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

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

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

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

EXPERIMENTAL

Preparation of HbV

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

Plasma expanders (water-soluble biopolymers)

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

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

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

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

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

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

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

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

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

Measurements

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

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

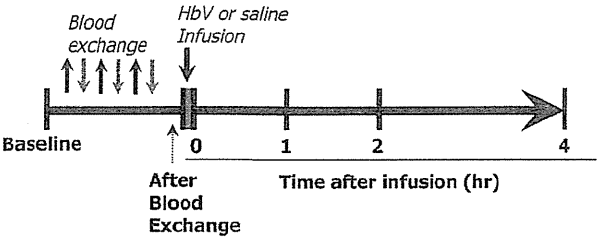

Figure 1. Time points for the measurements before and after 60% blood exchange with a plasma expander, and after infusion of HbV or saline.

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

RESULTS AND DISCUSSION

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

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

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

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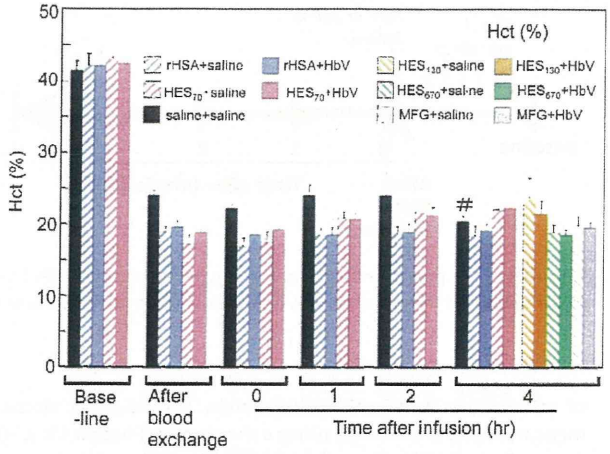

Figure 2. Changes in hematocrit (Hct) after blood exchange with rHSA and HES_{70} and for 4 hr after the subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other groups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

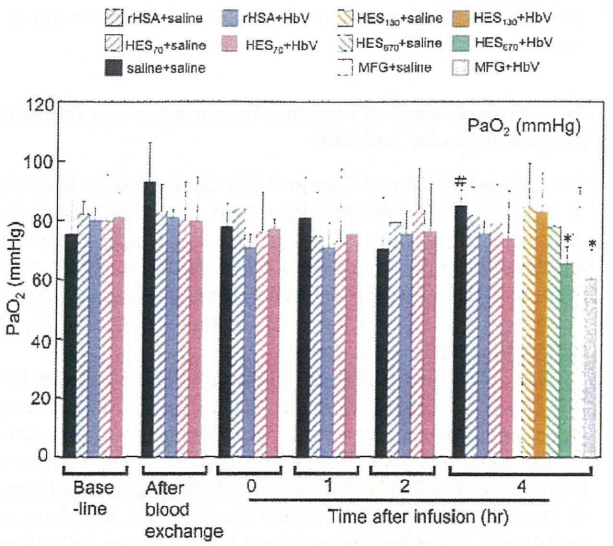

Figure 4. Changes in arterial blood oxygen partial pressure (PaO_2) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control.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

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

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

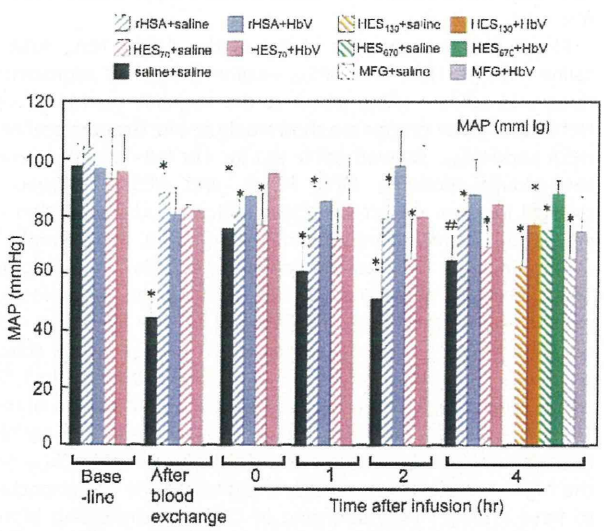

Figure 3. Changes in mean arterial pressure (MAP) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control. Data of other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

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

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

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

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

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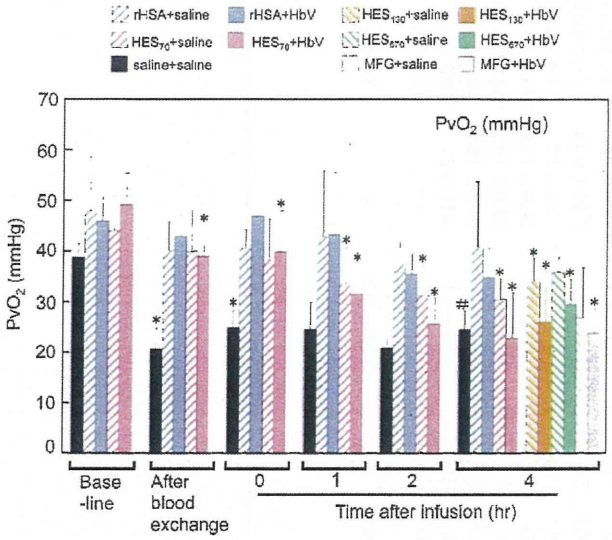

Figure 5. Changes in venous blood oxygen partial pressure (PvO_2) after blood exchange with rHSA and HES_{70} and for 4 hr after subsequent infusion of HbV or saline (the rHSA + HbV, rHSA + saline, HES_{70} + HbV, and HES_{70} + saline groups). The saline + saline group is also shown as a negative control.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

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

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

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

CONCLUSION

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

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原著

細胞型人工酸素運搬体の治験，第1相計画

Design of clinical trial (phase 1) for cellular artificial oxygen carrier

高折 益彦

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Abstract

We presented our protocol for the phase-1 clinical trial in this paper and appreciated to be given some criticisms by readers. This trial is separated into the first and second step. In the first step, it is scheduled that an artificial oxygen carrier, liposome encapsulated hemoglobin suspended in the physiological saline (HbV), of 10 ml, 50 ml, 100 ml, and 200 ml will be infused into 2 volunteers for each, respectively. In 400 E group of 6 volunteers, it is scheduled that 400 ml of volunteer's own blood will be withdrawn and then HbV of 400ml will be infused immediately. The blood will be stored at 4℃ and be transfused to the volunteer, if necessary. In the second step, 6 volunteers will be provided for 600 EG and 800 EG trial for each. In the 600 EG, blood exchange with HbV and HES (hydroxyethyl starch : 70 × 10^3 MW/0.55 DS) solution is scheduled as follows; Volunteer's own blood of 400 ml will be withdrawn and HbV of 400 ml and HES solution of 300 ml will be infused. At 20 min. after the above blood/HbV・HES exchange, volunteer's own blood of 200 ml will be withdrawn and HbV of 200 ml and HES solution of 150 ml will be infused. Blood/HbV・HES exchange in the 800 EG is scheduled as follows; Volunteer's blood of 400 ml will be withdrawn and HbV of 400 ml and HES of 300 ml will be infused as same as done in the 600 EG. Then 20 min. later, the same blood/HbV・HES exchange will be repeated. These volunteer's blood withdrawn at the first blood/HbV・HES exchange will be stored at 4 ℃ in a refrigerator for one week and will be transfused into each donor according to doctor's decision , if necessary. Tests which are accorded to the adverse effect report regulated by Japanese Ministry of Health, Labor and Welfare are scheduled to perform in this trial. And further tests on plasma ferritin level, lung functions (vital capacity, % FEV, arterial and central venous blood gas and pH), anaerobic metabolic parameters (plasma lactate, base excess), immunological tests (complement activities and antigen/antibody reaction) are added. Those tests will be performed before the HbV infusion, 20 min., 2 hr., 24 hr., and 7 days after the HbV infusion. This protocol is designed to minimize number of volunteers and to maintain their safety. Further it is designed to provide for the subsequent phase-2 of clinical trial according to the guideline by Japanese Ministry of Health, Labor and Welfare.

