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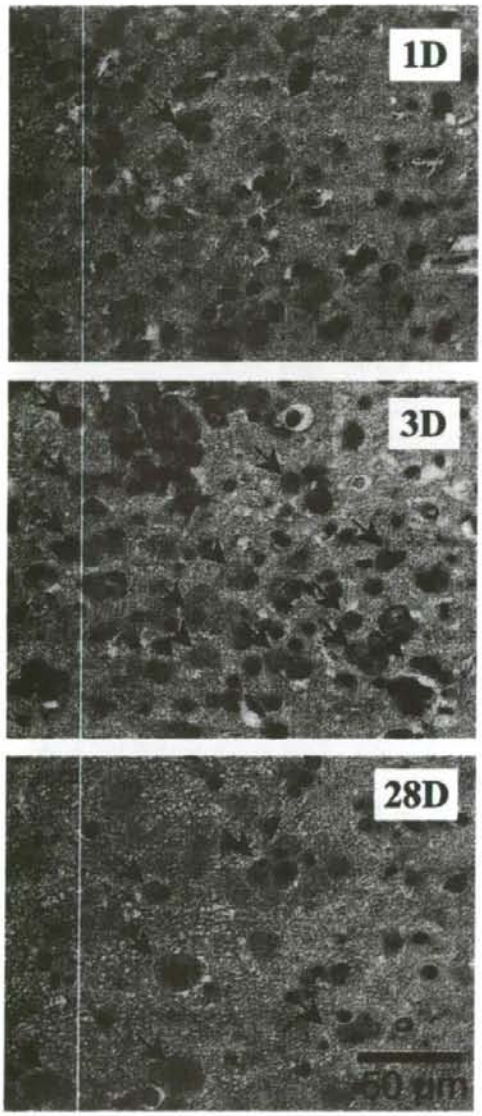

Figure 3. Immunochemical staining of rat brain tissue near the hematoma with anti-human Hb antibody. The round pink cells indicate the presence of HbV inside the cells. One day and three days after injection, the macrophages phagocytizing HbV are evident, as indicated with arrows. Even after 28 days, a large amount of HbV remained, although such cells were markedly fewer. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Figure 4. Transmittance electron micrographs of rat brain tissue in the HbV group 1 day and 3 days after intracerebral injection of HbV. The individual HbV particles are visible as black particles in the phagosomes of macrophages at 1 and 3 days, as indicated with white arrows. A macrophage phagocytizing not only HbV but also RBC (black arrows) is visible. Scale bar, 1 μm.

head injury.[4,27,28] Therefore, it is important to test possible adverse effects of HBOC when it is used for treatment of trauma such as in head injury involving brain hemorrhage.[20] Accordingly, examining the effects of HbV through a direct injection into the

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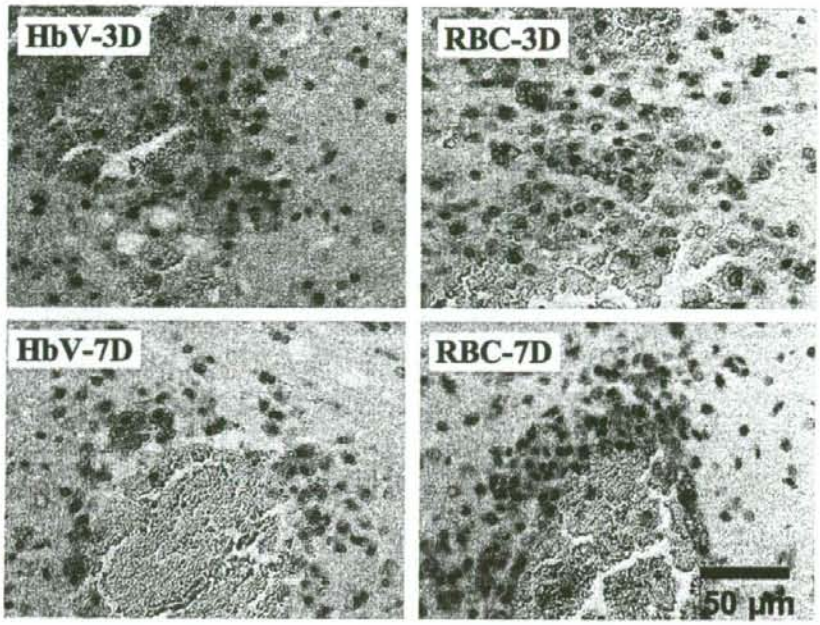

Figure 5. Immunochemical staining with anti-rat HO-1 antibody of rat brain 3 and 7 days after injection of HbV and RBC. Intense staining was confirmed especially at the rim of the perihematoma region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

brain can be a useful approach for fundamental characterization of HbV pathophysiology in ICH.[29] The vesicular size of HbV, 250 μm, is much larger than plasma proteins. It is noteworthy that, in a previous experiment, HbV did not infiltrate into the brain tissue compartment even when a severe hemorrhagic condition was produced experimentally by blood draining and the BBB was damaged while HbV solution was tested as a resuscitation fluid.[4] The current study further investigated possible HbV toxicity produced on brain tissue when it was injected directly into the brain compartment.

