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その他の刊行物

該当なし

# 研究成果の刊行物・別冊

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# HEMOGLOBIN VESICLES AND RED BLOOD CELLS AS CARRIERS OF CARBON MONOXIDE PRIOR TO OXYGEN FOR RESUSCITATION AFTER HEMORRHAGIC SHOCK IN A RAT MODEL

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ABSTRACT—Hemoglobin vesicles (HbVs) are artificial oxygen ($O_2$) carriers that encapsulate concentrated hemoglobin (Hb) solution in phospholipid vesicles (liposomes). Recent reports on cytoprotective effects of exogenous carbon monoxide (CO) urged us to test infusion of CO-bound HbV (CO-HbV) and red blood cells (CO-RBC) in hemorrhagic-shocked rats to improve tissue viability over that of $O_2$-bound HbV ($O_2$-HbV) and $O_2$-bound RBC ($O_2$-RBC). Male Wistar rats were anesthetized with 1.5% sevoflurane inhalation ($FiO_2$ = 21%) while spontaneous breathing was maintained. Shock was induced by 50% blood withdrawal from femoral artery. Fifteen minutes later, they received CO-HbV, CO-RBC, $O_2$-HbV, $O_2$-RBC, or empty vesicles (EV) suspended in 5% recombinant albumin. All groups showed prompt recovery of blood pressure and blood gas parameters just after resuscitation and survived for 6 h of observation period. However, only the EV group showed significant hypotension at 3 and 6 h. Plasma enzyme levels were elevated at 6 h, especially in the $O_2$-HbV, $O_2$-RBC, and EV groups. They were significantly lower in the CO-HbV and CO-RBC groups than in the $O_2$-bound fluids. Immunohistochemical staining of 3-nitrotyrosine exhibited less oxidative damage in the liver and lung for CO-HbV and CO-RBC groups. Blood carbonyl Hb levels (26%–39% immediately after infusion) decreased to less than 3% at 6 h while CO was exhaled through the lung. Both HbV and RBC gradually gained the $O_2$ transport function. Collectively, both CO-HbV and CO-RBC showed a resuscitative effect for hemorrhagic-shocked rats. They reduced oxidative damage to organs in comparison to $O_2$-HbV and $O_2$-RBC. Adverse and poisonous effects of CO gas were not evident for 6 h in this experimental model. Further study is necessary to clarify the neurological impact of a longer observation period for eventual clinical applications.

KEYWORDS—Blood substitutes, hemoglobin, liposome, resuscitation, carbon monoxide, reperfusion injury

ABBREVIATIONS—Hb–hemoglobin; HbV–Hb vesicles; CO-HbV–CO-bound HbV; RBC–red blood cell; CO-RBC–CO-bound RBC; EV–empty vesicles; HO–heme oxygenase; HBOC–hemoglobin-based oxygen carrier; rHSA–recombinant human serum albumin; Hct–hematocrit; $PaO_2$–arterial blood $O_2$ tension; $PaCO_2$–arterial blood $CO_2$ tension; HR–heart rate; AST–aspartate aminotransferase; ALT–alanine aminotransferase; LDH–lactate dehydrogenase; Mb–myoglobin; NOS–nitric oxide synthase

## INTRODUCTION

Carbon monoxide (CO), biliverdin, and bilirubin are produced during oxidative heme degradation that is catalyzed by a stress protein: heme oxygenase (HO; also termed *heat shock protein 32*) (1). They mediate antioxidative, antiproliferative, and anti-inflammatory effects. Endogenous CO shows a vasorelaxation effect, as does NO (2, 3). Many researchers have reported the importance of cytoprotective HO as a stress protein in animal models. However, the amount and the "source of heme" as a substrate and the amount of CO produced by induction of HO-1 remain unclear. These observations engender the concept of using exogenous, not endogenous, CO for therapeutic purposes. Motterlini et al. (4) synthesized a series of CO-releasing metal complexes; subsequent *in vivo* studies clarified some pharmacological effects. Despite the poisonous effect of CO gas, low-concentration CO inhalation (250 ppm) was tested in animal models of hemorrhagic shock, septic shock, and I/R (5, 6). Some cytoprotective effects were obtained, and the mechanism has been studied extensively. Cabrales et al. (7) recently reported CO-bound RBC (CO-RBC) injection to hemorrhaged hamsters and clarified its cytoprotective effect in subcutaneous microcirculation.

These studies have led us to test intravenous injection of CO as a ligand of heme in hemoglobin (Hb)-based oxygen ($O_2$) carriers (HBOCs) that have been extensively studied as transfusion alternatives. We are familiar with carbonyl Hb (HbCO) because we use stable HbCO for production of Hb vesicle (HbV) or liposome-encapsulated Hb as one HBOC (8–12). The stability constant of HbCO is approximately 200 times higher than that of $HbO_2$. Furthermore, autoxidation of Hb is preventable by carbonylation, which enables pasteurization of the HbCO solution at 60°C in combination with a subsequent encapsulation procedure without protein denaturation. In the final process, HbCO in HbV is photodissociated by irradiation of visible light under an $O_2$ atmosphere to convert $HbO_2$ (19). The $O_2$-bound HbV ($O_2$-HbV) can provide sufficient $O_2$-transport capacity that is comparable to that of RBC (11, 12).

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A traumatic hemorrhage might cause a shock state, which subsequently causes a systemic inflammatory response, in some cases leading to multiple organ failure. Resuscitation with transfusion or HBOCs with an $O_2$-carrying capacity induces reperfusion injury, as evidenced by elevations of plasma enzyme levels and tissue cytokine levels (12–15). Actually, we observed elevation of plasma enzyme levels 6 h after resuscitation from hemorrhagic shock by administration of $O_2$-bound RBC ($O_2$-RBC) and $O_2$-HbV in a rat model (11). It is expected that coinjection of cytoprotective CO would improve resuscitative effects. For this study, using the same experimental model, we tested injection of CO-bound HbV (CO-HbV) for the first time as an exogenous CO supplier for fluid resuscitation. In comparative experiments, we also tested empty vesicles (EV) that carry neither $O_2$ nor CO and CO-RBC. Carbonylation processes of RBC are quite simple; the resulting CO-RBC would be stable over a longer preservation time, which more than adequately compensates for the short shelf life of packed RBCs (16).

## MATERIALS AND METHODS

### Preparation of resuscitative fluids

For use in this study, HbV was prepared as reported in previous studies (8, 9. 17). 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 mM of pyridoxal 5′-phosphate (Sigma Chemical Co, St. Louis, Mo) as an allosteric effector to regulate $P_{50}$ to 25 to 28 torr. The lipid bilayer was a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5:5:1 (Nippon Fine Chemical Co Ltd, Osaka, Japan) and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG (0.3 mol%; NOF Corp, Tokyo. Japan). The HbVs were suspended in a physiologic salt solution at [Hb] = 10 g/dL and [lipids] = 6.8 g/dL and were deoxygenated in vials for storage. The HbV suspension (8.6 mL) was mixed with a recombinant human serum albumin (rHSA: 25%. 1.4 mL; Nipro Corp, Osaka. Japan) to regulate [rHSA] in the suspending medium to 5 g/dL and the colloid osmotic pressure to approximately 20 torr. Consequently. [Hb] was 8.6 g/dL, and HbV bound $O_2$ in an aerobic condition. This solution is designated as $O_2$-HbV.

Carbon monoxide gas was bubbled gently for 5 min to prepare CO-HbV into the deoxygenated HbV suspension in the vials. Similarly, the resulting CO-HbV was mixed with a 25% rHSA solution to regulate [Hb] at 8.6 g/dL. Before use, both $O_2$-HbV and CO-HbV were filtered (pore size, 0.45 μm; Dismic; Toyo Roshi Kaisha Ltd, Tokyo, Japan) to ensure a homogeneous dispersion state.

An EV suspension was prepared using the same lipids by hydration with a saline solution. The lipid concentration (6.8 g/dL), the particle diameter (ca. 250 nm), and the viscosity (ca. 3 cP) were almost identical to those of HbV. The suspension was mixed with the 25% rHSA solution to regulate colloid osmotic pressure.

To prepare a washed RBC concentrate, blood samples from donor rats were withdrawn into heparinized syringes (ca. 0.15 mL of 10,000 IU/mL heparin to 10 mL of blood) and centrifuged; it was then washed twice by resuspension in 5% rHSA and centrifugation (3000×*g*, 10 min). The [Hb] of $O_2$-RBC was adjusted to 8.6 g/dL, equivalent to that of HbV. The CO-RBC was prepared using gentle CO bubbling for approximately 5 min.

### Animal model and preparation

The entire experimental protocol was approved by the Laboratory Animal Care and Use Committee of the School of Medicine, Keio University. 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 59 male Wistar rats (274 ± 26 g body weight). The rats were anesthetized by 1.5% sevoflurane-mixed air inhalation (Maruishi Pharmaceutical Co, Osaka, Japan) using a vaporizer (Model TK-4 Biomachinery; Kimura Medical Instrument Co Ltd, Tokyo, Japan) throughout the experiment (fraction of inspired $O_2$: $FiO_2$ = 21%) while spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing, OD 0.8 mm, ID 0.5 mm; Natsume Seisakusho Co Ltd, Tokyo, Japan) filled with saline solution containing 40 IU/mL heparin were introduced through the right jugular vein into the right atrium and into the right common carotid artery. The arterial catheter was connected to a polygraph system (LEG-1000; Nihon Kohden Corp, Tokyo, Japan).

### Resuscitation from hemorrhagic shock

The systemic blood volume was estimated as 56 mL/kg body weight. Hemorrhagic shock was induced by withdrawing 50% of the blood (28 mL/kg, 1 mL/min) from the carotid artery. The rats were kept hypotensive for 15 min (MAP <40 mm Hg). Rats were resuscitated by infusion of $O_2$-HbV (n = 9), CO-HbV (n = 9), $O_2$-RBC (n = 9), CO-RBC (n = 9), or EV (n = 8) at a rate of 1 mL/min. The volume of the infused resuscitative fluid was identical to the shed volume: 50% of the blood volume at baseline. The severity of the shock state was confirmed with eight rats that received no resuscitative fluid. The survival rate decreased after 15 min; all the rats died within 45 min.

### Measurements of systemic responses

Systemic hemodynamics and blood gases were evaluated before hemorrhage (baseline), after hemorrhage, immediately after resuscitation, and 1, 3, and 6 h after resuscitation. Blood samples were collected in 70 IU/mL heparinized microtubes (125 μL; Clinitubes; Radiometer A/S. Copenhagen, Denmark) for blood gas analyses and in glass capillaries (Terumo Corp, Tokyo, Japan) for hematocrit (Hct) measurements. A pH/blood gas analyzer (models ABL 555 and 700; Radiometer A/S) was used for analyses of arterial blood $O_2$ tension ($PaO_2$), arterial blood $CO_2$ tension ($PaCO_2$), pH, and lactate. A recording system (Polygraph System 1000; Nihon Kohden Corp) was used for continuous monitoring of the MAP and the heart rate (HR).