Keywords

cellular artificial oxygen carrier, clinical trial, phase 1, hemodilution, single infusion, blood exchange, liposome encapsulated hemoglobin

1．緒言

人工酸素運搬体，いわゆる人工赤血球の開発が試みられてからすでに半世紀になる[1-3]．そして人工酸素運搬体の候補となったものの中で，perfluorocarbonを酸素結合体として用いたものは種々の合併症[4,5]の発生から現在ではその開発が頓挫している．一方，ヘモグロビンを酸素結合体として開発されたhemoglobin based oxygen carrier（HBOC：Hemopure™ [Biopure]）は南アフリカ共和国で鎌形赤血球症や，強度の急性貧血治療での使用が認められている[6]．しかし同じ製品についてもアメリカ合衆国では家畜を対象とした使用にのみ限られていて，ヒトを対象とした臨床での使用は認められていない．わが国においてはChang[1]，Djordjevich[7]らによって始められたcellular HBOC, liposome encapsulated hemoglobin vesicle (HbV) の開発が進められた．そしてすでに20数年となり[8]，現在，その前臨床試験

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はほぼ完成した状態にある．またその製造に関するGMP（good manufacture practice）の完成も遠くない状態にある．したがって将来を見据えてIIbVの臨床試験，第1相の施行体系をデザインするのは決して時期尚早とは思えない．これらの状況を踏まえここにIIbVの臨床試験，第1相の施行計画を提示し，人工酸素運搬体の開発にたずさわる方々からのご批判を頂きたいと本稿を発表した．

2．治験計画

対象治験薬であるIIbVは一般治療薬と異なりその臨床使用量はヘモグロビン内包リポソーム量として少なくとも約200グラム（薬液量として約600ml）を必要とする．今回予定している治験は第1相治験であって治験薬の安全性に主眼がおかれる．そしてそれをさらに2段階に分割，第1段階においてはIIbVに対する安全性を中心に，第2段階では第2相治験の施行も視野に入れて投与量増加にともなう生体反応を観察することを目的として計画した．

施行時期　：　GMP承認時
施行施設　：　臨床試験，治験施行民間施設
実施方法

対象者　：　報道機関を介した募集に対して応募した20～60歳の健康成人男女（妊娠中，およびその可能性のある女性を除く）に本治験の目的，その医療・社会的意義，前臨床試験での安全性，有効性，治験施行場所・時期，手順，検査項目，治験薬投与方法，その他の治験操作，それにともなう保証，ならびに手当てなどを記載した説明書，治験参加への同意を口頭ならびに文書として説明する．そして治験への参加の同意を得た応募者に過去・現在の病歴，健康状態，アレルギー等に関する問診，一般理学所見（身長 > 155cm，体重 > 50kg，血圧測定，胸部聴打診，腹部触診等），胸部X線検査，心電図検査を施行する．そしてこれらの検査において一応治験適合と予測された治験対象者を選出，自宅待機を依頼する．次ぎにこれらの選出者について適宜，治験施設での検査を依頼し，呼吸機能検査（肺活量，1秒率），肝機能検査（総蛋白，アルブミン，総コレステロール，遊離脂肪酸，ビリルビン，sGPT，sGOT，γGPT，LDH，アルカリホスファターゼ，尿酸，血糖，プロトロンビン値，血漿リパーゼ値），腎機能（尿色調・量・比重・沈渣，尿糖，BUN，creatinine，ウロビリノゲン），血液一般（Hb値，Ht値，赤血球数，白血球数・分類，血小板数，PT，aPTT），血清電解質（Na，K，Cl），免疫機能検査（補体活性）を追加検査する．治験対象者はこれらの検査後帰宅し，再び自宅待機する．治験当局はこれらの検査，すなわち選択検査の結果について検討し治験適任者を選出する．そして治験対象者と選出された被験者を第1段階治験群，第2段階治験群に無作為的に割り当て，それぞれの被験者に治験施行予定日を通知して以下の治験へと移行する．なお治験実施日は選別検査日から9±2日とする．

A．第1段階治験

上記の対象者の中，14名を第1段階治験対象者として以下の5群に区分する．

1）10ml群（10SG※）2名
2）50ml群（50SG）2名
3）100ml群（100SG）2名
4）200ml群（200SG）2名
3）400mlE群（400EG※）6名

※SG：single HbV infusion group
※EG：blood/HbV exchange group

これら5群への治験施行は10SG，50SG ..... 400EGの順に施行し，10SGにおいて予測せざる不祥事象（adverse effect）が発生しないことを確認後に50SGを施行，そして順次予測せざる不祥事象が発生しないことを確認して最終的に400mlEGを施行する．

対象被験者は治験前日に施設に赴き，一般理学的検査を受け，施設内での安静，食事，睡眠をとる．治験薬投与当日には所定の検査，治験薬の投与を受け，施設内での安静，食事，睡眠をとる．治験薬投与後1日目には所定の検査を受け，身体的に異常がないことを確認して帰宅する．さらに治験薬投与7日後に施設に赴き所定の検査を受ける．そして異常がないことを確認して治験を終わる．

検査：治験前日検査，治験薬投与前検査，治験薬投与2時間後検査，治験薬投与24時間後検査，治験薬投与7日後検査

検査項目：

治験前日検査は一般理学的検査にて健康状態に異常がないことの確認に留める．

治験当日，およびそれ以後の検査項目は以下のごとくである．

（1）一般理学的検査
（2）循環器機能（血圧，脈拍数，12誘導心電図，CPK-M）
（3）呼吸機能検査（胸部X線，肺活量，1秒率，PaO_2，$PaCO_2$，pHa）
（4）肝機能検査（総蛋白，アルブミン，総コレステロール，遊離脂肪酸，ビリルビン，sGPT，sGOT，γGPT，LDH，アルカリホスファターゼ，尿酸，血糖，プロトロンビン値，血漿リパーゼ値，血漿乳酸値）
（5）腎機能（尿色調・量・比重・沈渣，尿糖，BUN，creatinine，ウロビリノゲン）
（6）血液一般（IIb値，IIt値，IIbVcrit※，白血球数・分類，血小板数，PT，aPTT，FDP）※単位血液量中のIIbVリポソーム粒子量（%）
（7）血清電解質（Na，K，Cl）
（8）免疫機能検査（補体活性，血清IIbV抗原／抗体反応）
（9）神経・運動検査（歩行，握力，腱反射，眼球運動，知覚機能，視力，聴力）
（10）消化機能（食欲，腹部自覚症状，便所見）

なお被験者からの血液採取量は1回当たり15～17ml，治験当日検査から，治験薬投与1週間後の検査用血液を含めて総量

100 ml 以下を目標とする．

治験薬投与

Ⅰ．10 SG治験

1）被験者は治験前日午後に治験施設に赴き，一般理学的検査にて健康状態に異常がないことを確認，過激な運動を避け同施設にとどまり，治験施設より提供される食事を摂り，その夜は施設に泊まる（Fig.1）．

2）治験薬投与当日の朝食は絶食とし一般問診にて健康感に異常がないことを確認する．

3）上記（1）～（10）の検査を受ける．

4）診察台上にて仰臥位をとり，静脈確保を行ない5％ブドウ糖加乳酸リンゲル液0.5 ml/kg/minの点滴を20分間受ける．

5）上記静脈路の側管にIIbV製品点滴回路を接続，20分で10 ml量の輸注を受ける．この時点で5％ブドウ糖加乳酸リンゲル液を生理食塩液に変更して0.2 ml/kg/minの点滴を受ける．

6）20分後に上記（1）～（10）の検査を受ける．この際HbV注入前に施行した検査に加えて血液中HbV濃度（IIbVcrit）測定用の採血（2 ml）を受ける．この血液は測定所定機関に送付する．

7）HbV注入2時間後にHbV注入20分後に施行した同一項目の検査を受ける．

この間，0.2 ml/kg/minの生理食塩液の点滴は持続される．

8）検査後に異常ないことを確認して静脈路は抜去し，摂食，治験施設内にて安静を保ち，宿泊する．

9）翌日，被験者は朝食を絶食とする．

10）治験薬投与24時間後に上記（1）～（10）の検査，ならびにIIbVcrit測定用採血を受け，自覚的，他覚的に異常を認めない場合は2時間の安静後，昼食を摂って帰宅する．

11）被験者は一週後，治験施設に赴き，上記（1）～（10）の検査，ならびにHbVcrit測定用採血を受ける．

Ⅱ．50SG治験

10 SG治験を施行してその結果を解析して特に認めるべき合併症，副作用がなかった場合には50 SG治験に移行する．被験者は10 SG治験と同様にIIbV注入前日の検査を受け，施設内安静，食事，就眠をとる．またIIbV投与当日の朝食絶食，10 SG治験施行時のIIbV注入前諸検査［（1）～（10）］を受ける．また10 SG治験と同様の静脈確保，5％ブドウ糖加乳酸リンゲル液の0.2 ml/kg/minの速度の輸液を20分間受ける．次いで20分間で50 mlのIIbVを輸注を受ける．HbV注入後の輸液（生理食塩液），検査を10 SG治験と同時間に受ける．またHbV注入後の施設内での食事，宿泊も，10 SG治験と同様に行なう．そしてIIbV注入24時間後の検査，安静，摂食も10 SG治験同様に行なう．さらに被験者は一週後に治験施設に赴き，上記（1）～（11）の検査，ならびにIIbVcrit測定用採血を10 SG治験と同様に受ける．