Many reports have described the cellular, biochemical, and pathophysiological effects of ICH. In addition, the time course of the effects has been investigated using many animal model systems.[30–34] The intensity and the time course of these debilitating effects of experimentally induced ICH generally paralleled with the intracerebral injection volume.[16,35,36] In our experiment, the volume of injection into a rat brain was small: 20 μL. Precedent studies reported that 20 μL of RBC injection caused brain edema and BBB breakdown at 3 days,[37] and that the mortality rate increased with 30 μL injection.[38] We have anticipated, therefore, that HbV might cause some intense reactions as they were demonstrated with RBC.[35] Additionally, there has been a report demonstrating a close relationship between brain edema and forelimb placing deficit scores after the experimental ICH.[35] For both HbV and RBC groups, the 20 μL injection produced slight signs of motor dysfunctions, that is "miss-steps," "dragging" of the left foot, and the reduction of the forelimb "hanging-time," that is classical striatal motor dysfunctions. The current study used measurements of body weight gain, wet brain weight, and motor dysfunctions. However, all were mutually comparable with the HbV treated animals and with the RBC treated animals; HbV showed no notable adverse reactions over that seen with RBC.

Histopathological examination 1 day after the injection indicated that both HbV and RBC groups showed the infiltration of neutrophils surrounding the hematoma. Neutrophils release various inflammatory cytokines, such as tumor necrosis factor-α, interleukin-6, and interferon-γ, which play important roles in brain damage.[16] The neutrophil infiltration is a normal response to tissue damage such as brain ischemia and traumatic injury.[39] The number of neutrophils had decreased significantly by 3 days in both groups. Immunochemical staining with anti-human Hb antibody showed that a considerable amount of small HbV corpuscles were dispersed into surrounding tissue. In contrast, a large amount of RBC mostly remained in the hematoma. These differences between HbV and RBC might be attributable to the differences in sizes of two corpuscles, or other physicochemical properties for dispersion. It

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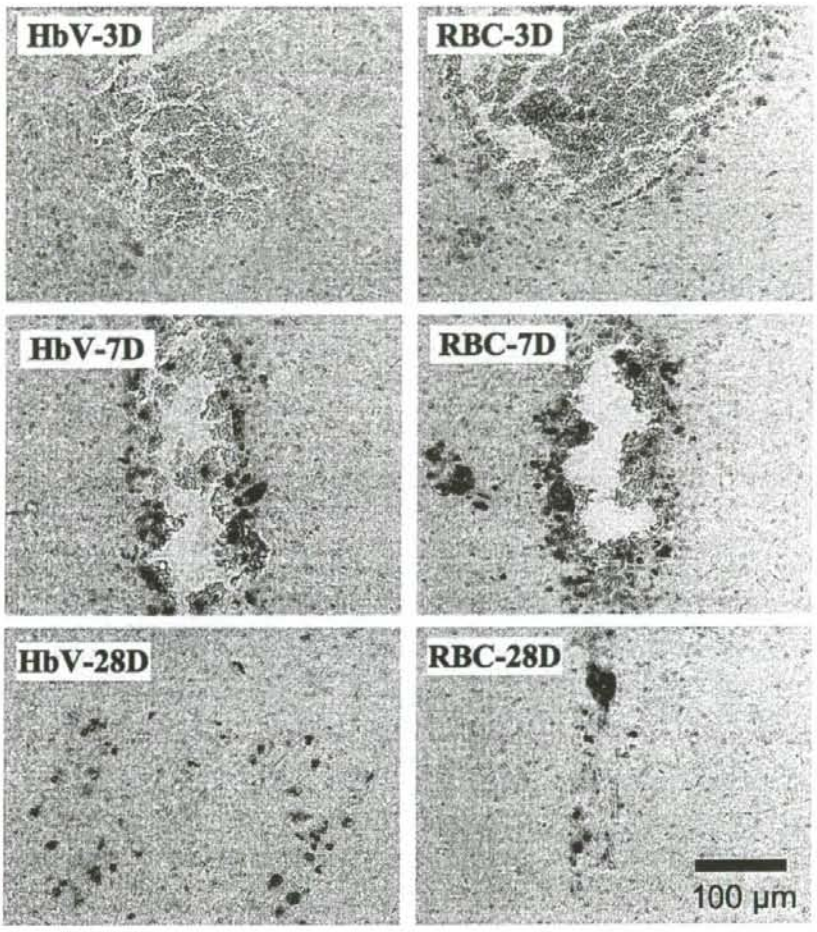

Figure 6. Berlin blue staining of rat brain 3, 7, and 28 days after injection of HbV and RBC. Large amount of hemosiderin deposition was confirmed just near the hematoma site for both groups. Hemosiderin deposition was confirmed at 28 days for both groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

will be quite important, therefore, to investigate these differences in future studies, in particular, to investigate how the HbV is biodegraded in the brain.

Perihematomal edema might involve in damage to the vascular endothelium. Iron is a potent catalyst of lipid peroxidation; the release of iron (a breakdown product of Hb) after RBC lysis might contribute to BBB damage, brain edema, reduced blood flow, and cell death.[16,18,28,31,40,41] Actually, TUNEL staining demonstrated that both groups had apoptotic cells during the entire period of observation. The strong perihematomal hemosiderin deposition observed in our current study also suggests that they are derived from RBC lysis. However, the fact that the surrounding tissue, infiltrated with HbV, showed no strong hemosiderin deposition compared to that of RBC, could indicate that HbV was more stable and degraded slowly by phagocytosis of the macrophages. This study did not identify whether the macrophages were microglia or those infiltrated from blood through BBB. The phagocytized HbV in the macrophages, however, were clearly present, even at 28 days, by staining with an anti-human Hb antibody.