The HbCO level in the CO-RBC group was monitored using a pH/blood gas analyzer (700; Radiometer A/S). The HbCO level in the CO-HbV group was monitored using a spectroscopic method from absorptions at 419 (HbCO) and 430 nm (deoxyhemoglobin) (10). The exhaled CO was measured using gas chromatography with a CO-analyzer (TRIlyzer mBA-3000; Taiyo Instruments Inc, Osaka, Japan) (18). One milliliter of exhaled gas was collected in 5 min in a gas-tight syringe connected with an indwelling needle (24-gauge; Nipro Corp) that was inserted directly into the trachea of a rat (CO-HbV, n = 4; CO-RBC, n = 3).

### Plasma clinical laboratory tests

Six hours after resuscitation, approximately 5 mL of arterial blood was withdrawn rapidly into a heparinized syringe. Then the animals were laparotomized and killed by desanguination. The organs were resected for histopathologic study. The blood samples were centrifuged at 3,000*g* for 5 min to obtain plasma. Plasma containing HbV or EV required further ultracentrifugation, at 50,000*g*. for 20 and 90 min, respectively, to remove the vesicles (19). The plasma samples were stored at −80°C until clinical laboratory tests (BML Inc, Kawagoe, Japan). The levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and its isozymes (LDH-1, LDH-2) were measured: AST and ALT reflect hepatic function: reportedly, LDH-1 and LDH-2 are indicators for early cardiac damage in a rat model (20. 21).

### Histopathologic examination

The organs were fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries Ltd. Tokyo), and the paraffin sections were stained using hematoxylin/eosin. Immunohistochemical analyses of liver and lung tissues were performed to detect 3-nitrotyrosine as the most direct indicator of oxidative damage (14, 22, 23). We did not examine the brain because it was influenced by the cannulation of the carotid artery for blood withdrawal and blood pressure monitoring (11).

Subsequently, 4-μm-thick paraffin sections were treated with 0.3% $H_2O_2$ in methanol for 20 min. After blocking nonspecific binding with an antibody diluent (S2022; DakoCytomation. Glostrup, Denmark), they were incubated overnight at 4°C with mouse monoclonal antibody against 3-nitrotyrosine (1/10 dilution NITT12-A: Alpha Diagnostic International. Inc, San Antonio, Tex). They were then incubated for 45 min at room temperature with goat antibodies against mouse immunoglobulins conjugated to the amino acid polymer (no dilution, Histofine Simple Stain MAX-PO(M); Nichirei Corp, Tokyo. Japan). Negative control was performed without the primary antibody against 3-nitrotyrosine. Color was developed using 3,3′-diaminobenzidine (16.7%; Sigma Chemical Co) in 0.05 M Tris-HCl. pH 7.4, containing 0.04% $H_2O_2$. Nuclei were stained with hematoxylin.

### In vivo *data analysis*

The *in vivo* data are given as the mean ± SD for the indicated number of animals. Data were analyzed using StatView (Ver. 5.0; Abacus Concepts, Inc.

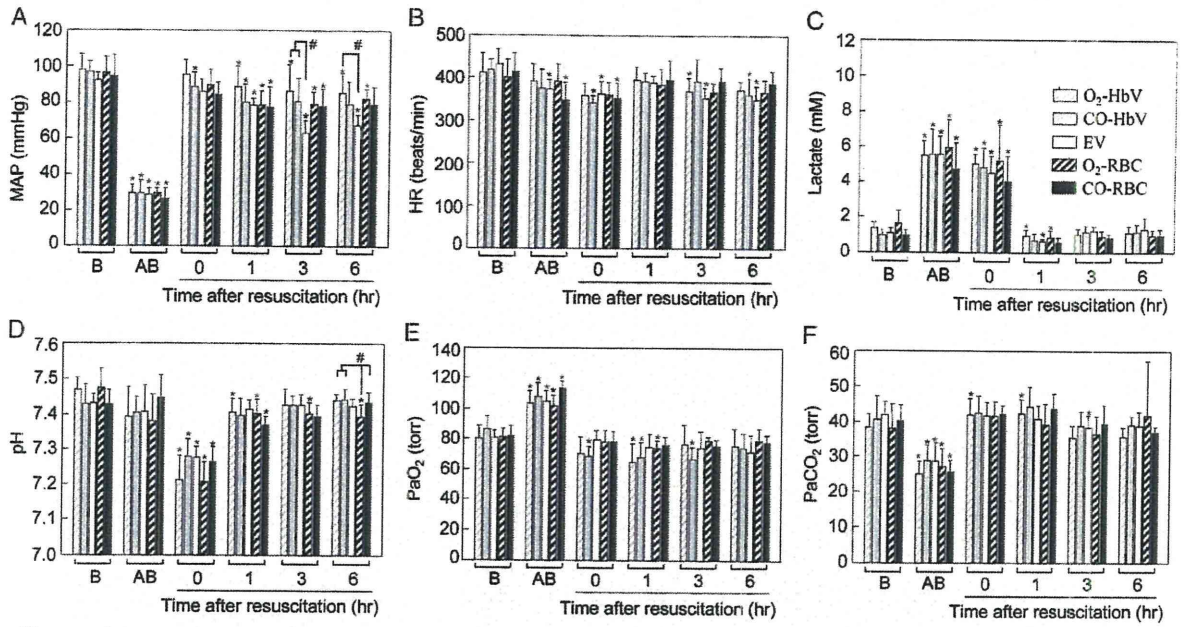


FIG. 1. **Changes in systemic parameters in rats before and after hemorrhage and resuscitation by injection of $O_2$-HbV, CO-HbV, EV, $O_2$-RBC, or CO-RBC suspended in 5% rHSA: (A) MAP, (B) HR, (C) lactate, (D) pH, (E) $PaO_2$, and (F) $PaCO_2$.** B indicates baseline; AB, after bleeding. $^*P < 0.01$ (Bonferroni correction) versus baseline; $^{\#}P < 0.05$ versus the indicated group. Repeated-measures ANOVA clarified that the profile of MAP (a) in the EV group was significantly different from the $O_2$-HbV and CO-HbV groups ($P < 0.01$), whereas there was no significant difference in the profiles of other parameters between the groups.

Berkeley, Calif). For systemic parameters, time-related differences compared with the baseline within each group were assessed by paired *t* test with Bonferroni correction to adjust multiple comparisons. Differences among the groups at the same time point were assessed by ANOVA followed by the Scheffe procedure. Repeated-measures ANOVA was used to assess differences in time-related profiles of a systemic parameter among the groups. Unpaired *t* tests were used for comparison of plasma enzyme levels among the groups. Differences were inferred as significant when $P < 0.05$.

### In vitro *CO exchange reaction from HbV to RBC*

A suspension of CO-HbV ([Hb] = 10 g/dL, 0.5 mL, in saline) was added to a rat $O_2$-RBC ([Hb] = 10 g/dL, 4.5 mL in saline) in a plastic tube at a volume ratio of 1:9; it was mixed immediately using a vortex mixer for 10 s at room temperature. Of that mixture, 0.5-mL quantities were transferred by pipette to a small plastic tube at 0.5, 1, 3, and 5 min then immediately centrifuged (5000*g*, 30 s) to obtain a supernatant containing HbV while RBC was precipitated. The HbCO level of the supernatant HbV was measured using the method described above.

## RESULTS

### *Systemic responses to hemorrhagic shock and resuscitation*

All rats survived for 6 h after resuscitation. The average MAP of the Wistar rats before hemorrhage was 96 ± 9 mm Hg, which declined to 29 ± 5 mm Hg after hemorrhage (Fig. 1). Immediately after resuscitation, the MAP of all groups increased to greater than 80 mm Hg. No significant difference was found between the $O_2$-HbV and CO-HbV and RBC for 6 h. All groups including $O_2$-RBC tended to slightly decrease MAP. The EV group showed significant hypotension in comparison to the $O_2$-HbV group at 3 and 6 h ($P < 0.05$). The profile of MAP for the EV group differed significantly from those for the $O_2$-HbV and CO-HbV groups ($P < 0.01$). The HR of the Wistar rats before hemorrhage was 415 ± 38 beats per minute. Slight reductions were apparent especially after hemorrhage and resuscitation, but all groups tended to sustain stable values for 6 h. Hemorrhagic shock induced anaerobic metabolism, as evidenced by an increase in average lactate from 1.2 ± 0.5 mM to 5.5 ± 1.4 mM. Metabolic acidosis was indicated by a delayed decrease to below 7.3 after

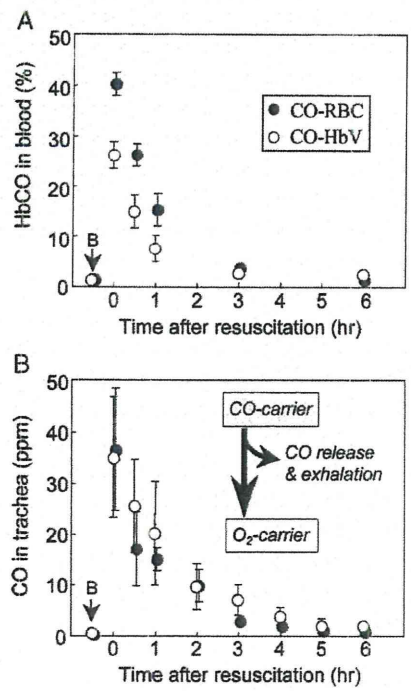


FIG. 2. **Time course of HbCO levels in blood (A) and CO levels in the trachea (B) before and after injection of CO-HbV or CO-RBC suspended in 5% rHSA as a resuscitative fluid for hemorrhagic shock in rats.** B indicates baseline. Both CO-HbV and CO-RBC released CO and gradually became $O_2$ carriers. In addition, a part of the released CO was exhaled through a lung and detected in the trachea. Most of the injected CO became undetectable in the body within 6 h.