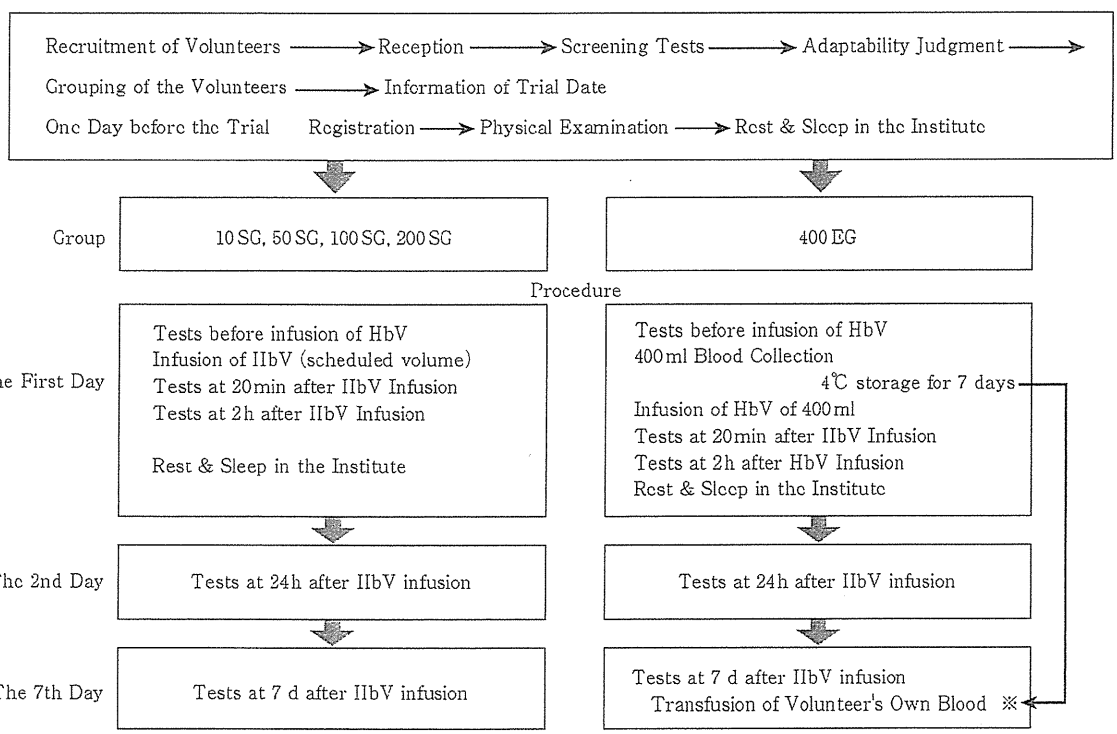

Fig.1. Diagram of the first step of trial. SG: single HbV infusion group. EG: blood/HbV exchange group. ※ : According to institute doctor's decision

Ⅲ．100SG治験

50SG治験を施行してその結果を解析して特に認めるべき合併症，副作用がなかった場合には100SG治験に移行する．被験者は50SG治験と同様にIIbV注入前日の検査を受け，施設内安静，食事，就眠をとる．またIIbV投与当日での朝食絶食，50SG治験施行時でのIIbV注入前諸検査を受ける．また50SC治験と同様の静脈確保，5％ブドウ糖加乳酸リンゲル液の0.2ml/kg/min の速度の輸液も受ける．次いで20分間で100mlのHbVを輸注を受ける．HbV注入後の輸液，検査は50SC治験と同様に受ける．またHbV注入後の施設内での食事，宿泊も，50SG治験と同様に行なう．そしてHbV注入24時間後の検査，施設内安静も50SG治験時と同様に行なう．さらに被験者は一週後に治験施設に赴き，上記（1）～(10) の検査，ならびにIIbVcrit測定用採血を50SG治験と同様に受ける．

Ⅳ．200SG治験

100SG治験を施行してその結果を解析して特に認めるべき合併症，副作用がなかった場合には200SG治験に移行する．被験者は100SG治験と同様にHbV注入前日の検査を受け，施設内安静，食事，就眠をとる．またHbV投与当日での朝食絶食，HbV注入前諸検査［(1）～(10)］を受ける．また100SG治験と同様の静脈確保，5％ブドウ糖加乳酸リンゲル液の0.2ml/kg/minの速度の輸液を20分間受ける．次いで40分間で200mlのHbVを輸注を受ける．IIbV注入後の輸液，検査は100SG治験と同様に受ける．またIIbV注入後の施設内での食事，安静，宿泊も，100SG治験と同様に行なう．そしてIIbV注入24時間後の検査，施設内安静も100SG治験施行時と同様に行なう．さらに被験者は一週後，治験施設に赴き，上記（1）～(11) の検査，ならびにHbVcrit測定用採血を100SG治験と同様に受ける．

Ⅴ．400EG治験

200SG治験を施行し，その結果を解析して特に認めるべき合併症，副作用がなかった場合には400EG治験に移行する．被験者は前治験（10SG～200SG治験）と同様にIIbV注入前日の検査を受け，施設内で安静を保ち宿泊する．またIIbV注入当日の朝食は絶食とし，IIbV注入前諸検査［(1）～(10)］を受ける．次ぎに静脈を確保し，5％ブドウ糖加乳酸リンゲル液の0.2ml/kg/minの速度の輸液も受ける．そして30分後に輸液剤を生理食塩液に替えてさらに同速度で20分間の輸液を受ける．次ぎに

1）治験者から400mlの血液を採血バック（日本赤十字社使用，献血採血バック）に十分な無菌操作下に採取する．採取した血液は1時間の室温放置の後に4℃の保冷庫に保存する．
2）採血終了とともに静脈路側管からIIbVの400mlを0.2ml/kg/minの速度で注入する．
3）被験者はIIbV注入後の検査を前治験と同様に受ける．HbV注入後の輸液，検査も前治験と同様に受ける．またIIbV注入後の施設内での食事，宿泊も前治験時と同様に行なう．そしてIIbV注入翌日の検査，生活も前治験と同様に行なう．
4）さらに被験者は一週後に治験施設に赴き，上記（1）～(10) の検査を受ける．またIIbVcrit測定用採血を受ける．
5）採取･保管した被験者自己血は治験施設内に保管するが，1週間後に被験者が施設に赴いた際に特に輸血を必要とすること（2 mg/dl以上のHb値の低下，被験者の倦怠･脱力感など医師による判定）が認められなかった場合には廃棄処分する．ただ輸血の必要性が認められた場合には被験者にそれぞれの自己血の必要量を10ml/minの速度で輸血する．
6）検査，あるいは輸血後，医師の判断で被験者に異常が認められない場合には被験者は帰宅し，治験は終了する．

B．第2段階治験

第1段階治験と同等の対象者，16名を以下の2群に区分する．

Ⅰ．600mlE群（600EG）　8名
Ⅱ．800mlE群（800EG）　8名

これら2群への治験施行は上記Ⅰ，Ⅱの順に施行し，600E群において予測せざる不祥事象（adverse effect）が発生しないことを確認後800E群の治験を施行する．

検査：治験前日検査，治験薬投与前，治験薬投与後20分後検査，2時間後検査，24時間後検査，治験薬投与7日後検査

治験操作，検査項目：

Ⅰ．600mlE群（600EG）での検査・IIbV注入

被験者はIIbV投与前日に検査施設に入り，一般理学的検査を受け，健康状態に異常がないことの診断を医師より受ける．そして施設内にて安静を保ち，夕食を摂取し，就眠する．IIbV注入当日での朝食絶食などは第1段階治験と同様に行なう（Fig.2）．次に末梢静脈から中心静脈カテーテルを挿入する（輸液剤は生理食塩液とし，3 ml/hで加圧注入）．そして以下の第2段階治験検査を行う．すなわち

（1）一般理学的検査
（2）循環器機能（血圧，脈拍数，12誘導心電図，CPK-M）
（3）呼吸機能検査（胸部X線，肺活量，1秒率，PaO_2, $PaCO_2$, pHa，中心静脈PvO_2，Base Excess）
（4）肝機能検査（総蛋白，アルブミン，総コレステロール，遊離脂肪酸，ビリルビン，sGPT，sGOT，γGPT，LDH，アルカリホスファターゼ，尿酸，血糖，プロトロンビン値，中心静脈血乳酸値）
（5）腎機能（尿色調･量･比重･沈渣･糖），BUN，creatinine，ウロビリノゲン）
（6）血液一般（IIb値，IIt値，IIbVcrit，赤血球数，白血球数・分類，PT，aPTT，FDP）
（7）血清電解質（Na，K，Cl）
（8）免疫機能検査（補体活性，血清IIbV抗原/抗体反応）
（9）神経･運動検査（歩行，握力，腱反射，眼球運動，知覚機能，視力，聴力）
（10）消化機能（食欲，腹部自覚症状，便所見）