Degradation of HbV in the brain seemed much slower than those observed in liver Kupffer cells and spleen macrophages after the HbV intravenous injection.[5,13] This gradual degradation might be advantageous clinically to prevent acute toxic effects of Hb molecules. It has been known that phospholipid vesicles (liposomes) are unstable capsules. However, the stability of liposomes depends on the selected physicochemical characteristics of lipids. Our HbV,

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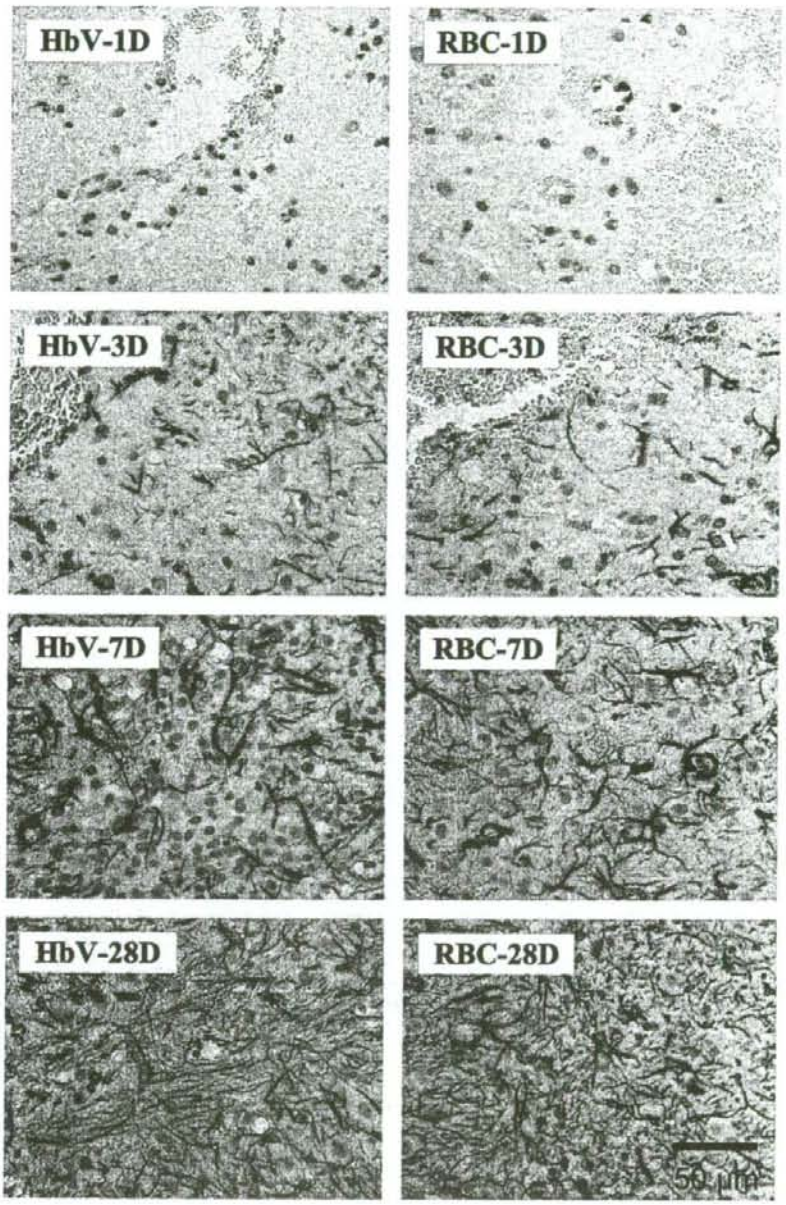

Figure 7. Immunochemical staining with anti-GFAP-staining 1, 3, 7, and 28 days after intracerebral injection of HbV and RBC. On day 1, the initial destruction of astrocytes in the perifocal zone for both groups, and from 3 days a dense network of hypertrophic processes reentered the perifocal zone. At 7 days, the hypertrophic and hyperplastic astrocytes were distributed in a large area. At 28 days, the presence of gliosis was evident in both groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

composed mainly of DPPC, cholesterol, and PEG-conjugated lipid, has been tested to be enormously stable, as presented in Table II, against physical stimuli by hypotonic shock and freeze-thawing. The process of phagocytosis involves the secretion of PLA_2.[42] The *in vitro* demonstration of HbV resistance to PLA_2 enzymatic lipolysis might support the notion that the HbV is also more resistant to PLA_2 in the brain and shows a lower rate of degradation than RBC. Moreover, it has been reported that

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TABLE II
Structural Stability of HbV in Comparison with RBC ($n = 3$)

Stimuli	Hemolysis (%)	
	HbV	RBC
Hypotonic lysis	0.4 ± 0.0	94.0 ± 0.7
Freeze-thawing	20.4 ± 1.2	77.6 ±1.2
PLA2, 30 min	0.0 ± 0.0	12.8 ± 0.3
PLA2, 2 h	0.1 ± 0.0	17.7 ± 0.7

Mean ± SE
PLA2, phospholipase A2.