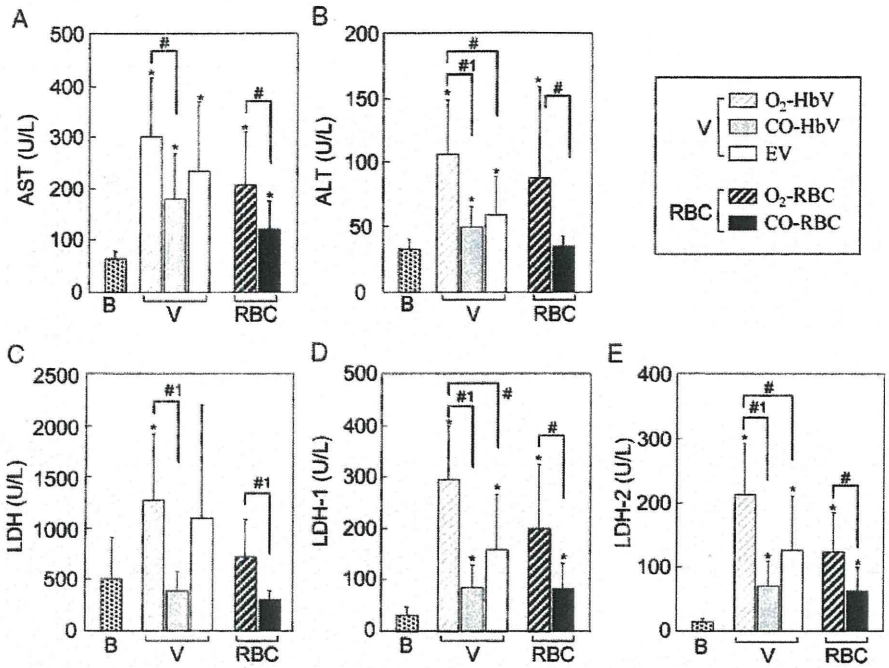


FIG. 3. **Plasma enzyme levels 6 h after injection of $O_2$-HbV, CO-HbV, EV, $O_2$-RBC, or CO-RBC suspended in 5% rHSA: (A) AST, (B) ALT, (C) LDH, (D) isozyme LDH-1, and (E) isozyme LDH-2.** B indicates baseline. $^*P < 0.05$ versus baseline group; $^{\#}P < 0.05$ versus the indicated group; $^{\#1}P < 0.01$ versus the indicated group.

fluid injection. Consequently, significant compensatory hyperventilation was observed as an increase in $PaO_2$ of 82 ± 8 torr to 106 ± 9 torr and a decrease in $PaCO_2$ of 40 ± 6 torr to 27 ± 4 torr. All groups tended to recover from hyperventilation immediately after resuscitation. The lactate and the pH values showed no immediate recovery after resuscitation but tended to recover within 1 h. The $O_2$-RBC group at 6 h showed statistically significant different pH ($P < 0.05$). However, no significant differences were found among groups in the pH profiles and other parameters (lactate, $PaO_2$, $PaCO_2$, and HR); no noteworthy difference was found between CO-HbV, $O_2$-HbV, CO-RBC, and the gold standard, $O_2$-RBC. Both CO-HbV and CO-RBC groups did not show any hypoxic sign after resuscitation.

The Hct before hemorrhage was 42% ± 2%; due to autotransfusion, it decreased to 34% ± 3% after bleeding (graphs not shown). After resuscitation, the Hct values in the $O_2$-HbV, CO-HbV, and EV groups were significantly reduced due to the blood dilution, respectively, to 19% ± 1%, 18% ± 1%, and 18% ± 1%. The HbV and the EV particles remained dispersed in the plasma phase in the glass capillaries for Hct measurements. The respective Hct in the $O_2$-RBC and CO-RBC groups were 34% ± 3% and 32% ± 2%. The total [Hb] in blood after resuscitation either with HbV or with RBC was estimated as 11 g/dL. The contribution of HbV was approximately 5 g/dL.

### *HbCO in blood and CO in exhaled air*

In a normal condition, the HbCO level in the rat blood was below 2% (Fig. 2). After injection of CO-RBC, the HbCO level increased to 39% ± 2%, which decreased to 15% ± 3% at 1 h. Injection of CO-HbV showed lower HbCO levels than that of CO-RBC. Immediately after injection, the HbCO level was 26% ± 3%, which decreased to 8% ± 3% at 1 h. The HbCO level of both groups diminished to less than 3% at 6 h. These data indicated that both CO-RBC and CO-HbV became $O_2$ carriers after releasing CO. The CO level in the trachea at the baseline was less than 1 ppm. After injection of CO-bound fluids, it increased to around 40 ppm and then decreased to 15 ppm at 1 h, in parallel with the change in the HbCO level in blood, and markedly diminished to less than 3 ppm at 6 h.

### *Clinical laboratory tests of blood serum*

Normal Wistar rats showed AST and ALT of 64 ± 13 U/L and 32 ± 8 U/L, respectively (Fig. 3). All groups at 6 h showed significantly higher AST levels than the baseline ($P < 0.05$). However, both the CO-HbV and the CO-RBC groups showed significantly lower values than the corresponding $O_2$-bound fluids ($P < 0.05$). Furthermore, CO-bound fluids showed significantly lower ALT levels than the corresponding $O_2$-bound fluids; particularly, ALT of CO-RBC diminished to the baseline level. The $O_2$-HbV and the $O_2$-RBC groups showed higher LDH than the baseline value of 504 ± 404 U/L: they were 1272 ± 645 U/L and 714 ± 373 U/L, respectively. Resuscitation with CO-HbV and CO-RBC showed significantly lower LDH levels to 384 ± 187 U/L and 300 ± 89 U/L, respectively. Similar higher values were observed for the LDH isozymes, LDH-1 and LDH-2, in the $O_2$-HbV and the $O_2$-RBC groups. Significantly lower values were noted in the cases of both CO-HbV and CO-RBC groups in comparison with the $O_2$-bound fluids groups ($P < 0.05$). The EV group showed significantly lower ALT, LDH-1, and LDH-2 than the $O_2$-HbV group ($P < 0.05$). However, the effects were smaller than those for the CO-HbV group.

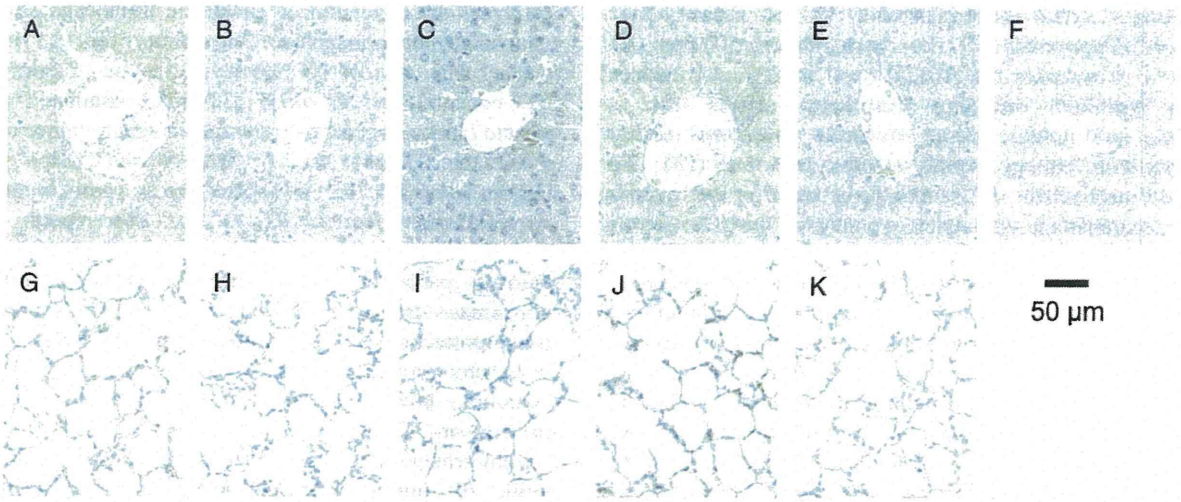


FIG. 4. **3-Nitrotyrosine detection in rat liver (A–E) and lung (G–K) tissues 6 h after resuscitation: (A) $O_2$-HbV, liver; (B) CO-HbV, liver; (C) EV, liver; (D) $O_2$-RBC, liver; (E) CO-RBC, liver; (F) $O_2$-RBC, liver, without primary antibody against 3-nitrotyrosine; (G) $O_2$-HbV, lung; (H) CO-HbV, lung; (I) EV, lung; (J) $O_2$-RBC, lung; and (K) CO-RBC, lung.** Nitrotyrosine adducts are shown as brown using immunohistochemistry, as described in Materials and Methods. F, the negative control of the immunohistochemical staining.

### *Histopathologic examination 6 h after resuscitation with HbV/rHSA*

Hematoxylin/eosin staining of the rat organs demonstrated no significant morphological abnormalities in the kidney or the heart (data not shown). The period of 6 h after hemorrhagic shock and resuscitation is not sufficiently long to cause morphological changes caused by hemorrhagic shock and resuscitation, except that the spleen macrophages and the liver Kupffer cells showed phagocytosis of HbV and EV (24). Immunochemical staining with antinitrotyrosine revealed marked changes in the liver and the lung. The livers of $O_2$-HbV, EV, and $O_2$-RBC group rats exhibited staining of nitrotyrosine (Fig. 4). In contrast, the degree of nitrotyrosine staining in the livers of CO-HbV and CO-RBC group rats was markedly less, especially in hepatocytes nearby the central veins. The lungs of $O_2$-HbV, EV, and $O_2$-RBC group rats showed nitrotyrosine staining. In contrast, the lungs of both CO-HbV and CO-RBC group rats showed markedly less staining. Negative control was performed without the primary antibody against 3-nitrotyrosine, and it showed no staining.

### In vitro *CO exchange reaction between HbV and RBC*

Immediately after mixing of CO-HbV with $O_2$-RBC suspension, CO was released rapidly from HbV and moved to RBC. The level of HbCO in HbV decreased to 35% at 0.5 min, 15% at 1 min, and 9% at 3 min; it eventually reached a plateau (Fig. 5). The final HbCO level coincided with the mixing ratio of CO-HbV/$O_2$-RBC = 1:9 by volume.

## DISCUSSION

The salient finding of this study is that both CO-HbV and CO-RBC showed a sufficient resuscitative effect when infused intravenously into anesthetized rats in a hemorrhagic shock condition. No meaningful difference between the CO- and the $O_2$-bound fluids was found in systemic parameters (i.e., MAP, HR, blood gas) during 6 h although some significant differences were found in comparison to the baseline. The plasma enzyme levels at 6 h, which reflect hepatic and cardiac functions, were significantly reduced using either CO-HbV or CO-RBC fluid in comparison to resuscitations with $O_2$-HbV and $O_2$-RBC despite the reduced $O_2$-carrying capacity. Hemorrhagic shock and resuscitation induces systemic reperfusion injury, a trigger of eventual multiple organ failure. Immunohistochemistry revealed that 3-nitrotyrosine, a marker of inflammatory oxidative damage, was attenuated significantly in the liver and the lung. These results demonstrate the cytoprotective effect of exogenous CO molecules. Reperfusion injury is attributable to the toxic effect of reactive oxygen species (ROS) that are generated once tissue is reperfused using an $O_2$-rich fluid (14, 22, 25). Resuscitation with EV, which carry neither $O_2$ nor CO, slightly reduced oxidative damage and sustained MAP for at least 1 h probably owing to the high viscosity of EV comparable to that of HbV (26), although hypotension became significant at 3 and 6 h. Our results imply that blood volume restoration is primarily important at the early stage of a fluid resuscitation; the prompt recovery of $O_2$ transport is pro-oxidative, although $O_2$ is eventually necessary to maintain MAP.