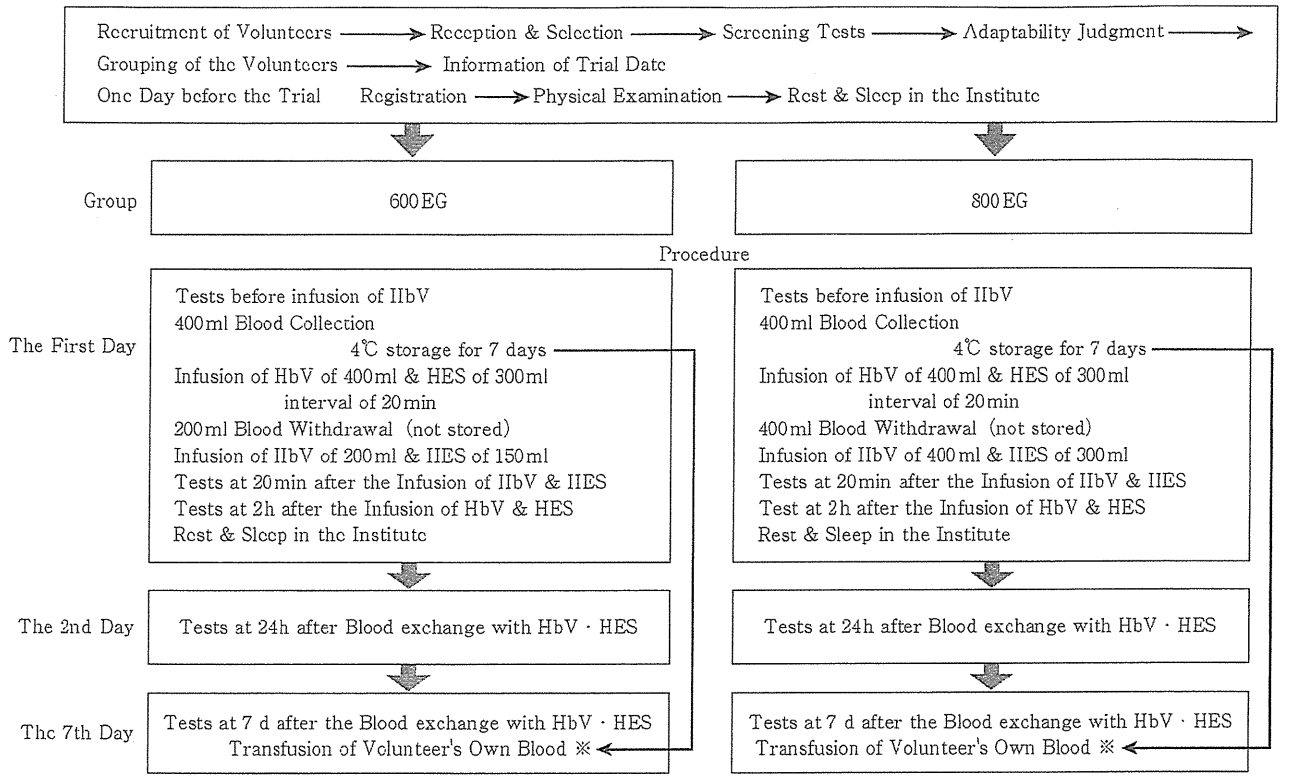

Fig.2. Diagram of the second step of trial. EG : blood/HbV exchange group. HES : 6% hydroxyethyl starch solution (Hespander™).
※ : According to institute doctor's decison

の検査を受ける．次ぎに静脈路を確保し，0.2 ml/kgの速度で2.5%ブドウ糖加乳酸リンゲル液の輸液も受ける．そして30分後に輸液剤を生理食塩液に替えてさらに同速度で20分間の輸液を受ける．

1）採血バック（日本赤十字社使用，献血採血バック）を使用して被験者から400 mlの血液を十分な無菌操作下に採取する．採取した血液は1時間の室温放置の後に4℃の保冷庫に保存する．

2）採血終了とともに静脈路側管からIIbVの400 mlを0.2 ml/kg/minの速度で注入する．

3）HbV注入完了と同時に300 mlのhydroxyethyl starch液（ヘスパンダー™）を0.2 ml/kg/minの速度で静脈内に投与する．

4）上記のIIbV注入20分後に被験者の自覚的，他覚的所見に異常が認められないことを確認して被験者から200 mlの採血を行う（この血液は廃棄処分する）．この採血終了と同時にHbVの200 mlを0.2ml/kg/minの速度で注入する．

5）HbV注入完了と同時に150 mlのhydroxyethyl starch液（IIES，ヘスパンダー™）を0.2ml/kg/minの速度で静脈内に注入する．その後，静脈路は0.2 ml/kg/minの速度の生理食塩液注入で維持する．

6）被験者はHES注入20分後，2時間後の検査受け，静脈路は抜去される．一方，中心静脈カテーテルはヘパリン充填で留置される．その後，被験者は施設内に留まり宿泊する．なおこの間，急性血液希釈に起因すると思われる症状が認められた場合には治験施設医師の判断で，確保されていた被験者の自己血を輸血し，治験は中止される．

7）翌日，被験者は朝食を絶食し，IIbV注入24時間後の検査を受ける．この間，中心静脈カテーテルは3 ml/hの生理食塩液の注入にて維持する．そして2時間の観察後，異常を認めなかった場合には中心静脈カテーテルは抜去され帰宅する．

8）被験者は一週後に治験施設に赴き，上記（1）～(10)［ただし中心静脈血について検査を除く］の検査，ならびにHbVcrit測定用採血を受ける．

9）採取・保管した自己血400 mlは1週間施設内に保管し，被験者が1週間後の検査が施行され，治験前のヘモグロビン値よりも2 g/dl以上の低下が見られた場合，あるいは医師の判断にもとずき被験者自己血の輸血が必要と認められた場合にはその必要量を10 ml/minの速度で被験者に輸血する．自己血の輸血の必要性が認められなかった場合，あるいは被験者の希望がなかった場合には保存した被験者自己血は廃棄処分される．

Ⅱ．800 ml E群（800EG）での採血・IIbV注入

被験者は600mlE群と同様にIIbV注入前日に治験施設に赴き検査を受け，施設内での安静，宿泊を行なう．IIbV注入当日の朝食は絶食とし，600EGで施行したのと同じHbV注入前諸検査［（1）～（10）］を受ける．次に静脈路を確保し，2.5%ブドウ糖加乳酸リンゲル液の0.2ml/kg/minの速度の輸液を受ける．そして30分後に輸液剤を生理食塩液に替えてさらに同速度で20分間の輸液を受ける．

1）被験者の400 mlの血液を十分な無菌操作下に採血バック（日本赤十字社使用，献血採血バック）に採取する．採取した血液は1時間の室温放置の後に4℃の保冷庫に保存する．

2）採血終了とともに静脈路側管からHbVの400 mlを0.2 ml/kg/minの速度で注入する．

3）HbV注入完了と同時に300 mlのhydroxyethyl starch液（HES，ヘスパンダー™）を0.2 ml/kg/minの速度で静脈内に注入する．

4）上記のIIbV，IIES液注入20分後に被験者は自覚的，他覚的所見に異常が認められないことを確認されて，再度400 mlの採血を受ける（この血液は廃棄処分する）．この採血終了と同時にHbVの400 mlを0.2 ml/kg/minの速度で注入する．

5）HbV注入完了と同時に300 mlのhydroxyethyl starch液（ヘスパンダー™）を0.2 ml/kg・minの速度で静脈内に注入する．その後，静脈路は0.2ml/kg/minの速度の生理食塩液注入で維持する．

6）被験者はIIES注入20分後，2時間後の検査受け，静脈路は抜去される．一方，中心静脈カテーテルはヘパリン充填で留置される．その後，被験者は施設内にて留まり宿泊する．なおこの間，急性血液希釈に起因すると思われる症状が認められた場合には治験施設医師の判断にで，確保されていた被験者の自己血を輸血し，治験は中止される．

7）翌日に被験者は朝食を絶食し，IIbV注入24時間後の検査を受ける．そしてこの間，中心静脈カテーテルは3 ml/hの生理食塩液の注入にて維持される．2時間の観察で異常を認めなかった場合は中心静脈カテーテルは抜去され帰宅する．

8）被験者は一週後に治験施設に赴き，上記（1）～（10）［ただし中心静脈血について検査を除く］の検査，ならびにHbVcrit測定用採血を受ける．

9）採取・保管した自己血400 mlは1週間施設内に保管し，被験者が1週間後の検査が施行され，治験前のヘモグロビン値よりも2 g/dl以上の低下が見られた場合，あるいは医師の判断にもとずき被験者自己血の輸血が必要と認められた場合にはその必要量を10 ml/minの速度で被験者に輸血する．なお自己血の輸血の必要性が認められなかった場合，あるいは被験者の希望がなかった場合には保存した被験者自己血は廃棄処分される．

3．考察

1）HbV，hydroxyethyl starch注入量

Abeら[9]，藤原ら[10]の研究ではラットにおいてIIbV注入に対する即時型アレルギー反応も遅延型のアレルギー反応も認められていない．また補体活性反応，免疫抑制反応も認められていない．しかしヒトを対象としたIIbVの注入は本試験が最初である．そのため段階的に増量する方法をとり，安全性を確保するように計画した．とくに補体を介した反応では極めて少量の投与でも生体反応が現れる．そのため初めて投与する際の投与量を10 ml，2回目での投与量は50 mlとした．