DPPC, which comprises saturated acyl chains, is less subject to lipid peroxidation than the unsaturated phospholipids in biomembranes.[43,44]

Acellular intramolecularly crosslinked Hb solution was clinically tested in stroke patients; production of significant adverse events was reported.[45] The same material was also tested for the treatment of severe traumatic hemorrhage. The mortality rate seemed increased, especially in patients with head injury.[19,26] Even though the mechanism for this adverse response has not been clarified, it is anticipated that Hb molecules (6 nm) smaller than HbV (250 nm) easily infiltrate into the perifocal tissues when the BBB breaks down. The extravasated Hb would directly interact with the cells and potentiate brain damage.[46,47]

Significant morphological changes of astrocytes were observed in our study. Astrocytes are the most numerous cells in the central nervous system. They provide structural, trophic, and metabolic support to neurons and modulate synaptic activities. Impairment in the astrocyte functions during brain ischemia and other insults can critically influence neuron survival.[48] After traumatic injury, surviving astrocytes are well known to begin to exhibit hypertrophy and proliferation.[49,50] In our study, GFAP-immunoreactivity showed that, from 3 days, a dense network of hypertrophic processes appeared in the perifocal zone. At 7 days, the hypertrophic and hyperplastic astrocytes distribute in a large area, replacing the hematoma. At 28 days, the hematoma scar remained as gliosis. This process closely resembles the observations of the RBC group in the current study, and in the reported pathological profile after ICH.[51]

Aside from blood substitute research, phospholipid vesicles or liposomes encapsulating or embedding functional drugs or biological materials have been investigated aggressively for use in drug delivery systems or controlled release; some were subsequently approved for antifungal or anticancer therapy.[52] The BBB presents an obstacle for efficient drug administration using nanocarriers to brain tissue.[53] In fact, intracerebral injection of antitumor drugs encapsulated in liposomes is documented.[54,55] From the viewpoint of biomaterial science, results of the present study assure the safety of the present lipid formulation used for the HbV, and show it to be applicable for drug release systems for intracerebral injection.

In conclusion, intracerebral injection of HbV caused infiltration of HbV into the perihematomal brain tissue and the inflammatory reaction that consist of neutrophil accumulation at the site of injury, subsequent gradual degradation of HbV in macrophages, and hypertrophy of astrocytes to reconstruct the injured tissue. These are all expected to be normal reactions to the injury. Actually, there was no aberrant reaction in comparison to the injection of RBC. On the other hand, the delayed degradation of HbV might benefit tissue reconstruction after treatment with HbV infusion as a resuscitative fluid. Further investigations should follow to show the neurological safety of the lipid components of HbV. Because the HbV remained in the brain at 28 days, further investigations should also include longer period of observation. Our present data provided valuable information related to the safety of HbV and encourage us to proceed to clinical research of HbV as a transfusion alternative.

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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 Mg/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), 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.

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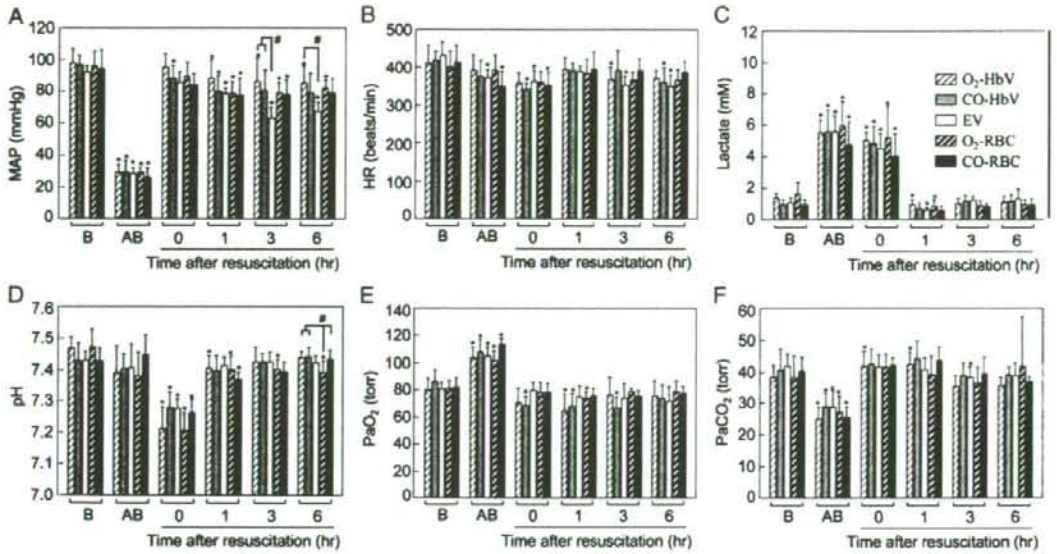

Fig. 1. **Changes in systemic parameters in rats before and after hemorrhage and resuscitation by injection of O_2-HbV, CO-HbV, EV, O_2-RBC, or CO-RBC suspended in 5% rHSA: (A) MAP, (B) HR, (C) lactate, (D) pH, (E) PaO_2, and (F) $PaCO_2$.** B indicates baseline; AB, after bleeding. $^*P < 0.01$ (Bonferroni 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

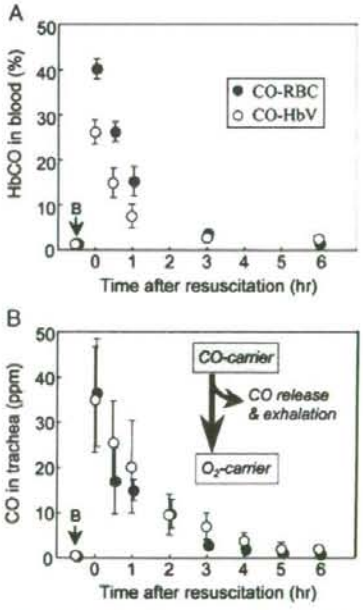

Fig. 2. **Time course of HbCO levels in blood (A) and CO levels in the trachea (B) before and after injection of CO-HbV or CO-RBC suspended in 5% rHSA as a resuscitative fluid for hemorrhagic shock in rats.** B indicates baseline. Both CO-HbV and CO-RBC released CO and gradually became O_2 carriers. 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.