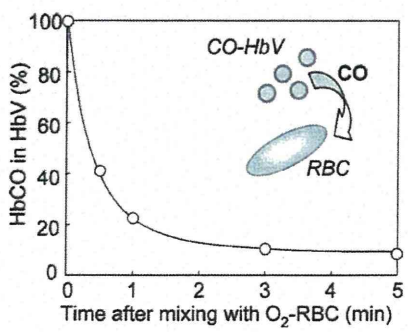


FIG. 5. ***In vitro* CO release of CO-HbV after rapid mixing with $O_2$-RBC.** A CO-HbV ([Hb] = 10 g/dL, 0.5 mL) and an $O_2$-RBC suspension (10 g/dL, 4.5 mL) were mixed immediately. The level of HbCO of HbV fraction after centrifugation was monitored. Results indicate that the CO release rate is unexpectedly rapid.

Inhaling a certain concentration of CO gas causes anthracemia or CO poisoning (27). For a man, inhaling 500 ppm CO gas for 4 h increases the HbCO level to 40% and induces various symptoms including neurological effects such as headache and fatigue. More advanced symptoms include dizziness with lethargy, coma, seizures, and death (28). The proposed mechanism of CO toxicity is based on the hypoxia theory accompanied with apnea or asphyxia due to a narcotic action of CO gas. In our experiment, respiratory function was preserved well, and blood gas and hemodynamic parameters were stable. We confirmed that CO was exhaled promptly; no accumulation of CO in the body was apparent. Another mechanism is the cellular theory based on the chemical reaction of mitochondrial cytochromes, myoglobin (Mb), and nonspecific heme-containing enzyme (29). Cytochrome oxidases have a greater affinity for $O_2$ than CO in contrast to Hb. Myocardial $O_2$ consumption is preserved at up to 77% of carbonyl Mb saturation (30). In our experiment, the myocardial function was apparently preserved, as evidenced by the sustained MAP, HR, and the LDH levels. The CO affinity of Mb is approximately one fifth of that of Hb, and the level of CO saturation is apparently not deteriorative. The CO affinity of cytochrome oxidase is approximately 1/1.7 of that of Mb; there is no evidence that the HbCO level in our experiment would impair $O_2$ metabolism under the condition that $PaO_2$ is sufficiently maintained (31). Cabrales et al. (7) recently tested injection of CO-RBC of 25% blood volume into conscious hamsters and observed stable systemic parameters for 90 min. Our more severe experimental condition, injection of CO-RBC of 50% blood volume to anesthetized rats and observation for 6 h, further supports the effectiveness of CO injection.

In our experiment, the HbCO levels in the CO-HbV and the CO-RBC groups immediately after infusion were 26% to 39%, but they decreased rapidly and became less than 3% within 6 h. It is well known that the equilibrium constant of HbCO is 200 times greater than that of $HbO_2$. However, a rapid ligand-exchange reaction from HbCO to $HbO_2$ occurs because the rats inhale atmospheric air; fundamentally, the amount of $O_2$ is much greater than that of CO in the rat circulating blood. The *in vitro* rapid CO exchange reaction, which is shown to occur within 1 min from HbV to RBC in Figure 5, also supports an *in vivo* rapid ligand exchange among Hbs in HbV and RBC and heme proteins in tissues. The maintained respiratory function is necessary for emission of CO gas in the exhaled air. According to the gradual release of the CO gas, both HbV and RBC become $O_2$ carriers. The faster CO release of HbV than that of RBC coincides with the profiles of $O_2$ release, as observed in our previous study; it is putatively related to the larger surface-to-volume ratio of HbV than that of RBC (32).

Various reports have described the pharmacological effects of CO molecules. Reportedly, CO inhalation induces significant hypotension and reduces total peripheral resistance at HbCO levels as low as 7%, putatively due to the vasodilatory effect of CO (33). In fact, CO is a vasorelaxation factor in hepatic and subcutaneous microcirculation (3, 34). Although both NO and CO bind soluble guanylate cyclase, which catalyzes the conversion of guanosine triphosphate to cyclic guanosine monophosphate, the affinity for CO is much weaker than that of NO. However, in our experiment, the CO concentration in blood (3.1 mM, estimated from the injected heme concentration) would be much higher than that of NO (ca. 100 nM) (35). The MAP of the CO-HbV group is slightly lower than that of $O_2$-HbV group, probably due to the vasorelaxation effect of CO (3, 36). Other reports describe that CO might cause a downregulation of proinflammatory cytokine production through the p38 mitogen-activated protein kinase–dependent pathway leading to anti-inflammatory tissue protection. The p38 mitogen-activated protein kinase is not a heme protein and has no binding target of CO; a heme-containing protein is believed to be involved in the upstream mechanism (37).

Hemorrhagic shock and resuscitation typically entail systemic I/R injury. Activated neutrophils and macrophages produce ROS (38), in which nicotinamide adenine dinucleotide phosphate oxidase is involved as a major source of ROS (14). This enzyme is a flavohemoprotein containing two hemes that catalyze the nicotinamide adenine dinucleotide phosphate–dependent reduction of $O_2$ to form superoxide ($O_2^-$) (39). However, CO can bind to the hemes and modulate the enzymatic activity (40). In myocardium, Mb autoxidation and $O_2^-$ generation are enhanced at a condition of I/R in which $O_2$ supply recovers spontaneously, although a delay of pH recovery is observed (41), just as in the conditions according to our results in Figure 1. It is expected that the injected CO spontaneously binds to myocardial Mb to reduce Mb autoxidation.

During hemorrhagic shock, there should be an initiation of inflammatory cytokine production and NO release from the inducible form of NO synthase (NOS) in organs such as the liver and the lung (23, 42). In fact, CO gas potently inhibits the conversion of L-arginine to NO and citrulline by neuronal and macrophage NOS because two heme moieties are contained in the active enzymes (43). CO would modulate overproduction of NOS-derived NO (44).

Together, $O_2^-$ and NO react to form peroxynitrite, $ONOO^-$, a potent cytotoxic molecule that promotes nitration of tyrosyl residues in proteins (45). The possibility exists that the injected CO reduces production of both NO and $O_2^-$ and its resultant $ONOO^-$. Actually, our immunohistochemical observations of the liver and the lung clarified that injection of CO-HbV and CO-RBC reduced the formation of nitrosotyrosine on the proteins. This effect closely resembles those of Tempol (a scavenger of $O_2^-$) and GW274150 (an iNOS inhibitor), which reduce both nitrotyrosine formation and plasma enzyme levels after hemorrhagic shock and resuscitation (22, 23).

Our data indicate no acute toxicity of CO in the anesthetized rats, probably due to the rapid CO emission and species dependence (33). However, it is proposed that delayed neurological damage of CO poisoning is caused by polymorphonuclear leukocytes that might be activated by CO and interact with endothelial cells and diapedese. Such damage might include brain lipid peroxidation and encephalopathy even after the CO is withdrawn (28, 46). A longer term of

observation would be necessary to optimize CO concentration and to clarify more detailed mechanism, potential neurological toxicity, and possibility of clinical applications.

A precedent report describes the possible utilization of an HBOC for detoxification of a lethal CO poisoning model (47) to transport $O_2$ efficiently in an anoxic condition. To our knowledge, the present study is the first to use an HBOC to administer CO in a shock state for a pharmacological effect. Although further research is definitely necessary to clarify the mechanism and the clinical relevance of our experimental results using small animals, the data would suggest that both RBCs and HBOCs can be an effective CO carrier and might improve their resuscitative effect in pathological situations of not only systemic hemorrhagic shock but also local ischemia. Advantages of CO-HbV and CO-RBC injection are as follows: (i) both CO-HbV and CO-RBC are stable for a longer term storage (8, 16); (ii) the special equipment to inhale CO gas is not necessary in an emergency situation; (iii) the CO dosage is strictly definable; and (iv) the fluid functions initially as a CO carrier to prevent pro-oxidative damage and functions in succession as an $O_2$ carrier.

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# Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model

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ABSTRACT

Hemoglobin-vesicles (HbV) are liposomal artificial oxygen carriers that may be useful as a resuscitation fluid during hemorrhagic shock (HS). It is well-known that the pharmacokinetic properties of liposome change in response to both pathological conditions and repeated administration. Therefore, we compared the pharmacokinetics of single versus repeated administration of HbV during HS. HS was induced by withdrawal of 40% of total blood volume. The normal (non-HS) and $HS_1$ group was received an injection of $^{125}$I-labeled HbV ($^{125}$I-HbV). The $HS_2$ group was resuscitated with non-labeled HbV, and 1 h later, it received an injection of $^{125}$I-HbV. The half-life was shorter in $HS_1$ rats, but it returned to non-HS levels after the second HbV injection. During 12 h after administration of HbV, tissue distribution of HbV was greatest in the $HS_1$ group; however, the $HS_2$ group had the greatest tissue distribution at subsequent time points. Excretion into urine, major elimination pathway, did not differ between non-HS and $HS_1$ rats, but was significantly reduced in the $HS_2$ group. Furthermore, the half-life of HbV in humans was estimated to be approximately 3–4 days using an allometric equation. This suggests that HbV may be a useful artificial oxygen carrier in HS based on HbV pharmacokinetics.



## 1. Introduction

Before new drugs are approved for clinical use, they must undergo many types of evaluation, including physicochemical tests, animal studies and clinical trials. Among these assessments, pre-clinical pharmacokinetic studies in various mammalian species are essential, as the results of such studies can be extrapolated to humans, allowing estimation of appropriate dosing regimens in humans. In fact, Kennedy et al. reported that inadequate pharmacokinetic data accounts for approximately 40% of the failed attempts to develop new drug-administration protocols during the clinical-trial stage of drug development [1].

To date, hemoglobin-based artificial oxygen carriers (HBOCs), which include cross-linked [2], polymerized [3] and polymer-conjugated Hbs [4], have been developed to overcome the problems associated with blood transfusion, such as cross-matching, blood-bone infection (human immunodeficiency virus, hepatics virus), and shortage of donated blood. Several of these HBOCs are currently in the final stages of clinical evaluation. Unfortunately, however, some HBOCs cause vasoconstriction, and consequently, hypertension, due to scavenging of nitric oxide (NO) by Hb. This phenomenon may occur because, unlike red blood cells (RBCs), HBOCs lack a cellular structure [5].

In contrast, hemoglobin vesicles (HbVs) are artificial oxygen carriers with a cellular structure similar to that of RBCs: highly concentrated Hb encapsulated in a phospholipid bilayer membrane. Thus, HbV does not cause the adverse effects that are associated with HBOCs [6,7], and HbV can control oxygen release to adjust the amount of allosteric effector [8]. Recently, Sakai et al. reported that the resuscitative effect of HbV is equivalent to that of autologous blood when injected into rats with hemorrhagic shock (HS) induced by withdrawal of 50% of total blood volume [9]. Yamasaki et al. also demonstrated that use of HbV as a cardiopulmonary bypass (CPB) priming solution improved neurologic and neurocognitive outcomes in a rat model of CPB [10].