Sakaiらは[11]ラットに20 ml/kgのHbVを連続14日投与して動物が生存しうることを確認している．したがってヒトを対象とした本治験において成人を対象とした200 mlのIIbV単独注入はとくに循環血液量負荷とは考えられない．またSchnabelら[12]は僧帽弁疾患患者でも20 ml/kgの膠質液注入に耐えうると報告している．しかしHbVの30%はヘモグロビンを内包した固形のリポソームであり血管壁を通過することがなく，膠質液よりも血管内滞留時間は延長される．その後リポソームは時間経過とともに肝臓，次いで脾臓に補足される[13]．宗ら[14]によるとヒトにおけるHbVの血液中半減期は7日と推定されている．したがって循環血液量の10%以上の付加は避けるべきと考えてHbV単独投与量は200 mlとした．そしてこの量を超えて投与する場合には血液交換を採用した．すなわち400 mlの採血を行い，それに対応した量のIIbVを輸注することとした．またその際採血した自己血は1週間後にIIb値として2 g/dlの低下があって，治験施設の医師の判断により，あるいは被験者の希望によては返血することとした．

一方，厚生労働省の赤血球輸血施行の基準では成人で600 mlの血液喪失があった場合となっている[15]．したがって人工酸素運搬体であるHbVを赤血球輸血の代替として使用する適応は600 ml以上の血液喪失があった場合となり，400 mlでの投与基準では人工酸素運搬体として投与する価値が認められない．それゆえに本治験の第2段階施行は必要と考えた．ヘモグロビンを内包したリポソーム粒子は生理食塩液に浮遊された状態にあり，IIbVcrit（ヘモグロビン内埋したリポソーム粒子体積のIIbV全容量に対する比率）は30%と推定されている[16]．上述のごとくIIbV粒子の血管内半減期7日と予想されているが，溶媒である生理食塩液は注入後1時間以内にその約2/3が血管外に移行して[17]血液量維持効果に乏しい．そのため400 ml以上の血液交換においては生体が代償しうる限界の血液量を超える危険性がある．そのため膠質液（hydroxyethyl starch液）を投与することが必要であると考えた．

一方，hydroxyethyl starch液などの人工膠質液を用いた血液希釈に関してはすでに自己血800～1200 ml採取による希釈式自己血輸血が臨床において施行され，その安全性は確立されている[18,19]．膠質液としてのhydroxyethyl starch液（ヘスパンダー™）も，投与3～4時間でその70～80%が血管外に移行する[20]．しかし少なくともその20～30%は数時間血管内に留まる[21]．さらにすでに脱血量と同等量のHbVが投与されている．そのた

め投与HES液は脱血量の3/4量とした．

2）検査項目

酒井ら[13]がラットでその循環血液量の50%とHbVとの血液交換を行ない2週間観察した際に血液中のコレステロール値に特徴的な上昇を認めた．この上昇は投与したIIbVのリポソームが代謝され，あるいは遊離したものと推測された．またその機序は明かにされてはいないが血漿中リパーゼ値がIIbV投与とともに上昇している．今回の治験においてはHbVの投与量は少ないものの同様な変化がみられるか血液中コレステロールを中心とした脂肪代謝の変化を観察することが重要と考えられ総コレステロール，遊離脂肪酸，γGPT，アルカリホスファターゼ，血漿リパーゼ値を厚生省薬務局通達項目[22]に加えた．

IIbVと同様なリポソームを使用した人工酸素運搬体に関してPhillipsら[23]はラットでリポソーム膜が多重相となると血清中の補体を活性させることを報告している．また藤原ら[10]も軽度ながらラットで同様な現象が見られることを報告している．したがって本治験においてはIIbV投与にともない血清中の補体活性を観察するように計画した．そして補体活性にともなう生体反応として気管支平滑筋の反応を診るために肺活量，一秒率，血液ガス所見を検討するように計画した．さらにHbV投与にともないHbVに対して抗体産生がないか観察するためにIIbV投与後1日目，7日目の血清について抗原/抗体反応を検査することを計画した．

頻回の輸血は生体内に鉄分子の蓄積を来たしヘモクロマトーシスを来たす[24]．そしてその際には血液中のフェリチン値が上昇することが認められている．IIbVは生体に投与されて生体内の網内系に捕捉され，その後脾臓に転送され分解されて遊離の鉄分子が生じると推測される．一般に遊離された鉄分子は蛋白と結合してフェリチンとして血液中に放出される[25]．ただ頻回の輸血にともなう生体に付加される鉄分子の総量に比較すればHbVとして投与される鉄分子の量は少ない．しかしHbVの場合にはその分解速度が輸血された赤血球のそれよりは速やかである[11]．そのためIIbVから鉄分子の遊離を観察するために血清フェリチンの測定は必要と考えた．

第2段階治験においては中心静脈カテーテルを挿入し，全身的な組織嫌気性代謝の指標としての中心静脈血のPvO_2，Base Excess，血漿乳酸値の測定を行なう．第1段階治験での400EGでは日本赤十字血液センターが行なっている400ml献血量と同等量の血液とHbVとを交換するもので，それによる各臓器・組織への酸素供給面での安全性は保証されている．しかし第2段階治験での600EG，800EGではその保証が得られていない．しかし短時間での急性血液希釈に関しては十分量の膠質液を使用した希釈性自己血輸血での臨床成績[18,19]が示すごとく安全性は確認されている．今回の治験においては赤血球の代替役を果たすIIbVは投与されてはいるが800mlの血液交換後の血液希釈状態が1週間におよぶ．それ故にこの面での安全性の確認が必要と考えられた．またhydroxyethyl starch液の投与のみ行なうものを対照として検討すればHbVの酸素運搬機能面での優位性が得られるかも知れない．しかしそのような対照群を設置することは倫理的な立場から好ましいとは思われない．ただ治験薬との血液交換24時間後の中心静脈血のPvO_2，Base Excess，血漿乳酸値の観察から1週間の推移が想定される．それによってもしなんらかの全身的な組織嫌気性代謝が危惧されれば確保した自己血の輸血を行なうことも考えている．中心静脈血に関する測定を治験薬投与当日，その翌日にのみに限定するのは中心静脈カテーテル留置にともなう合併症を最小限に留めることを目的とした．そして所定の検査・測定が完了次第，中心静脈カテーテルを速やかに抜去して被験者の負担を少なくすることに努めた．

3）被験者数と検査期間

健康成人被験者を対象とした治験第1相試験ではその結果を統計的に処理する十分な被験者を整えることも重要である．しかし倫理的観点から対象者を限定することも考慮しなければならない．第1段階治験での被験者数を10SG，50SG，100SG，200SGでは各群ともそれぞれ2名としている．HbVに対する反応性，とくにアレルギー，アナフィラキシー様反応がマウス[26]，ラット[27]，ビーグル犬[28]を使用したいずれの前臨床試験でも認められていない．またこれらの反応は治験薬投与量には関係なく[29]，もし反応が生じる場合には少量，たとえば皮内投与量でも顕れる．それ故に被験者数を少なくするためにも各群2名，計8名に留めた．一方，400mlの治験薬を投与してそれが生体機能に及ぼす影響を検討するためには，統計的観察が最小限可能な6名の被験者を採用した．同様に第2段階治験でも各群6名を対象者とした．

検査・観察期間に関して選別検査からHbV投与当日までの期間を検査数値が著しく変化しない9±2日と定め，被験者の負担を軽減するために努めた．さらに治験薬投与前後の施設内拘束期間も3日と最小限に留めた．

4．おわりに

わが国において，また外国においても人工酸素運搬体の臨床使用に課せられた社会的責務は赤血球輸血の代替をすることにある．したがって本稿において検討したIIbVに関する第2段階治験はそれに即するように計画された．すでに述べたごとく，赤血球輸血のトリガー値は12ml/kgの循環血液量の喪失とされている[15]．それをもとに今後第2相治験，あるいは第3相治験において用いられるIIbV量は成人を対象として少なくとも600～800mlと想定される．そのためここに企画した第2段階治験では最大800mlの使用量と設定した．

また冒頭にも述べたごとく，この第1相治験は安全性に重点をおかなければならない．そして第2相治験を円滑に施行することにも配慮すべきである．本計画では被験者の心身的な負担を最小限にすることに注意した．すでに行われた前臨床試験の結果から推測してこの計画の実施での安全性は十分確保されているものと思われる．またHbV投与にともなう生体変化も確実に把握できるものと思われる．したがってGMPの完成とともに

一日も早く本治験が実施されることに期待する．そしてわが国が指導して進めてきた細胞型人工酸素運搬体の開発がわが国の医療に，そして世界の医療に貢献することを切望する．

謝辞

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Hemoglobin Vesicle Improves Recovery of Cardiac Function After Ischemia–Reperfusion by Attenuating Oxidative Stress in Isolated Rat Hearts

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Abstract: Hemoglobin vesicle (HbV) could be a useful blood substitute in emergency medicine. The aim of this study was to clarify the effects of HbV on cardiac function after ischemia–reperfusion (I/R) ex vivo. Isolated rat hearts were perfused according to the Langendorff method. An ischemia–reperfusion group (n = 6) was subjected to 25 minutes of global ischemia and 30 minutes of reperfusion. HbV (hemoglobin, 0.33 g/dL) was perfused before ischemia–reperfusion for 10 minutes (HbV group, n = 6). Hemodynamics were monitored, and tissue glutathione contents were measured. The redox state of reactive thiols in cardiac tissues was assessed by the biotinylated iodoacetamide labeling method. Left ventricular developed pressure was significantly recovered in the HbV group after 30 minutes of reperfusion (56.3 ± 2.8 mm Hg vs. ischemia–reperfusion group 27.0 ± 8.0 mm Hg, $P < 0.05$). Hemodynamic changes induced by HbV were similar to those observed when N^G-nitro-L-arginine methyl ester was perfused for 10 minutes before ischemia–reperfusion (L-NAME group). The oxidized glutathione contents of cardiac tissues significantly decreased, and biotinylated iodoacetamide labeling of thiols was maintained in both the HbV and the L-NAME groups. HbV improved the recovery of cardiac function after ischemia–reperfusion in isolated rat hearts. This mechanism is dependent on functional protection against thiol oxidation.