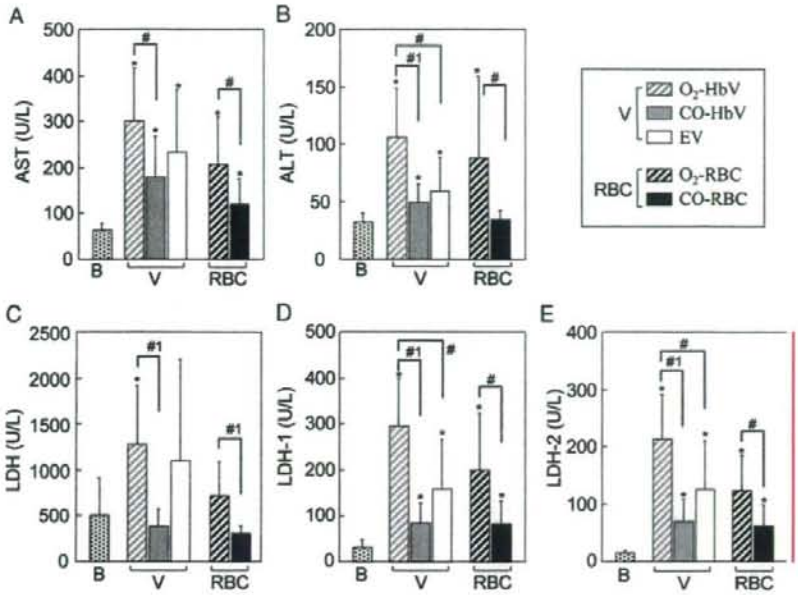

FIG. 3. **Plasma enzyme levels 6 h after injection of O_2-HbV, CO-HbV, EV, O_2-RBC, or CO-RBC suspended in 5% rHSA: (A) AST, (B) ALT, (C) LDH, (D) isozyme LDH-1, and (E) isozyme LDH-2.** B indicates baseline. $^*P < 0.05$ versus baseline group; $^{\#}P < 0.05$ versus the indicated group; $^{\#1}P < 0.01$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

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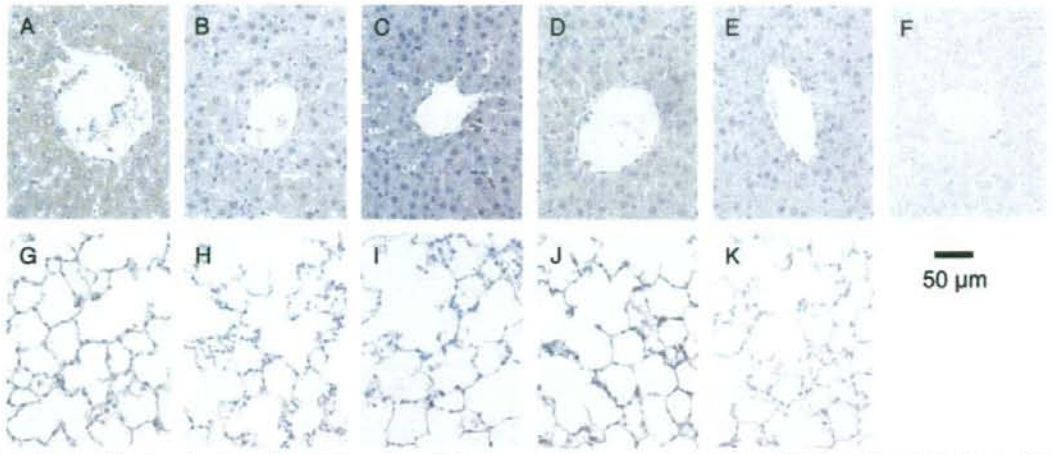

Fig. 4. **3-Nitrotyrosine detection in rat liver (A–E) and lung (G–K) tissues 6 h after resuscitation: (A) O_2-HbV, liver; (B) CO-HbV, liver; (C) EV, liver; (D) O_2-RBC, liver; (E) CO-RBC, liver; (F) O_2-RBC, liver, without primary antibody against 3-nitrotyrosine; (G) O_2-HbV, lung; (H) CO-HbV, lung; (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.

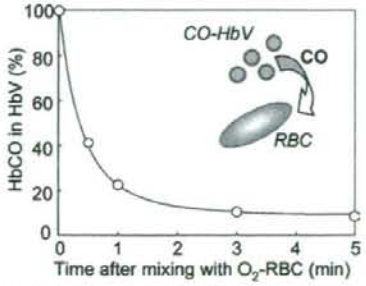

Fig. 5. ***In vitro* CO release of CO-HbV after rapid mixing with O_2-RBC.** A CO-HbV ([Hb] = 10 g/dL, 0.5 mL) and an O_2-RBC suspension (10 g/dL, 4.5 mL) were mixed immediately. The level of HbCO of HbV fraction after centrifugation was monitored. Results indicate that the CO release rate is unexpectedly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

ACKNOWLEDGMENTS

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学会報告

FDA Workshop on Hemoglobin Based Oxygen Carriersに参加して

Report on "FDA Workshop on Hemoglobin Based Oxygen Carriers"

酒井宏水[1]，堀之内宏久[2]，小林紘一[2]

Hiromi Sakai[1], Hirohisa Horinouchi[2], Koichi Kobayashi[2]