Despite evidence for the effectiveness of HbV, the pharmacokinetics of HbV has not been well characterized. Thus far, pharmacokinetic studies of HbV have evaluated only changes in serum concentration and organ distribution in normal rats and rabbits administered $^{99m}$Tc-labeled hexamethyl-propylenamine oxime (HMPAO) encapsulated in HbV [11]. However, resuscitation after

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massive hemorrhage resulting from clinically relevant causes, such as injury, accidental blood loss or major surgery, has not been studied in detail. Clinical conditions affect the pharmacokinetics of numerous drugs [12,13]. For example, clinical trials have shown that the pharmacokinetics of liposome-encapsulated amphotericin B differ between normal individuals and patients [14,15]. Therefore, clarifying the pharmacokinetics of HbV in animal models of HS should provide useful information, such as dosing regimens, for future clinical applications.

Moreover, clinical use of HbV in patients with HS would require repeated administration of HbV. Recently, PEGylated liposomes were shown to lose their long-circulation half-life after being administered twice to the same animals (referred to as "accelerated blood clearance (ABC) phenomenon") [16]. Therefore, the pharmacokinetics of HbV, which has a liposomal structure, might be altered by repeated administration.

In the present study, we hypothesized that the pharmacokinetics of HbV would be altered by the presence of a pathological condition and by repeated administration. We directly labeled the internal Hb of HbV with iodine to create $^{125}$I-labeled HbV ($^{125}$I-HbV) and subsequently investigated the changes in HbV pharmacokinetics resulting from a pathological condition and repeated administration using a rat model of HS induced by massive hemorrhage.

## 2. Materials and methods

### 2.1. Materials and animal

Hb solution, which was purified from outdated donated blood, was provided by the Japanese Red Cross Society (Tokyo, Japan). Pyridoxal 5′-phosphate (PLP) was purchased from Sigma Chemical Co. (St. Louis, MO). 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1,5-bis-*O*- hexadecyl-*N*-succinyl-L-glutamate (DHSG) were purchased from Nippon Fine Chemical Co. Ltd. (Osaka, Japan). 1,2-distearoyl-*sn*-glycero-3- phosphatidyl- ethanolamine-*N*-PEG (PEG-DSPE) was purchased from NOF Corp. (Tokyo, Japan). Recombinant human serum albumin (rHSA) was given by Nipro Corp. (Osaka, Japan).

Sprague–Dawley (SD) rats (180–210 g) were purchased from Kyudou Co. (Kumamoto, Japan). SD rats were maintained in a temperature-controlled room with a 12-hr dark/light cycle and *ad libitum* access to food and water. All animal experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Kumamoto University.

### 2.2. Preparation of HbV

HbVs were prepared under sterile conditions as previously reported [17]. Briefly, the encapsulated Hb (38 g/dl) contained 14.7 mM of PLP as an allosteric effector to regulate $P_{50}$ to 25–28 Torr. The lipid bilayer was a mixture of DPPC, cholesterol, and DHSG at a molar ratio of 5/5/1, and PEG-DSPE (0.3 mol%). The HbVs were suspended in a physiological salt solution at [Hb] 10 g/dL, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and bubbled with $N_2$ for storage.

### 2.3. HbV labeling with $^{125}$I ($^{125}$I-HbV) and preparation of HbV solution

$^{125}$I-HbV was prepared by incubation of HbV with Na$^{125}$I (Piscataway, NJ, USA) in an Iodo-Gen (1, 3, 4, 6-tetrachoro-3, 6-diphenylglycoluri) tube for 30 min at room temperature. Thereafter, $^{125}$I-HbVs were isolated from free $^{125}$I by passage through a PD-10 column (GE Healthcare Bio-Sciences AB). The $^{125}$I-HbVs were then filter-sterilized (pore size, 450 nm) to remove aggregates. Over 97% of iodine was bound to internal Hb in HbV. Three different HbV suspensions were prepared: non-radiolabeled HbV and $^{125}$I-HbV for HS model rats; and, $^{125}$I-HbV for non-HS model rats. All suspensions were mixed with rHSA to adjust the albumin concentration of the vesicle-suspension medium to 5 g/dL. Under these conditions, the colloid osmotic pressure of the suspension is kept constant at approximately 20 mm Hg [17].

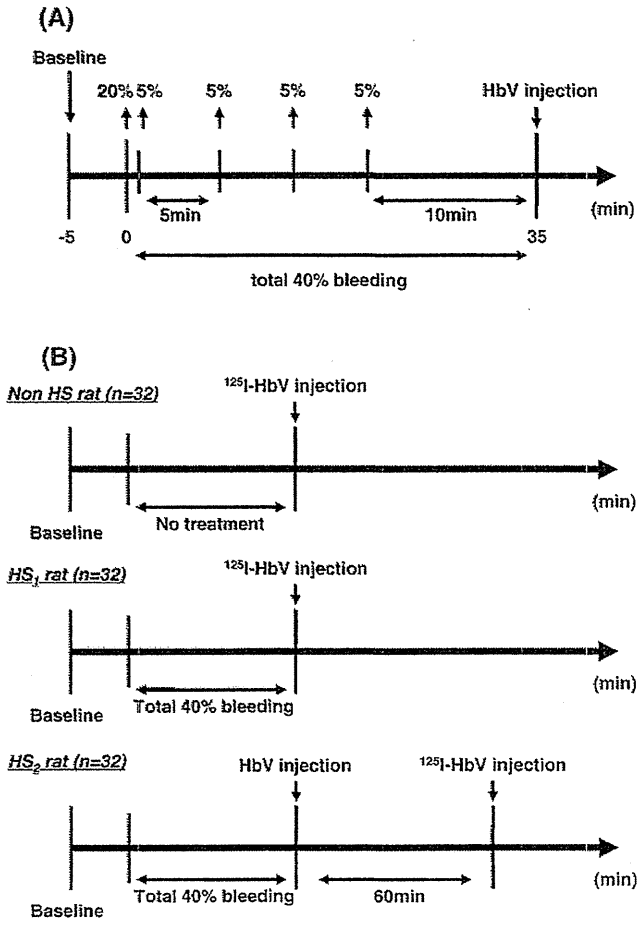


Fig. 1. Scheme illustrating (A) the method of preparing HS model and (B) the pharmacokinetic experimental protocol.

### 2.4. Preparation of hemorrhagic shock model rats

SD rats were anesthetized with pentobarbital. Subsequently, polyethylene catheters (PE 50 tubing, outer diameter equal to 0.965 mm, and inner diameter equal to 0.58 mm; Becton Dickinson and Co., Tokyo, Japan) containing saline and heparin were introduced into the left femoral artery for infusion and blood withdrawal, and into the right femoral artery to monitor mean arterial pressure (MAP), heart rate (HR) and pressure pulse. HS was induced by removal of 40% of total blood volume (22.4 mL/kg). Systemic blood volume was estimated to be 56 mL/kg [9]. Briefly, blood was removed as follows: constant withdrawal of 20% (1 mL/min); and, removal of 5% (0.5 mL/min) of total blood volume four times over a period of 5 min. After the last withdrawal of blood, animals were allowed to recover for 10 min at room temperature (Fig. 1A).

### 2.5. Measurement of cardiocirculatory dynamic parameters and plasma clinical chemistry

Ten HS rats were resuscitated by infusion of either isovolemic HbV (1400 mgHb/kg, 1 mL/min; $n=5$) or saline (22.4 mL/kg, 1 mL/min; $n=5$). After resuscitation, the polyethylene catheter of right femoral

artery was removed and the monitoring finished. Blood samples were collected immediately prior to blood withdrawal, after bleeding, and 7 days after resuscitation. Immediately after withdrawal, the pH of all blood samples was measured using a pH meter (HORIBA, Kyoto, Japan). An aliquot of blood was treated with perchloric acid to remove blood proteins for lactate analysis. The remaining blood was centrifuged (3000 g, 5 min) to obtain plasma for analysis of albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyltransferase (γ-GTP), amylase, lipase, total-cholesterol, free-cholesterol, HDL-cholesterol, phospholipids, urea nitrogen (BUN), creatinine (CRE) and iron (Fe). All plasma samples were stored at −80 °C prior to analysis by a commercial clinical testing laboratory (SRL, Tokyo, Japan).

### 2.6. The pharmacokinetic experimental protocol

All rats were given water containing 5 mM sodium iodide (NaI) for the duration of the experiment to avoid specific accumulation in the glandula thyreoidea. Ninety-six male SD rats were anesthetized with pentobarbital and polyethylene catheters were inserted into the left femoral artery. The rats were divided into three groups. Two groups (one-injection group ($HS_1$), $n=32$; two-injection group ($HS_2$), $n=32$) of rats underwent experimental HS. Both the non-HS (non-HS group, $n=32$) and $HS_1$ rats were injected with the $^{125}$I-HbV suspension (1400 mg Hb/kg, 1 mL/min). 1 h after the first injection of non-labeled HbV suspension (1400 mg Hb/kg, 1 mL/min), the $HS_2$ rats received the $^{125}$I-HbV suspension (1400 mg Hb/kg, 1 mL/min). The volume of the infused fluid was identical to the volume of blood withdrawn to induce HS (22.4 mL/kg) (Fig. 1B). In each group of rats, five rats were randomly selected to undergo the plasma concentration test. The rats were anesthetized using ether, blood samples were collected at multiple time points after the $^{125}$I-HbV injection (3 min, 10 min, 30 min, 1 h, 6 h, 12 h, 24 h, 48 h, 72 h) and the plasma was separated by centrifugation (3000 g, 5 min). Degraded HbVs and free $^{125}$I were removed from plasma by centrifugation in 1% bovine serum albumin and 40% trichloroacetic acid (TCA). Three rats in each treatment group ($n=27$/group) were euthanized at each time point (3 min, 30 min, 1 h, 2 h, 6 h, 12 h, 24 h, 48 h, 72 h). The organs were excised (kidney, liver, spleen, lung, heart), rinsed with saline, and weighed. The levels of $^{125}$I radiation in the excised organs, urine and feces, collected at fixed intervals (2 h, 6 h, 12 h, 24 h, 48 h, 72 h in each group; urine samples at 2 h and 6 h were not collected in the $HS_1$ group because of the reduction of renal function) using metabolic cages, were determined using a γ-counter (ARC-5000, Aloka, Tokyo, Japan). A two-compartment model was used to determine the pharmacokinetic parameters after HbV administration. Pharmacokinetic parameters were estimated by curve-fitting using MULTI, a normal least-squares program. Pharmacokinetic analysis was described in the supplementary materials and methods in detail.

### 2.7. Interspecies scaling of pharmacokinetic parameters

Allometric relationships between various pharmacokinetic parameters ($P$) and body weight ($W$) were plotted on a log–log scale. Linear regression of the logarithmic values was calculated using the least-squares method using Eq.(1) to obtain the coefficient ($\alpha$) and exponent ($\beta$) values [18].

$$P = \alpha W^{\beta} \quad (1)$$

To calculate pharmacokinetic parameters, such as $V_1$ and CL for humans using Eq.(1), the total blood volume of rabbits and humans were assumed to be 5.7% and 7% of body weight, respectively [11]. The CL of rabbits and humans, which were previously reported by Sou et al., were calculated using $V_1$ and half-life (see supplementary methods Eq. (8)) [11]. The $V_1$ and CL of mice were 1.75 mL and 0.116 mL/h, respectively (*submitted in separate manuscript*). After prediction of $V_1$ and CL for humans using Eq.(1), the half-life for human was estimated.