Key Words: reduced and oxidized glutathione, thiol oxidation, hemoglobin vesicle, ischemia-reperfusion

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INTRODUCTION

Blood transfusion is an essential therapy in emergency and critical care medicine, disaster medicine, and surgery with large amounts of bleeding. However, insufficiency in the amounts of isolated blood available for transfusion is a major problem worldwide. So the need for blood substitutes has increased, and they started to be developed in the 1960s.[1] Two kinds of blood substitute have been developed, polymerized hemoglobin solution and hemoglobin vesicle (HbV). The former is a simple blood substitute for oxygen supply, but its renal toxicity and high colloidal osmotic pressure have been problems in clinical usage. The latter is a cellular type blood substitute. In 1996, Sakai et al[2] developed a method for producing HbV in which hemoglobin molecules are encapsulated with a lipid bilayer membrane, which overcomes the adverse effects of hemoglobin solution.

In many types of shock, various organs are exposed to ischemia, and the basic principle of therapy for shock is maintaining the oxygen supply to important organs, such as the brain and heart. Even in hemorrhagic shock, myocardial ischemia is induced, which can worsen cardiac function.[3] Several studies reported that the hemodynamic effects induced by HbV transfusion in animal hemorrhagic shock models are comparable with those observed in autologous blood transfusion.[4–6] When resuscitation from ischemia is prolonged, the degree of ischemia–reperfusion injury increases systemically, and protection against ischemia–reperfusion injury is crucial in such a situation.

Among various factors, which can modulate ischemia–reperfusion injury in heart tissues, the main focus has been on ischemic preconditioning over the past 2 decades. Adenosine[7] and metabolites of arachidonic acid[8] have been reported to protect cardiac tissue against ischemia–reperfusion injury via ischemic preconditioning. It has been clarified that mitochondrial adenosine triphosphate–sensitive potassium channels (mito-K_{ATP}-channel) play a central role in ischemic preconditioning.[9] Moreover, various neurohumoral factors modulate ischemia–reperfusion injury. Among cardiovascular modulators, nitric oxide (NO) has 2 types of effect on cardiac function. NO generated at the physiological level works as a normal signaling molecule and plays a protective role via its vasodilation effects. However, when an excess of NO is present, such as in ischemia–reperfusion, it can have a deleterious effect when reactive nitrogen species are produced during reperfusion.[10]

The aim of this study was to clarify the effects of HbV on cardiac functions in ischemia–reperfusion. We employed the Langendorff perfusion mode to produce our ischemia–reperfusion model, and HbV was added to the perfusion

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solution. Metabolic changes and oxidative stress were estimated by measuring lactate and pyruvate release into the perfusate, cardiac tissue glutathione (GSH), the amount of reactive thiols on proteins, and redox-related enzyme activities.

MATERIALS AND METHODS

Materials

N^G-nitro-L-arginine methyl ester (L-NAME) was purchased from Dojin Chemical (Kumamoto, Japan) and was dissolved with modified Krebs–Henseleit (mKH) buffer at the concentrations described below.

HbV is a small sphere (diameter, approximately 250 nm) formed from a lipid bilayer membrane,[2] which includes hemoglobin as a saline suspension containing 10 g/dL of hemoglobin. The HbV suspension was diluted with mKH buffer to a final hemoglobin concentration of 0.33 g/dL. Beforehand, we tested various concentrations of HbV without changing the ionic concentration, and this was determined as the maximum concentration.

Heart Sample Preparation and Perfusion Method

Male Wister rats (Charles River Japan, Inc), which were 9–12 weeks old and weighed 350–400 g, were treated with heparin (1000 IU, intraperitoneally) and 10 minutes later anesthetized with pentobarbital (60 mg/kg, intraperitoneally). The heart was excised quickly, the aorta cannulated, and the heart perfused retrogradely with mKH buffer according to the Langendorff perfusion mode. Perfusion was conducted at a constant perfusion pressure of 100 cm H_2O at 37°C. The mKH buffer contained 116 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, and 11 mM glucose. The experimental buffer solutions were gassed with 95% O_2–5% CO_2.

Cardiac functions were monitored and recorded using a fluid-filled left ventricular balloon connected to a transducer (P-50; Gould, Inc) and a WS-641G multichannel recorder (Nihon Kohden, Tokyo, Japan). Initially, balloon volume was adjusted to produce a left ventricular end-diastolic pressure (LVEDP) of 0–5 mm Hg. Left ventricular developed pressure (LVDP) was calculated by subtracting LVEDP from left ventricular systolic pressure.

Coronary effluent was collected at 5-minute intervals throughout the experiment and stored at −80°C until chemical analysis for lactate and pyruvate. After the experiment, heart samples were stored at −80°C until chemical analysis was performed. All experiments were in accordance with the National Defense Medical College Institutional Animal Care and Use Committee Guidelines.

EXPERIMENTAL PROTOCOLS

Experiment 1

Isolated hearts were assigned to 3 groups. Each group was subjected to the following experimental protocols (Fig. 1): ischemia–reperfusion (I/R) group (n = 6), hearts were perfused with mKH buffer for 30 minutes (control perfusion) and then subjected to 25 minutes of global ischemia by stopping the perfusion, followed by 30 minutes of reperfusion (I/R); HbV group (n = 6), hearts were perfused with HbV (0.33 g/dL) suspension for 10 minutes after 20 minutes of control perfusion and then subjected to the same I/R procedure; L-NAME group (n = 6), hearts were perfused with L-NAME (100 μM) for 10 minutes after 20 minutes of control perfusion and then subjected to the same I/R procedure.

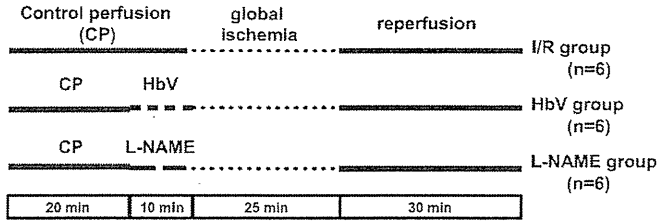

FIGURE 1. Protocol of experiment 1. Study for verifying the effect of HbV and L-NAME on cardiac function and cardiac tissue redox state in ischemia–reperfusion.

Fifteen heart samples in the I/R (n = 4), HbV (n = 5), and L-NAME (n = 6) groups were used in chemical analysis for GSH and oxidized glutathione (GSSG) contents. Biotinylated iodoacetamide (BIAM) labeling of reduced protein thiols was performed for each of the 4 heart samples in the I/R, HbV, and L-NAME groups.

Experiment 2

To measure the activity of redox-related enzymes, another set of experiments was performed (Fig. 2). Hearts were freeze-clamped with aluminum tongs precooled in liquid nitrogen after 30 minutes of control perfusion (control group, n = 5), after 20 minutes of control perfusion and 10 minutes of HbV perfusion (C + HbV group, n = 5), after 30 minutes of control perfusion + the I/R treatment mentioned above (I/R group, n = 7), and after 20 minutes of control perfusion and 10 minutes of HbV perfusion + the I/R treatment (I/R + HbV group, n = 6) and stored at −80°C until chemical analysis for enzyme activities.

Biochemical Analysis

Measurement of Lactate and Pyruvate Levels in Coronary Effluent

The lactate and pyruvate contents of the coronary effluent were analyzed enzymatically by the method of Lowry

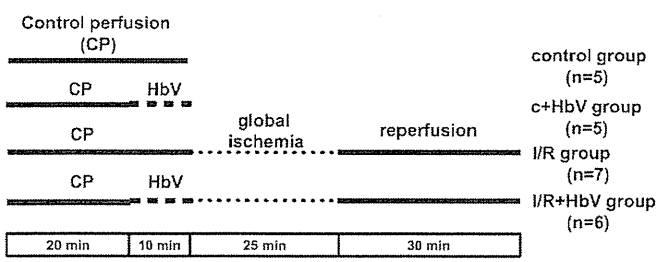

FIGURE 2. Protocol of experiment 2. Study for verifying the effect of HbV on redox-related enzyme activities in ischemia–reperfusion.

and Passonneau.[11] The lactate–pyruvate ratio (L/P ratio) was calculated for each sample.