2008年4月29-30日に米国Maryland州Bethesdaにある《米》国立衛生研究所（National Institute of Health, NIH）にて，《米》食品医薬品局（Food and Drug Administration, FDA）主催のワークショップ（Hbを用いる人工酸素運搬体）が開催された．これに参加したので，その概要を報告したい．

一番の話題は，Journal of American Medical Association（JAMA）に報告の，欧米で開発されたCell-free Hb-based oxygen carriers（HBOCs）の臨床試験結果のメタアナリシスについてである[1]．北米の5社が開発したHBOCsの臨床試験結果の論文13報，その他3件の公開データをもとに3,711人分のデータを解析している．Cell-free HBOCs投与群と対照群の比較では，死亡率および心筋梗塞の発症率がHBOCs群で高く，臨床試験とはいえヒトに投与するには極めて危険な製剤であり，何らかの改良がなされ，十分に動物試験で安全性を確認すべき，とNatanson博士（NIH researcher）らは主張している．JAMAの論文は4月28日（Workshopの前日）にOn lineで公表されたばかりであったが，Workshop当日の朝に見た現地の新聞（USA Today）には，JAMA誌の内容の一部と，これがFDA Workshopで議論されるであろうとの予告が掲載されていたことには驚いた．しかし偶然とは思えなかった．

会合ではNatanson博士らの見解に対し，当然の事乍ら反論もあった．この分析には，Baxter社のHemassistをはじめ，Hemosol社のHemolink，Biopure社のHemopure，Northfield社のPolyHeme，Sangart社のHemospanの臨床試験結果について，それぞれが物性の特徴に違いがあるにもかかわらず，ひとまとめにして解析をしていることである．反面，動物実験の段階で十分な安全性評価が為されていなかった為に，今になって副作用が明らかになったことも問題である．ICH（International Conference on Harmonization）ガイドラインの非臨床動物実験は，HBOCsの安全性試験には向いていないこと，企業も臨床試験の全データを公表しておらず，これをFDAも知らないデータがあり得ること，またFDAに対しても，患者（被験者）を救うために臨床試験を早期に中止すべきではなかったのかとの批判もあった．

過去にBaxter社が分子内架橋Hbを展開し，以前から血管収縮と血圧亢進のことが問題となり，Phase IIIで漸くそれを重篤な副作用として認め，撤退に至った経緯がある[2]．誰もが重合Hbであれば，このような副作用は低減されると考えていたにもかかわらず，今になって重合Hbでも副作用が明らかになり，間違いをまた繰り返しているように思える．二日目のセッションでは，HBOCsによるNO捕捉と血圧亢進の対処策として，NO吸入，NO_2の投与や，ハプトグロビンでHbを捕捉して副作用を低減するような話題提供があったが，どれも対処療法であり，新しい物質が誕生しない限り問題解決は期待できないのではないかと感じた．

今回の騒ぎで懸念されることは，HBOCs開発の全体的な停滞である．しかし，幸いにもNatansonらの論文の標題は，"Cell-free Hemoglobin-Based Blood Substitutes and Risk of Myocardial Infarction and Death"であり，意図的かどうか解らないが「非細胞型」のHBOCsにはっきりと限定している．論文および議論の中では，いわゆる「細胞型」のHb-vesicles（HbV）については全く触れていないのであるが，Cell-free HbとCellular Hbとの違いをどれだけの人が理解して下さっているか．日本の「細胞型」HbV開発者は，細胞型の利点を明確にし，Cell-free Hbとは全く違うことを主張していかなければならない．

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本ワークショップには300名以上の参加者があった．日本からは，早慶から酒井，堀之内，小林が，また，東海大学医学部の川口章先生が参加した．昨年北京で開催された第11回国際血液代替物学会（11th-ISBS）では，中国でのHBOCsの研究が国家プロジェクトとして推進されていることが良く解ったが，今回も中国からの参加者も散見され，HBOCsの開発の結末がどうなるのか静観しているように思えた．北米の非細胞型の化学修飾HBOCsの開発で生じた問題点を凝視し，分子デザインから新たな展開を開始する時期に差し掛かったのかもしれない．

以下にプログラムに従って，各発表者の内容について，パワーポイント発表および小生のメモをもとに，重要なことを書き出した．しかし，小生の英語力の無さのため，聞き落としや誤解もあるし，時差ぼけの影響もあり，物足りないと感じられることもあろう．また，空欄の箇所もできてしまったことには，お詫び申し上げたい．詳細について知りたい読者には，FDAのホームページにTranscriptが掲載されているので，これを参照して頂きたい（http://www.fda.gov/cber/blood/hboc042908.htm）．

Tuesday, April 29, 2008

Opening remarks

Jesse L. Goodman (Center for Biologics Evaluation and Research, FDA)

Simone Glynn (National Heart, Lung and Blood Institute, NIH)

Jerry A. Homberg (Office of the Secretary and Office of Public Health and Science, HHS)

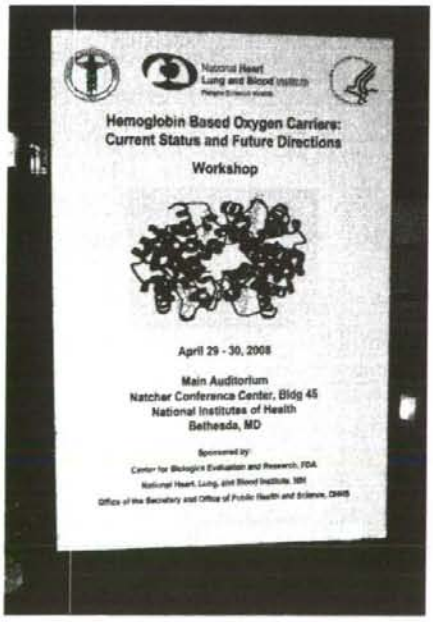


写真1．会場入口の案内

Session I, Workshop Overview and HBOCs Update

Overview of the Workshop

Moderator: Joseph C. Fratantoni (Maxcyte)