### 2.8. Data analysis

A two-compartment model was used in the pharmacokinetic analyses after HbV administration. Each parameter was calculated by fitting using MULTI, a normal least-squares program [19]. Data are means ± SD for the indicated number of animals. Dunnett's test was used for comparison of baseline and subsequent values within each group. Significant differences among each group were examined using a Student's $t$-test. A probability value of $p<0.05$ was considered to indicate statistical significance.

## 3. Results

### 3.1. Systemic response to hemorrhagic shock

MAP of the SD rats before induction of HS by bleeding was 79.9 ± 4.3 mm Hg; MAP declined significantly to 30.1 ± 1.8 mm Hg after induction of HS. During blood withdrawal to induce HS, MAP remained less than 40 mm Hg (hypotension) for 30 min. After resuscitation with HbV, MAP immediately increased to 81.4 ± 5.6 mm Hg, which was similar to MAP measured at baseline, and apparently HbV did not induce hypertension (Fig. 2A). Average HR before and after hemorrhage was 369 ± 44 and 290 ± 43 beats/min, respectively. However, during the interval between 10-min-post-bleeding and initiation of resuscitation, HR increased slightly (Fig. 2B). Pressure pulse, which reflects cardiac

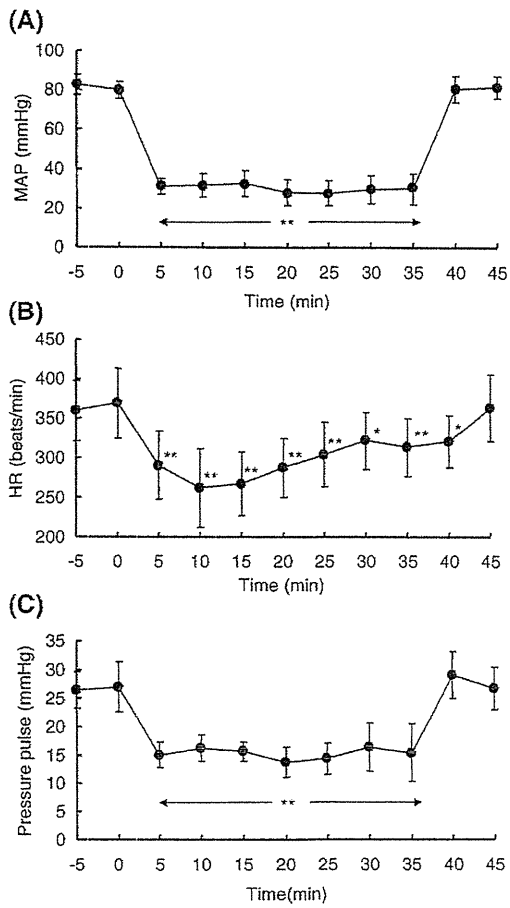


Fig. 2. Change in (A) mean arterial pressure (MAP), (B) heart rate (HR), (C) pressure pulse from −5 min (5 min before bleeding) to 45 min (10 min after resuscitation). Each point represents the mean ± SD ($n=5$). *<0.05 or **<0.01 vs. 0 min.

**Table 1**
Plasma clinical chemistry analyses after withdrawal of 40% of total blood volume and 7 days after administration of HbV or saline.

| | | | 7 days after resuscitation | |
|---|---|---|---|---|
| | Baseline | After bleeding | HbV | Saline |
| Albumin, g/dL | 3.38±0.13 | 2.96±0.15 | 3.34±0.28 | 3.32±0.26 |
| Phospholipids, mg/dL | 124.4±15.4 | 103.6±12.0 | 139.2±12.9 | 129.4±14.2 |
| Total-cholesterol, mg/dL | 75.0±11.3 | 59.4±8.8 | 80.8±10.3 | 75.2±9.5 |
| Free-cholesterol, mg/dL | 20.2±2.0 | 16.2±1.9 | 20.2±2.8 | 18.8±2.3 |
| BUN, mg/dL | 13.1±1.9 | 13.7±0.6 | 16.0±1.0 | 15.6±2.0 |
| Creatinine, mg/dL | 0.17±0.01 | 0.29±0.02[a] | 0.19±0.02 | 0.19±0.01 |
| AST, IU/L | 74.0±9.3 | 65.6±8.7 | 119.3±28.8[a] | 101.8±11.4[b] |
| ALT, IU/L | 42.4±4.7 | 37.2±2.4 | 57.0±9.9 | 56.0±9.7 |
| γ-GTP, IU/L | <2.8 | <2.8 | <2.8 | <2.8 |
| HDL-cholesterol,mg/dL | 23.0±3.2 | 19.2±2.6 | 28.2±1.5 | 25.4±2.9 |
| Fe, μg/dL | 153.5±36.5 | 171.4±54.7 | 148.7±22.5 | 123.7±66.3 |
| Lipase, U/L | 6.8±0.4 | 6.8±0.8 | 5.4±0.5 | 5.8±0.4 |
| Amylase, U/L | 669.8±28.5 | 638.8±34.0 | 604.0±67.7 | 624.4±66.0 |

The values are mean±S.D. ($n=3$–5). [a]$p<0.05$, [b]$p<0.01$ vs. baseline.

**Table 2**
Pharmacokinetic parameters of HbV in non-HS rats, $HS_1$ and $HS_2$ rats.

| | Non-HS | $HS_1$ | $HS_2$ |
|---|---|---|---|
| $t_{1/2\alpha}$, h | 5.3±3.9 | 0.58±0.05[a] | 5.0±1.7 |
| $t_{1/2\beta}$, h | 30.6±4.0 | 18.1±3.7[b] | 32.4±1.1 |
| $C_0$, % of dose/mL | 9.1±0.2 | 9.9±0.5 | 9.8±1.9 |
| $V_1$, mL | 10.9±0.2 | 10.1±0.5 | 10.4±2.1 |
| $V_2$, mL | 5.5±1.2 | 11.9±1.6[b] | 5.9±2.0 |
| Vss, mL | 16.7±1.1 | 21.6±2.3[b] | 17.0±3.1 |
| ke, $\times 10^{-3}$ $min^{-1}$ | 0.70±0.06 | 1.4±0.1[b] | 0.60±0.07 |
| $k_{12}$, $\times 10^{-3}$ $min^{-1}$ | 0.41±0.10 | 6.6±2.3[b] | 7.4±3.5[b] |
| $k_{21}$, $\times 10^{-3}$ $min^{-1}$ | 0.69±0.23 | 5.3±1.2[b] | 13.9±4.7[b] |
| AUC, h% of dose/mL | 210.3±22.9 | 126.5±12.8[b] | 259.0±48.6 |
| CL, mL/hr | 0.47±0.04 | 0.80±0.08[b] | 0.40±0.09 |
| $k_{12}/k_{21}$ | 0.60±0.14 | 1.2±0.1[b] | 0.55±0.13 |

$t_{1/2\alpha}$, the distribution-phase half-life; Vss, the steady-state distribution volumes; $k_{12}$, the rate constants for the transfer from $V_1$ to $V_2$; $k_{21}$, the rate constants for the transfer from $V_2$ to $V_1$.
Values are means±S.D. ($n=5$). [a]$p<0.05$, [b]$p<0.01$ vs. non-HS rats.

output, was 26.4±3.2 and 15.3±3.1 mm Hg before and after hemorrhage, respectively. After resuscitation, pressure pulse returned to baseline values (Fig. 2C). Acute bleeding resulted in a non-significant decrease in pH from 7.55±0.06 to 7.51±0.05, and a significant increase in lactate from 21.4±4.0 to 62.8±17.5 mg/dL ($p<0.01$). In addition, skin pallor and hyperpnea developed during hypotension. These results indicate that acute blood withdrawal in rats is an adequate model of clinical HS.

### 3.2. Plasma clinical chemistry

All rats survived the complete experimental protocol. Table 1 summarizes the clinical chemistry parameters measured in plasma. Immediately after hemorrhage, the concentration of CRE in plasma, which reflects kidney function, was two-fold greater than the concentration at baseline ($p<0.01$). In contrast, BUN, which is also a marker of kidney function, and other parameters were not affected by hemorrhage. We also performed clinical chemistry analyses in plasma that was collected from HS rats after 7 days of treatment with HbV or saline. Only AST increased significantly, reaching 119.3±28.8 IU/L in the group given HbV ($p<0.01$ vs. baseline) and 101.8±11.4 IU/L in the group administered saline ($p<0.05$ vs. baseline). CRE decreased from 0.29±0.02 mg/dL to 0.19±0.02 and 0.19±0.01 mg/dL in the HbV and saline groups, respectively. Although HbV contains many lipids and iron, lipid-related parameters, such as total-cholesterol, free-cholesterol, HDL-cholesterol, phospholipids, and Fe did not differ between the HbV- and saline-treated rats. These results show that neither organ function nor serum parameters were affected by HbV administration, despite the temporary disruption of circulatory function by HS.

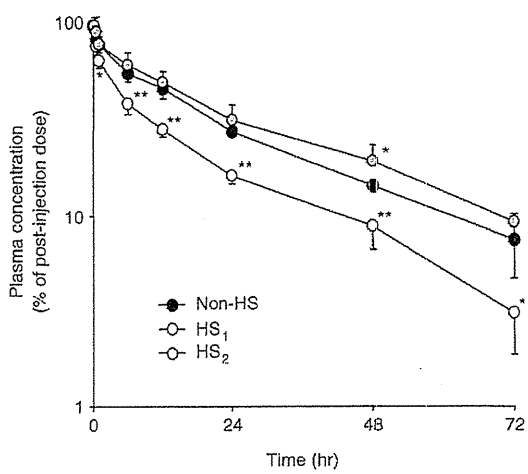


**Fig. 3.** Relative plasma concentration of $^{125}$I-HbV after administration of 1400 mgHb/kg via injection of non-HS, $HS_1$ or $HS_2$. Each point represents the mean±SD ($n=5$). *<0.05 or **<0.01 vs. non-HS rats.

