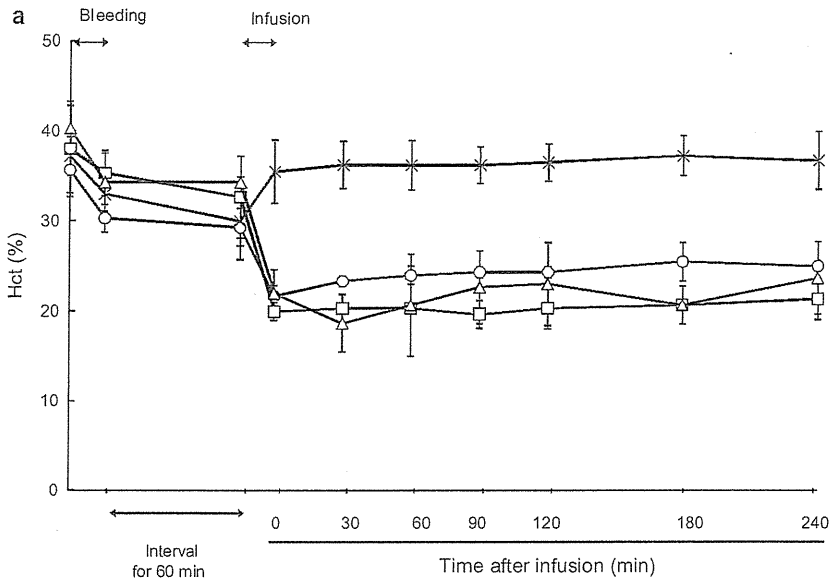

Figure 5a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on hematocrit (Ht) in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the HSA and LR groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

the blood was centrifuged and the HbV dispersed in the plasma component was measured separately. The change in Hb concentration is shown in Figure 5b. The Hb derived from RBC was 7.2 ± 1.33 g/dL immediately post-resuscitation and decreased to 6.0 ± 0.69 g/dL at 30 minutes post-resuscitation, then gradually increased to 7.7 ± 1.2 g/dL at 240 minutes post-resuscitation. The concentration of Hb derived from HbV was 3.86 ± 0.4 g/dL immediately after resuscitation and decreased to 3.24 ± 0.14 g/dL at 30 minutes after resuscitation, but there were no significant change in total Hb concentration.

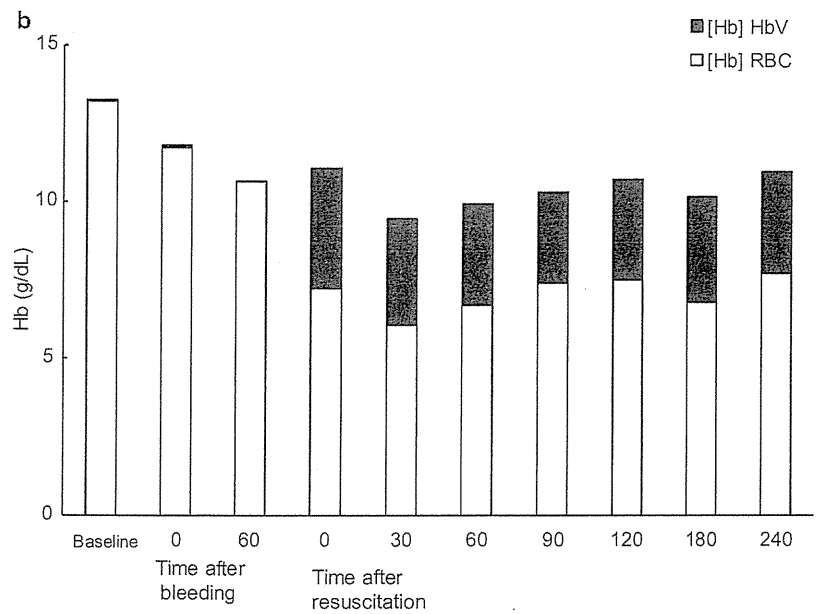

Figure 5b. Contribution of HbV to the total hemoglobin concentration after administration of HbV. (Black bar: Hb derived from HbV; blank bar: Hb derived from RBC).