Overview of Oxygen Physiology

H.F. Bunn (Harvard Medical School)

血行動態が維持されなければ，酸素は運搬できない．NO捕捉を抑制すること，生理的に適したP_{50}値，膠質浸透圧の調節が重要であることを主張．また，StamlerらのSNO-Hbの説について，Hbのβ93CysがSNO-Hbの発生部位なので，これをアラニンに置換したマウスを使って検討したところ，血管収縮などは全く無かったとの論文[3]が最近出ており，SNO-Hbの説を否定している．

HBOCs: Biochemical and Physiological Perspectives

A.I. Alayash (CBER, FDA)

Hbの毒性について詳細に説明した．先ず，Hemosol社のo-raffinose polymerized Hbについて．分子量分布が広く，monomeric Hbも含有する，ヘム構造が歪んだrhombic hemeの存在，T構造が固定されていて，酸素親和度が異様に低い（P_{50}が高い），様々な有用性が言われているβ93Cysが化学修飾のときの架橋点になっている．H_2O_2との反応により毒性の高いフェリルHbを産生する．心筋に対する毒性が組織病理から明らか．NO反応と自動酸化によるメト化を抑える方法が必要．Biopure社のOxyglobinについて，アスコルビン酸を体内で産生する動物種（例えばラット）であれば，血中でmetHbが還元される．しかし，ヒトではそうはいかない．HbとSOD/Catalaseの架橋，trolox, glutathioneなど抗酸化剤の利用を検討すべき．

Nitric Oxide and Nitrate Ions: Physiology, Pathology and Pharmacology

Alan N. Schechter (Molecular Medicine Branch, NIDDK, NIH)

HbとNOの反応の概説．NO吸入によるHbNOの増大と同時に，Nitrate（NO_3^-）が顕著に増大する．Nitrite（NO_2^-）はさほど増大しない．NitriteはdeoxyHbで還元されてNOになることが明らかになった．Nitriteの投与試験から血圧の降下を実証．NitriteをNOドナーとして利用できる．

Non-Clinical Testing: Strengths and Limitations

George P. Brio (Univ. of Ottawa and Univ. of Toronto)

従来のICH基準，およびGLP基準の非臨床動物実験（健常動物への単回投与，反復投与）では，安全性について十分な評価ができないのではないか．見落とす項目が出る可能性あり．疾病モデル動物の使用が必要．アカデミックな研究では，一般に効能についての研究が多く，また観察項目が限定されているため，一般性が持てない場合がある．血管内皮傷害は，様々な病態と

関係している．HBOCを投与するとNOが捕捉されるので，血管障害を助長する可能性がある．

Session II , Clinical Experience with HBOCs

Introduction to the Issues

Toby A. Silverman (CBER, FDA)

臨床試験の考え方について概説．Traumaに対する投与試験の難しさ．死亡率は明白なendpointであるが，短期生存と長期生存の結果が一致しない．QOLのある長期生存が患者およびその家族に最も重要である．輸血の出来ない場合（遠隔地など）を想定した臨床試験の倫理的・技術的問題．これまでの各社臨床試験の結果を解析し，その副作用をまとめた一覧表を提示（左心室心筋への影響，心筋虚血，血管収縮，消化器系の不快感/嘔吐/下痢/嚥下傷害/腹痛/腸管からのバクテリア侵入）．警告（Caveats）として，臨床試験の全てが公表されている訳ではない．今回ここに公表されているデータは，FDAに最終報告されたものとは違う．データ解析法や，解釈に問題がある場合もある．血中酵素濃度の上昇を，副作用とみなしていない場合がある．最後に課題として，もっとHBOCsの特徴について科学的に検証する必要あり．臨床上の利益を得る点ではendpointを明確にする必要あり．Survivalのみでは十分な評価ができない可能性があり，Surrogate markerを設定して，安全性と有効性を評価する必要がある．臨床的安全性の理解（最大投与量など），投与の損益の理解，論理的な臨床開発プログラムの決定，など．

Risk: Benefit considerations in Clinical Trials

Sara F. Goldkind (Office of the Commissioner, FDA)

FDAは，その薬剤を使用することの利益が，既知の毒性または副作用の可能性よりも重要であるかを検討する．また，病態が重篤であるか，他の治療法が無いかも考慮して，薬剤の危険性について回答するか判断する．

Presentations from Industry: Proposed Clinical Indications for HBOCs and Clinical Trial Experience to Date

Moderator: Barbara Alving (National Center for Research Resources, NIH)

Development of Hemospan

Peter Keipert （Sangart Inc.)