### 3.3. Plasma concentration

The fate of the $^{125}$I-HbV administered to non-HS, $HS_1$ and $HS_2$ rats was evaluated by determination of residual TCA-precipitable radioactivity in plasma. Fig. 3 shows the time course of the plasma concentration of $^{125}$I-HbV in non-HS, $HS_1$ and $HS_2$ rats and Table 2 lists the pharmacokinetic parameters for these groups. The elimination-phase half-life ($t_{1/2\beta}$) of $^{125}$I-HbV in $HS_1$ rats was reduced significantly–by 0.66-fold–compared with the $t_{1/2\beta}$ of $^{125}$I-HbV in non-HS rats. In addition, the AUC was decreased, while the elimination rate constant (ke) and clearance (CL) were increased in $HS_1$ rats compared with non-HS rats. Notably, the initial plasma concentration ($C_0$) and distribution volume of the central compartment of HbV ($V_1$) were identical between the two groups, whereas the distribution volume of the peripheral compartment ($V_2$) in $HS_1$ rats was nearly two-fold greater than non-HS rats. Interestingly, the plasma concentration of $^{125}$I-HbV and pharmacokinetic parameters, except $k_{12}$ and $k_{21}$, of $HS_2$ rats were similar to the results observed in non-HS rats. Furthermore, $V_1$ (approximately 10 mL) was nearly equivalent to blood volume (56 mL/kg).

### 3.4. Organ distribution of HbV

Fig. 4 shows the time course of organ distribution of $^{125}$I-HbV (% of ID) after $^{125}$I-HbV administration. Shortly after $^{125}$I-HbV administration, the amount of $^{125}$I-HbV was significantly greater in the kidney, lung and heart of $HS_1$ rats compared with non-HS rats; however, there were no differences in distribution of $^{125}$I-HbV in other organs between groups. As time passed, HbV was distributed to all observed organs (kidney, liver, spleen, lung and heart) of $HS_1$ rats, compared with the non-HS rats. In contrast, less $^{125}$I-HbV was distributed in the liver, spleen, lung, and heart of the $HS_2$ rats compared with both non-HS and $HS_1$ rats. However, past 12 h after administration of HbV, $HS_2$ rats exhibited the greatest distribution of $^{125}$I-HbV in kidney and liver. Furthermore, during the first 12 h after HbV administration, the total amount of $^{125}$I-HbV distributed in all observed organs (kidney, liver, spleen, lung and heart) was greatest in $HS_1$ rats, while after 12 h post-administration of HbV, the greatest amount of total radioactivity was observed in the $HS_2$ rats.

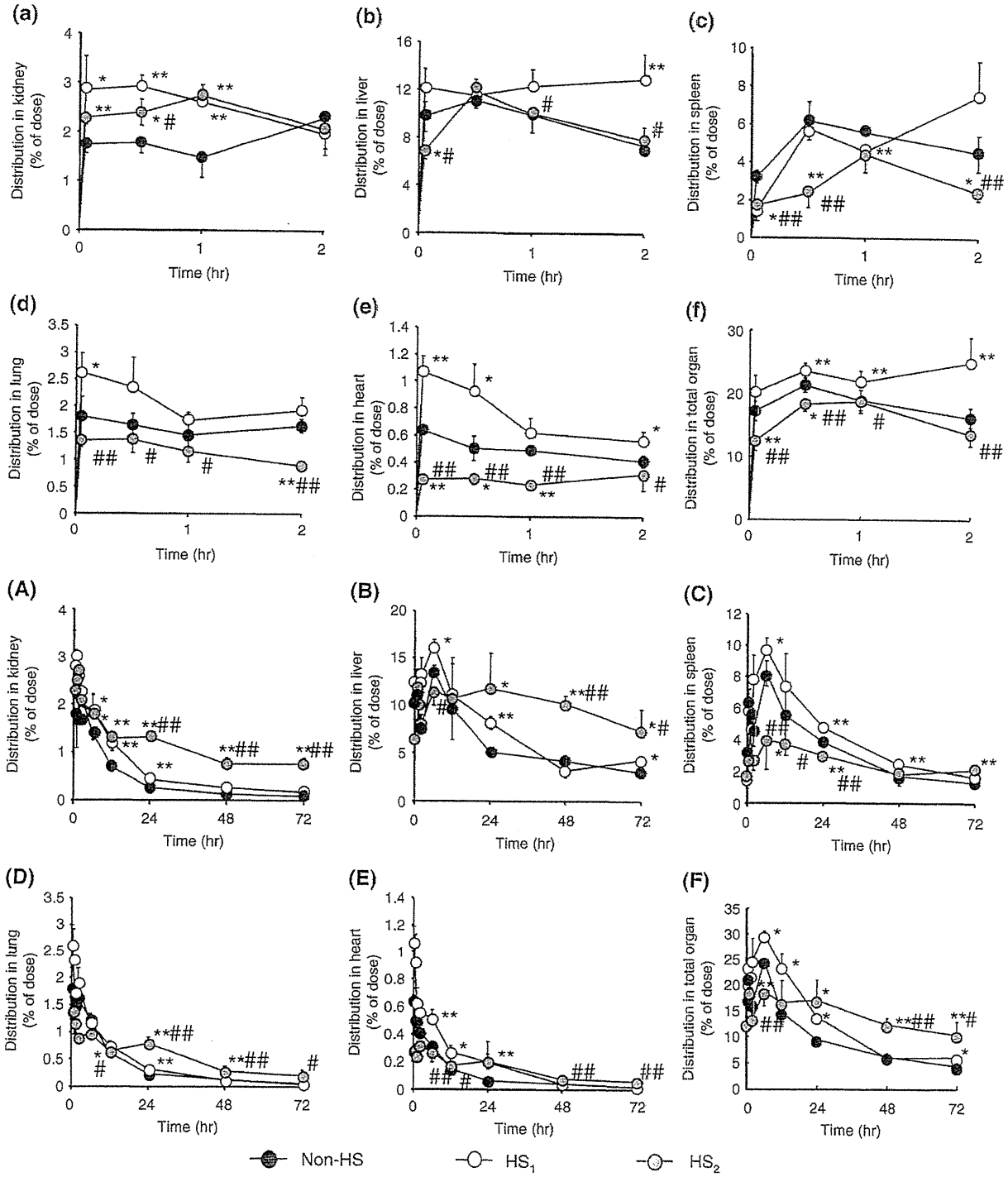


Fig. 4. Distribution of HbV in kidney (a), liver (b), spleen (c), lung (d), heart (e), total organ (f) after injection of non-HS or $HS_1$ or $HS_2$ from the time of administration until 2 h post-administration and in kidney (A), liver (B), spleen (C), lung (D), heart (E), total observed organ (the sum of kidney, liver, spleen, lung and heart) (F) from the time of administration until 72 h post-administration. Each point represents the mean ± SD ($n=3$). *<0.05 or **<0.01 vs. non-HS rats. #<0.05 or ##<0.01 vs. $HS_1$.

The tissue distribution of $^{125}$I-HbV in non-HS, $HS_1$ and $HS_2$ rats was also evaluated based on both percentage of the infused dose/organ weight (ID/g organ) and the tissue-to-plasma partition coefficient (*Kp*), which can be used to compare the kinetics of plasma and tissue HbV concentrations. Table 3 shows the ID/g organ and *Kp* data for the kidney, liver, spleen, lung and heart at 1 h, 12 h and 72 h after administration of HbV. The *Kp* data in spleen became more than 1 (mL/g of tissue), but that in other organ less than 1 (mL/g of tissue) at 1 h and 12 h. At 72 h post-administration of HbV, the % of ID and % ID /g organ values were greatest, the *Kp* value was lowest, and there was an increase in the plasma concentration of HbV in the $HS_2$ group.

### 3.5. Urinary and fecal excretion of HbV

To identify the pathways by which HbV is excreted, the amount of HbV in urine and feces was measured in non-HS, $HS_1$ and $HS_2$ rats (Fig. 5). We found that HbV was excreted primarily in the urine of all groups (58.9 ± 5.6, 65.5 ± 10.5 and 44.2 ± 2.9% of ID for non-HS, $HS_1$

**Table 3**
Dose/gram of organ weight (dose/g) and tissue-to-plasma partition coefficient (Kp) for non-HS rats, $HS_1$ and $HS_2$ rats.

| | | Dose/organ weight (dose/g) | | | Kp (mL/g of tissue) | | |
|---|---|---|---|---|---|---|---|
| | | Non-HS | HS1 | HS2 | Non-HS | HS1 | HS2 |
| 1 h | | | | | | | |
| | Kidney | 0.86 ± 0.18 | 1.32 ± 0.09[b] | 1.65 ± 0.14[b,c] | 0.14 ± 0.04 | 0.32 ± 0.02[b] | 0.37 ± 0.01[b,c] |
| | Liver | 1.15 ± 0.13 | 1.09 ± 0.14 | 0.93 ± 0.44[a] | 0.19 ± 0.01 | 0.26 ± 0.02[b] | 0.21 ± 0.03[c] |
| | Spleen | 7.87 ± 0.56 | 7.11 ± 0.42 | 9.30 ± 0.44[a,d] | 1.30 ± 0.09 | 1.72 ± 0.10[b] | 2.09 ± 0.19[b,c] |
| | Lung | 1.47 ± 0.42 | 1.48 ± 0.11 | 1.11 ± 0.16[c] | 0.24 ± 0.06 | 0.36 ± 0.06[a] | 0.25 ± 0.03[c] |
| | Heart | 0.81 ± 0.07 | 0.90 ± 0.17 | 0.52 ± 0.10[b,c] | 0.13 ± 0.01 | 0.22 ± 0.03[b] | 0.12 ± 0.02[d] |
| 12 h | | | | | | | |
| | Kidney | 0.37 ± 0.05 | 0.64 ± 0.14[a] | 0.78 ± 0.09[b] | 0.16 ± 0.02 | 0.29 ± 0.07[a] | 0.25 ± 0.03[b] |
| | Liver | 0.87 ± 0.11 | 1.07 ± 0.31 | 1.29 ± 0.67 | 0.39 ± 0.05 | 0.47 ± 0.02[a] | 0.39 ± 0.16 |
| | Spleen | 7.23 ± 1.32 | 10.9 ± 2.69[a] | 6.49 ± 1.27[c] | 3.19 ± 0.61 | 4.89 ± 0.72[a] | 2.01 ± 0.08[a,d] |
| | Lung | 0.54 ± 0.08 | 0.71 ± 0.07[a] | 0.55 ± 0.10[c] | 0.24 ± 0.04 | 0.32 ± 0.11 | 0.17 ± 0.01[a,c] |
| | Heart | 0.24 ± 0.07 | 0.39 ± 0.09[a] | 0.30 ± 0.04 | 0.10 ± 0.03 | 0.18 ± 0.02[a] | 0.09 ± 0.01[d] |
| 72 h | | | | | | | |
| | Kidney | 0.05 ± 0.01 | 0.08 ± 0.01[b] | 0.47 ± 0.15[b,d] | 0.21 ± 0.07 | 0.63 ± 0.01[b] | 0.52 ± 0.10[b] |
| | Liver | 0.33 ± 0.07 | 0.40 ± 0.07 | 0.75 ± 0.26[a,c] | 1.29 ± 0.28 | 3.12 ± 0.73[b] | 0.81 ± 0.15[a,d] |
| | Spleen | 1.52 ± 0.45 | 1.11 ± 0.16 | 1.75 ± 0.41[c] | 5.74 ± 0.23 | 7.06 ± 0.11[b] | 1.93 ± 0.39[b,d] |
| | Lung | 0.05 ± 0.01 | 0.05 ± 0.01 | 0.18 ± 0.07[b,d] | 0.18 ± 0.02 | 0.36 ± 0.09[a] | 0.19 ± 0.01[c] |
| | Heart | 0.03 ± 0.01 | 0.02 ± 0.01 | 0.09 ± 0.03[b,d] | 0.13 ± 0.02 | 0.19 ± 0.03[a] | 0.10 ± 0.01[d] |

Values are means ± S.D. ($n = 3$).
[a]$p < 0.05$, [b]$p < 0.01$ vs. non-HS rats, [c]$p < 0.05$, [d]$p < 0.01$ vs. $HS_1$ rats.

and $HS_2$ rats, respectively), while radioactivity was virtually undetectable in feces (2.0 ± 0.5, 3.2 ± 1.3 and 2.8 ± 0.5% of ID, for non-HS, $HS_1$ and $HS_2$ rats, respectively). Urinary and fecal levels of radioactivity were not significantly different between the non-HS and $HS_1$ groups. Meanwhile, urinary radioactivity in $HS_2$ rats was significantly less than that in non-HS and $HS_1$ rats, and the half-life of HbV was significantly longer in $HS_2$ rats compared with the other groups.