Measurement of GSH/GSSG by High Performance Liquid Chromatography With Coulometric Electrochemical Detection

Tissue GSH and GSSG contents were determined by high performance liquid chromatography with coulometric electrochemical detection.[12] Cardiac tissues were homogenized in 10% trichloroacetic acid containing 100 μM diethylenetriamine pentaacetic acid. After centrifugation, the GSH and GSSG concentrations of the supernatants were measured using a high performance liquid chromatography system with a coulometric chemical detector consisting of 2 pumps (ESA Coulochem, model 520; ESA, Inc., Chelmsford, MA). An MCM C18 analytical column (inner diameter, 250 × 4.6 mm; LMS Co, Tokyo, Japan) was used for reversed-phase chromatography. The GSH and GSSG peaks, occurring at 750 mV at 9 minutes and 840 mV at 18 minutes, respectively, were monitored using the coulometric detector. Protein concentrations of the centrifuged sediments were measured by the bicinchorinic acid method (Thermo Scientific, Rockford, IL).

BIAM Labeling of Reduced Protein Thiols

BIAM labeling of the reactive thiols on proteins was performed by the following previously reported method[13] with minor modifications. Briefly, tissues were lysed in buffer A (50 mM piparazine-1,4-bis(2-ethansulfonic acid) pH 6.5, 150 mM NaCl, 5 mM $MgCl_2$, 50 μM diethylenetriamine pentaacetic acid, 2 mM phenyl methyl sulfonyl fluoride and 0.5% Triton X-100) with 100 μM of N-(biotinoyl)-N′-(iodoacetyl)-ethylenediamine in the dark at 25°C for 30 minutes. Lysed homogenates were mixed with Laemmli buffer containing 5% β-mercaptoethanol and boiled at 100°C for 5 minutes. Proteins were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to the polyvinylidene difluoride membrane, and the reduced reactive thiols on proteins were detected using horseradish peroxidase–conjugated streptavidin and enhanced chemiluminescence solution.

Measurement of Redox-Related Enzyme Activity in Heart Tissues

Heart samples (obtained from experiment 2) were homogenized in a Tris buffer containing 50 mM Tris–HCl (pH 7.5), 5 mM EDTA, 1 mM DTT, 1 μg/mL leupeptin, and 0.2 mM phenyl methyl sulfonyl fluoride. The homogenates were centrifuged at 4°C at 10,000*g* for 15 minutes, and the resulting supernatants were used for analysis. The activities of the enzymes glutathione peroxidase, glutathione reductase, Cu/Zn-superoxide dismutase, Mn-superoxide dismutase, and catalase were measured using assay kits supplied by Cayman Chemicals (Ann Arbor, MI). The protein concentrations of the supernatants were measured by the bicinchorinic acid method.

Statistical Analysis

Data were expressed as mean ± standard error. Data for LVDP, LVEDP, coronary flow, and L/P ratio were analyzed by 2-factor analysis of variance with repeated measures followed by the post hoc *t* test with Bonferroni correction for multiple comparisons. Data for GSH, GSSG, GSH/GSSG ratio, and enzyme activity were analyzed by 2-way analysis of variance followed by the post hoc *t* test with Bonferroni correction for multiple comparison. Correlations between tissue GSSG content and LVDP or LVEDP at 30 minutes after reperfusion were tested with Pearson product–moment correlation. $P < 0.05$ was considered statistically significant.

RESULTS

Comparison of Effects of HbV and L-NAME on Cardiac Function and Cardiac Tissue Redox State

Cardiac Function

In the I/R group, LVDP was sustained at 120–140 mm Hg for the 30 minutes of control perfusion, and there was poor recovery in LVDP during the 30 minutes of reperfusion after 25 minutes of ischemia (27.0 ± 8.0 mm Hg, 18.7 ± 5.1% of the preischemic value). In the HbV and L-NAME groups, LVDP rapidly decreased just after the start of the HbV and L-NAME perfusion, and their mean values at 5 and 10 minutes were significantly lower than the corresponding values in the I/R group.

In the HbV and L-NAME groups, LVDP had recovered significantly after 30 minutes of reperfusion (56.0 ± 3.1% and 52.8 ± 8.1% of the pre-ischemic value, respectively; Fig. 3A). In all the groups, LVEDP increased gradually during global ischemia and continued increasing after the start of reperfusion. Although it stayed at a high level during the 30 minutes of reperfusion in the I/R group, in the HbV and L-NAME groups, it decreased gradually until the end of reperfusion. As a result, the mean values of LVEDP in the HbV and L-NAME groups at 20 and 30 minutes of reperfusion were significantly lower than the corresponding values in the I/R group (Fig. 3B).

The coronary flow was mostly recovered after reperfusion in the I/R group. However, in the HbV and L-NAME groups, the coronary flow after reperfusion was lower than the I/R group (Fig. 3C).

The heart rate changed similarly in the 3 groups throughout the time course of this experiment (Fig. 3D).

Metabolic State

After 5 minutes of reperfusion, the L/P ratio had slightly increased in the L-NAME group. This increase had erased after 30 minutes of reperfusion (Fig. 4).

Cardiac Tissue GSH and GSSG Contents

The cardiac tissue GSH content was slightly lower in the HbV and L-NAME groups than that in the I/R group, but differences among the 3 groups were not statistically significant (Fig. 5A). The cardiac tissue GSSG content was lower in the HbV and L-NAME groups than that in the I/R group, and the differences between the I/R group and the HbV and L-NAME groups were statistically significant (Fig. 5B). As a result, the GSH/GSSG ratio was higher in the HbV and L-NAME groups than that in the I/R group, and the difference

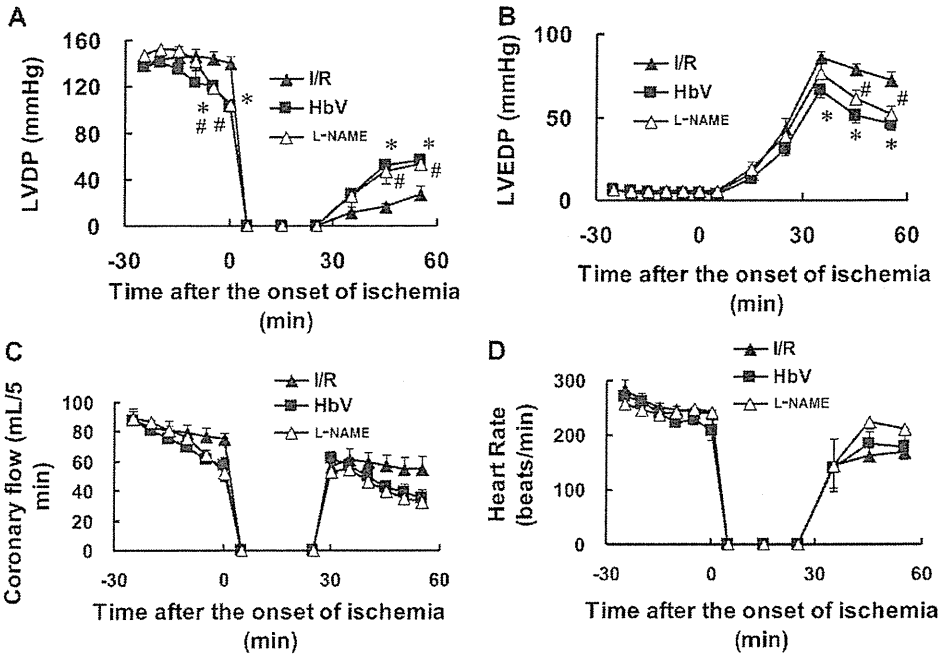

FIGURE 3. Effect of L-NAME (100 μM for 10 minutes of perfusion) and HbV (0.33 g/dL hemoglobin concentration, for 10 minutes of perfusion) on LVDP (A), LVEDP (B), coronary flow (C), and heart rate (D) in isolated rat hearts. ▲, I/R group (n = 6); ■, HbV group (n = 6); △, L-NAME group (n = 6). *$P < 0.05$ between I/R and HbV groups, and #$P < 0.05$ between I/R and L-NAME groups.

between the I/R group and the HbV and L-NAME groups in this respect was significant (Fig. 5C).

Furthermore, after 30 minutes of reperfusion, the cardiac tissue GSSG content was found to be negatively correlated with LVDP ($r = -0.683$, $P = 0.005$) (Fig. 6A) and positively correlated with LVEDP ($r = 0.564$, $P = 0.028$) (Fig. 6B).

Protein Thiol Oxidation After Ischemia–Reperfusion

In Figure 7, the first 2 lanes of BIAM labeling blotting patterns are for the I/R group. The strong bands indicated by arrows are nonspecific bands thought to be carboxylase/decarboxylase containing biotin molecules as inherent prosthetic groups. The other stained bands are proteins, which contain reduced reactive thiols. Although the number and intensity of these bands decreased in the I/R group, they were maintained at high levels in the HbV and L-NAME groups, suggesting that thiol oxidation was prevented in these 2 groups.