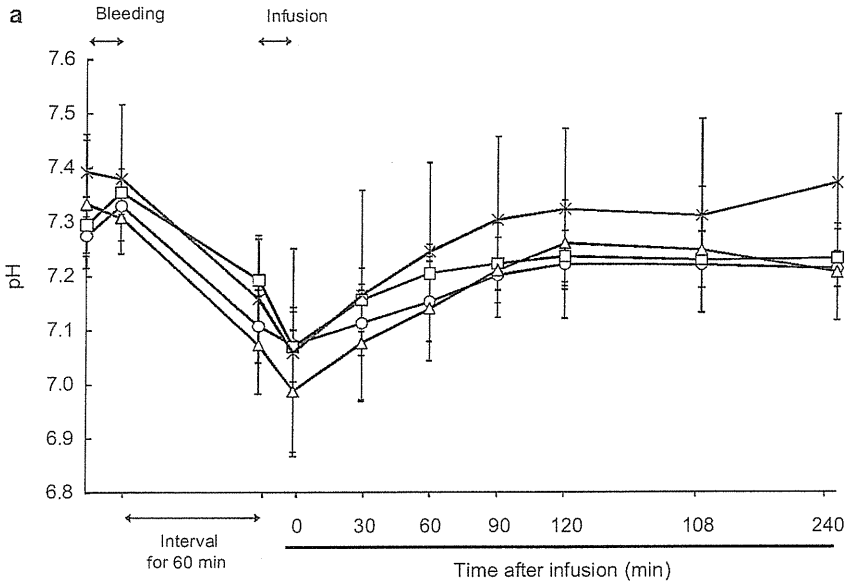

Figure 6a. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on pH in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).

Acid-base Balance

Blood pH decreased and serum lactate increased after exsanguination. After resuscitation, the pH level was restored in every group with no significant differences between groups (Figure 6a, 6b). The serum lactate level also decreased after resuscitation in the ASB, HSA, and HbV groups. In the LR group, lactate did not recover to the baseline level and serum lactate actually increased 90 minutes after resuscitation, although we cannot evaluate the statistical difference between the other groups because one animal died 150 minutes after resuscitation.

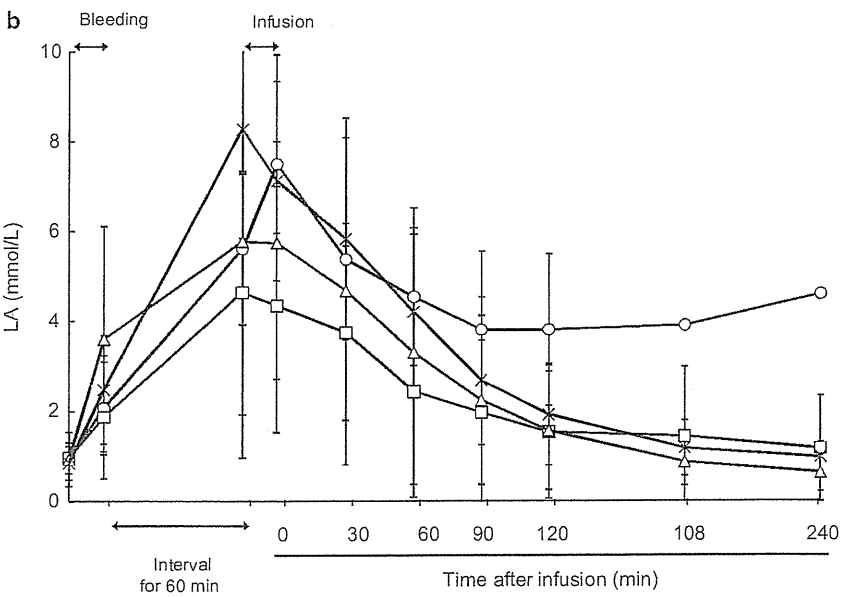

Figure 6b. Effects of Lactated Ringer's solution (LR), human serum albumin (HSA), autologous shed blood (ASB), and hemoglobin vesicles (HbV) on serum lactate level in anesthetized dogs subjected to 50% exsanguination. There were no significant differences between the HbV group and the other groups. (○: LR group; □: HSA group; ×: ASB group; △: HbV group).