### 3.6. Prediction of human pharmacokinetics

As mentioned in the "*Plasma concentration*" section, we found that $V_1$ was equivalent to arteriovenous blood flow. To predict the CL in humans, we examined the allometric relationship between $V_1$ and body weight in mice, rats, rabbits and humans. As shown in Fig. 6A, there was a good correlation between $V_1$ and body weight, with $r^2$ values of 0.99.

Using the relationship observed for both mice and rats, the CL of HbV in humans was predicted to be approximately 36.6 mL/h. In addition, we applied the rabbit [11] and human CL values for HbV to this relationship. We calculated the human CL (47.2 mL/h) using $k_e$, which is from Sou *et al.* [11], and our estimated $V_1$. These values fit our allometric equation well (Fig. 6B). The half-life of HbV in humans was extrapolated to be approximately 96 h.

## 4. Discussion

HbV is a cellular-type, artificial oxygen carrier that consists of a highly concentrated Hb solution coated with a phospholipid bilayer membrane. Recent findings have demonstrated that HbV restores tissue and plasma oxygenation [9], and improves survival [20] in HS animal models. To exert these pharmacological effects and enhance oxygen-carrying capacity, HbV, or any other RBC substitute, must have a long half-life in circulation ($t_{1/2}$; free Hb and PEG-Hb in rats for 1.5 and 10.9 h, respectively [21,22], while RBC in human for up to 60 days [23]).

In the present study, the elimination-phase half-life ($t_{1/2\beta}$) of $^{125}$I-HbV in the $HS_1$ rats was shorter than that in non-HS rats. This result does not seem to support the therapeutic use of HbV, because the most important determinant of HbV efficacy is HbV-derived Hb concentration in blood. However, the shorter half-life in the $HS_1$ group may reflect transfer of HbV from arteriovenous blood to the organ capillary beds, thereby normalizing distribution of blood to the organs after bleeding. This scenario would result in an apparent reduction in HbV in arteriovenous circulation. Consistent with this hypothesis, HbV has been shown to both reach microcirculation in critically ischemic peripheral tissue and promote recovery from capillary pathological hemodynamic conditions [24]. In addition, the results of the present study also support our hypothesis: 1) the initial plasma concentration of HbV ($C_0$) and the amount of HbV eliminated in $HS_1$ rats were similar to the values observed in non-HS rats, such that the amount of residual HbV in non-HS and $HS_1$ rats were similar; 2) the time course of HbV distribution in the kidney, liver, spleen, lung and heart in the $HS_1$ rats was significantly greater than that of the non-HS rats; and, 3) the plasma concentration of $^{125}$I-HbV and pharmacokinetic parameters in the $HS_2$ rats were similar to the values observed in the non-HS rats.

For HbV to keep long circulating retention, the enhanced tissue distribution of HbV is a concern, as it may reflect both increased scavenging of HbV by cells of the mononuclear phagocyte system (MPS), such as Kupffer cells, red pulp zone splenocytes, and mesangial cells [7], and functional changes in MPS after HS. However, alterations in HbV, such as PEG-modification and controlled particle size (250 nm), suppress scavenging by the MPS relative to scavenging of Hb encapsulated in other liposomes [21,25]. Moreover, MPS scavenging of liposomal preparations is influenced by the dose administered [26], while mild hemorrhage does not affect phagocytic activation of Kupffer cells [27]. Therefore, because we used a high dose of HbV in

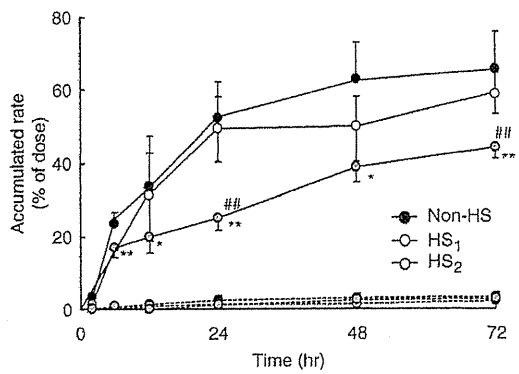


**Fig. 5.** Time-dependence of urinary and fecal radioactivity after administration of $^{125}$I-HbV to non-HS or $HS_1$ or $HS_2$. The solid line represents urinary excretion, and the dotted-line represents fecal excretion. Each point represents the mean ± SD ($n = 3$). *<0.05 or **<0.01 vs. non-HS rats. ##<0.01 vs. $HS_1$.

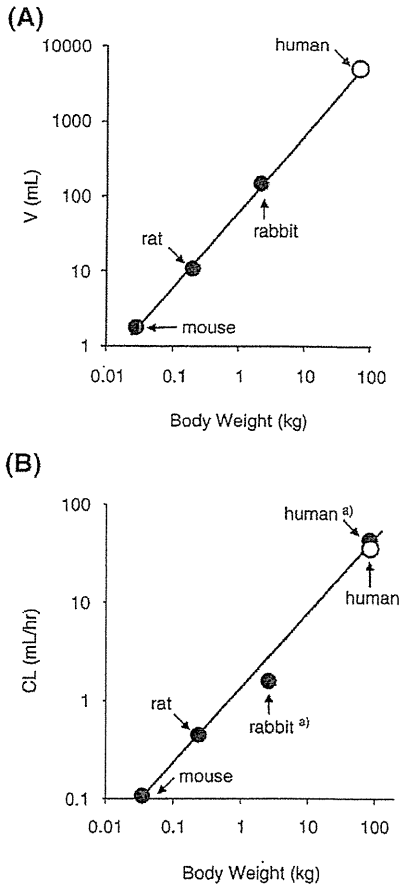


**Fig. 6.** Allometric relationships between body weight and distribution volume ($V_1$) (A) and body weight and clearance (CL) (B). The linear regression of the logarithmic values was calculated using the least-squares method (A, $y = 61.48 \times^{1.027}$, $r^2 = 0.99$; B, $y = 1.466 \times^{0.764}$, $r^2 = 0.984$). The extrapolated human values based on a body weight of 70 kg (open circle) are also shown. [a]Data from reference [11] for Rabbit, and we calculated the human CL using $k_e$, which is from Sou *et al.*[11], and our estimated $V_1$.

this study, it is unlikely that scavenging of HbV by MPS contributed significantly to the large distribution of HbV to organs during HS. On the contrary, HbV did not induce hypertension just after resuscitation as shown in Fig. 2, which is contrast to the cell-free Hb-based oxygen carriers such as crosslinked Hb and polymerized Hbs [28,29]. This indicates that HbV would not induce vasoconstriction and the blood flow distributes promptly to the capillary bed in all the organs.

In addition, we also were concerned that repeated administration of HbV would shorten circulation half-life by induction of the ABC phenomenon. However, because the half-life of HbV was longer in the $HS_2$ group compared with the $HS_1$ group and was not different from the half-life in non-HS rats, the ABC phenomenon did not occur nor did it contribute significantly to the pharmacokinetics of HbV. The dose and dosing interval used in the present study likely prevented induction of the ABC phenomenon. Ishida et al. reported that a low dose and dosing interval of approximately 5 days induced the ABC phenomenon [16]. Furthermore, anti-PEG IgM, which elicits a response by the spleen, plays an essential role in induction of the ABC phenomenon [30,31]. In fact, anti-HbV IgM was detected at 5 days after HbV administration to non-HS rats at a dose of 0.1 mg/kg (low dose), but not detected at 1 h after HbV administration to HS rats at a dose of 1400 mg/kg (data not shown). Therefore, acquisition of immune tolerance and inhibition of anti-PEG IgM production in the present study by use of a high dose and short interval seemed to prevent induction of the ABC phenomenon. In contrast, phagocytic activity, which was studied by measurement of carbon clearance, reportedly was transiently enhanced 3–7 days after administration of HbV at a dose of 2000 mg/kg body weight [32]. Therefore, the altered HbV pharmacokinetics observed in the $HS_2$ rats might be due to the longer dosing interval. However, in a clinical setting, the dosing interval for treatment of HS would be less than 1 day, so that the effect of phagocytic activity on HbV pharmacokinetics would be minimal.

From the viewpoint of future clinical applications, the allometric prediction of human pharmacokinetics based on data obtained from animal studies–so called, "animal scale-up"–is important for determination of optimal doses and intervals [33]. In the present study, we used an allometric equation that is generally used in animal scale-up to extrapolate the half-life of HbV in humans to be approximately 96 h In contrast, based on half-life and % ID values obtained from pharmacokinetic studies of HbV in rats and rabbits, Sou et al. predicted that the half-life of HbV in healthy humans is approximately 72 h [11]. Differences in predicted HbV half-life may be due to differences in experimental conditions, such as animal species, radiolabeled form and the analytical approach. In fact, the half-life of liposomal doxorubicin (Doxil formulation) in rats and humans is 35 h and 56–90 h, respectively [34]. Furthermore, the half-life of liposomal preparations is 2–3-fold greater in humans than in rats [34]. Therefore, the half-life of HbV in humans seems to be expected 3–4 days from our and previous study [11,34]. These results suggest that HbV functions as an oxygen carrier temporarily until a blood transfusion is available or until autologous blood is recovered after a massive hemorrhage.

## 5. Conclusion

The pharmacokinetic properties of HbV differed among non-HS, $HS_1$ and $HS_2$ rats. To the best of our knowledge, this is the first study to examine changes in the long-term pharmacokinetic characteristics of HbV resulting from a pathological condition and repeated administration using a rat model of HS induced by massive hemorrhage. Our results support that HbV efficacy in HS, which is long enough to meet oxygen-delivery demands until autologous blood volume and oxygen-carrying capacity are restored. Based on the pharmacokinetics of HbV described in this paper, we conclude that HbV might be a useful artificial oxygen carrier during HS.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jconrel.2009.02.009.

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