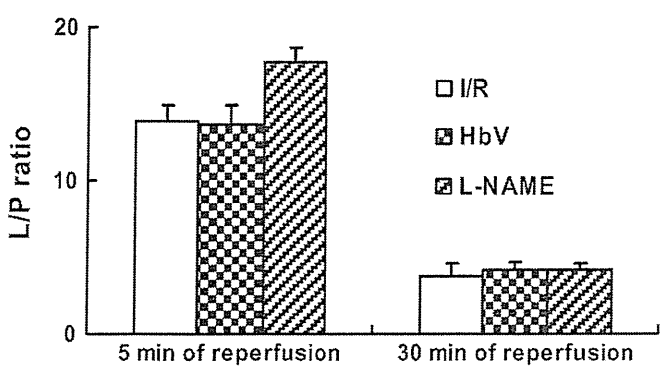

FIGURE 4. Effect of L-NAME (100 μM for 10 minutes of perfusion) and HbV (0.33 g/dL hemoglobin concentration, for 10 minutes of perfusion) on L/P ratio for perfusate during reperfusion.

Effect of HbV on Redox-Related Enzyme Activities

Ten minutes of HbV perfusion had not influenced redox-related enzyme activities (glutathione peroxidase, glutathione reductase, Cu/Zn-superoxide dismutase, Mn-superoxide dismutase, and catalase) either before ischemia or after reperfusion (Table 1).

DISCUSSION

In this study, we found that HbV improved recovery of cardiac function after ischemia–reperfusion in isolated rat hearts, and the effects of HbV closely resembled those of L-NAME under our experimental conditions. The cardioprotective effect of HbV and L-NAME did not depend on the preservation of coronary flow or the decrease in cardiac lactate production. Both HbV and L-NAME maintained the redox state of cardiac tissue in ischemia–reperfusion, which was implicated with the significant recovery of cardiac function.

Nitric oxide synthase inhibitors have shown diverse and controversial effects on cardiac function depending on the species of animal and experimental conditions used in isolated and perfused rat hearts.[14] N^G-nitro-L-arginine[15] and N^G-monomethyl-L-arginine[10] have been reported to improve myocardial function after ischemia–reperfusion. Andelova et al[16] reported that L-NAME improves functional recovery (LVDP, LVEDP, and +dp/dt) after 25 minutes of ischemia and 35 minutes of reperfusion when it was perfused at 100 μM for 15 minutes just before ischemia. The effects of L-NAME we noted seem to be mostly in accordance with those observed by Andelova et al.[16] L-NAME inhibited endothelial nitric oxide synthase (eNOS) activity and lowered the NO concentration in the perfusate during the control perfusion. Both L-NAME and HbV decreased coronary flow, supporting our speculation that they attenuated the effects of NO derived from eNOS or NO-independent effects of L-NAME.[17]

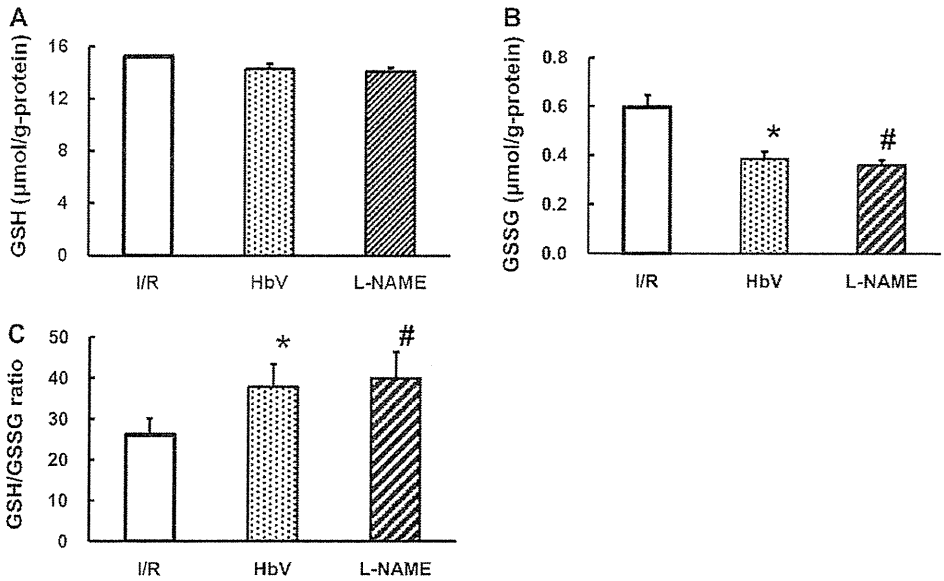

FIGURE 5. Effects of L-NAME (100 μM for 10 minutes of perfusion) and HbV (0.33 g/dL hemoglobin concentration, for 10 minutes of perfusion) on cardiac tissue GSH content (A), GSSG content (B), and GSH/GSSG ratio (C). *$P < 0.05$ between I/R and HbV groups, and #$P < 0.05$ between I/R and L-NAME groups.

L-NAME possibly inhibited NO synthesis to some extent during reperfusion through inhibition of eNOS because it penetrated into the vascular endothelium during the control perfusion and ischemic period and may have remained there in the active form during reperfusion.[18] It is well known that oxyhemoglobin captures NO molecules in the blood and controls their level under physiological conditions.[19] Also heme iron in HbV can bind NO.[20] Although HbV captures NO synthesized by eNOS in the perfusate during the control perfusion, it would not have been able to penetrate into the cardiac tissue and have been rapidly washed out from the vascular space just after the onset of reperfusion and therefore could not have been involved in NO synthesis in cardiac tissue during reperfusion.[21]

It has been demonstrated that in ischemia–reperfusion, especially in the reperfusion phase, peroxynitrite ($ONOO^-$) is excessively produced from the superoxide anion ($O_2^{\cdot-}$) and nitric oxide (NO) and prevents the recovery of cardiac function during reperfusion.[10,22] Thus, it was considered that L-NAME and HbV suppress the increase in oxidative stress during reperfusion, resulting in improved recovery of cardiac function. This idea is supported by the fact that L-NAME and HbV had a similar effect on changes in biochemical redox markers (GSH/GSSG and BIAM labeling) in cardiac tissues after ischemia–reperfusion. GSH is the principal intracellular low-molecular weight thiol and plays a crucial role in cellular defense against oxidative stress by directly scavenging reactive oxygen species.[23]

It has been reported that tissue total GSH and GSSG contents are not changed by ischemia alone, although the former decreases and the latter increases after reperfusion in isolated perfused rat hearts.[24] Considering our finding that the GSSG content of cardiac tissue was inversely and positively correlated with LVDP and LVEDP, respectively (Figs. 6A, B), it is interesting that L-NAME and HbV suppressed the increase in GSSG content after reperfusion (Fig. 5B). During reperfusion, the large amount of hydrogen peroxide generated is detoxified through a glutathione peroxidase reaction and GSH is concurrently oxidized to GSSG in the reaction. Because the activities of antioxidant enzymes were largely unchanged between groups (Table 1), the suppression of the increase in GSSG after ischemia–reperfusion was supposed that the production of reactive oxygen species was attenuated in the HbV and L-NAME groups. Li et al[25] reported that

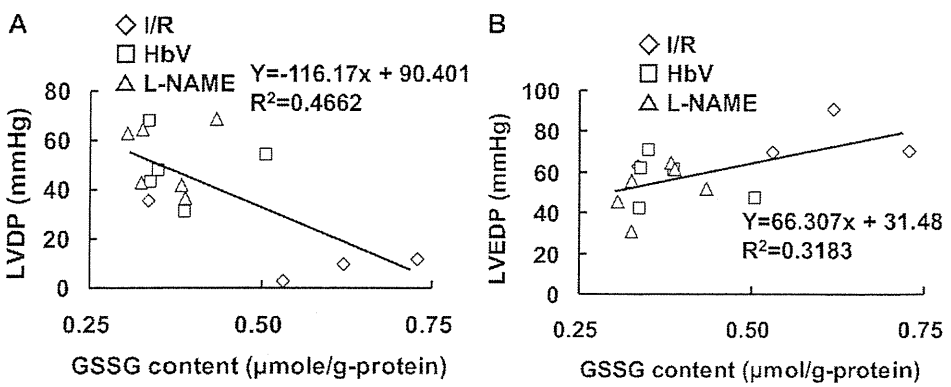

FIGURE 6. Correlation between cardiac tissue GSSG content and LVDP (A) or LVEDP (B) was estimated using 15 data points [4 in I/R group (◇), 5 in HbV group (□), and 6 in L-NAME group (△)]. The GSSG content was inversely and positively correlated with LVDP ($r = -0.683$, $P = 0.005$) and LVEDP ($r = 0.564$, $P = 0.028$) at 30 minutes after reperfusion, respectively.