Dr. Robert M. Winslow 欠席のため代理としてDr. Keipertが発表した．様々なHBOCsがあるが，同じではない（Polymerized Hbsと一緒にして欲しく無いという意味）．Hemospanは若干の血圧亢進はあるが心拍出量を増大させ，末梢血管抵抗を低下させる．Autoregulatory Theoryの観点から重要なことは，分子サイズが大きいこと，酸素親和度が高いこと（P_{50}値が低い，5 mmHg），低Hb濃度（4.3 g/dL），膠質浸透圧が高いこと．NO結合速度と血管収縮は関係がない．Phase II（Sweden）では，整形外科手術（関節形成術）における投与試験を実施．血圧維持効果を実証．二例の死亡例あったが，Hemospanの投与とは無関係と判断．Phase III（欧州）を開始する予定．低血圧の予防（n = 370）と治療（n = 460）の試験について被験者登録名簿が今年完了する見込．Hemospanの用途は，ショックの蘇生，術中の血圧安定化，血液が利用できない場合の輸血迄のつなぎ，虚血領域への酸素ターゲティング．限界は，Hb濃度が低いので，血液希釈度が高いときに対応できない，膠質浸透圧が高いので，体液量過剰が起こりうる．

写真2．会場風景．左から，Dr. De Angelo, Dr. Estep, Dr. Greenburg, Dr. Abuchowski, Dr. Gould, Dr. Keipert, そしてDr. Goldkind

Clinical Development of Polyheme

Steven A. Gould (Northfield Laboratories, Inc.)

「Unmet medical needs」（いまだ満たされていない医学的ニーズ），つまり赤血球輸血が出来ない状況での対処法として，Polyhemeが有効である．I U =500 mL, [Hb] = 10 g/dL，1年間保存可能．Hospital Trauma Trial（n= 171）では，投与量に応じて血中Hb濃度が高くなること，死亡率の減少を確認[1]．Pivotal Phase III Trial（n = 720）重篤な外傷出血患者を対象とした試験では，過去に問題点が指摘された．実際には，124名の患者の登録が正しく行われなかったので，再度解析を行った．その結果，PolyHeme投与の方が，副作用の発症率が高い（肺炎，MOF，出血性ショック，呼吸障害，凝固亢進，敗血症，心筋梗塞）．それでも，赤血球輸血が出来ない状況での対処法として，Polyhemeは有効である．

HBOCs: Current Status and Future Directions

Abraham Abuchowski (Prolong Pharmaceuticals)

PEG-bovine Hbを開発したEnzon社の設立者．PEG修飾蛋白質3種類が既にFDAの認可を得ている（Adagen, Oncaspar, PEG-Intron）．PEG-HbについてはEnzon社の時代に，Phase Ib（n = 60）を実施．PEG-Hbの特徴: P_{50} = 14 - 20 mmHg, [Hb] = 4 - 6 %, met Hb < 10%, -20℃で6ヶ月以上安定，4℃で7日間安定．イヌ投与試験（30%血液交換）では特に問題なし．ブタ投与試験（80%血液交換）では，腎臓と脾臓に空包変性があるほかは特に副作用なし．Phase I試験（ n = 34），1.67 - 8.33 ml/kgを投与．食道痙攣がみられた例があったが，鎮痙薬（levesinex）の投与で解消した．適応としては，放射線増感剤

があり，転移性疾患の患者に対するPhase Ib試験（n=33）では，2－8ml/kgを投与した．副作用としては，緩慢な血圧上昇（24%），不全失語症（24%），吐き気（24），嘔吐（12%）．製品名AfterShock™は，PEG-Hbを高張食塩水溶液に溶解させた溶液．ショックの蘇生液として期待される．

Biopure HBOC-201

A. Gerson Greenburg (Biopure Corp.)

終始，過去のFDAの見解に対する反論．HBOCsの副作用について，全てのHBOCsが同じではない．安全性のシグナルが毒性と解釈されることがある．血管活性と主要臓器に対する毒性の因果関係は無い．HbOC-201は生命を維持するに十分な酸素運搬が出来る．FDAが要求した補足実験（ブタ10-30%交換輸血後の臓器血流量と酸素分圧の測定）の結果，主要臓器の血管収縮の証拠は無し．骨格筋では血管収縮あり．冠動脈の血管収縮なし．Pittmanらの微小循環計測の結果，血圧亢進と骨格筋の血管収縮はあったが，腸管では血管収縮なし．臨床試験でも冠動脈の収縮は無い．血圧亢進はあっても，冠動脈性心臓病の患者に影響を与えない．動物実験では，血液が無い状態でも，左心室系の機能を維持できた．臨床試験において患者の管理方法に問題があったため，効能の評価に不整合が生じた．重篤な虚血状態に対しては，赤血球であろうとHBOCsであろうと副作用を低減させる事はできない．血液が無い時，HBOCsは利用できる利点がある．HBOCsの臨床試験は継続すべきである．今行うべきことは，安全性シグナルを正確に解釈する事，現在のマテリアルの最も適した用途を考える事，そしてHBOCsの開発を継続する事．

Lessons Learned from the Baxter Experience in the development of HBOCs

Timothy N. Estep (Chart Biotech Consulting)

Baxterの分子内架橋Hb（DCLHb）の副作用の一つに心筋損傷がある．実はpreclinical studyから解っていた．IND（investigational new drug）申請までの2年間にこの副作用の解析に費やした．その結果は，既に論文で公表されている[3]．サル，ブタで投与後24-28時間で発症するが，時間と共に治癒する．CK_{mb}，LDH_1はさほど上昇しなかった（但しoverallのCK，LDHは，ブタで上昇した）．心機能的には問題は無かった．心筋損傷は，SFHbでも認められた．またHbを重合，或はNO結合を遅くする事で低減された．NOS阻害剤であるL-NAMEの投与で同様の心筋損傷が認められた．しかしヒトでの確認は生検に困難を伴う．次に血管収縮について，NO反応性の制御，血管外漏出の抑制によって低減されるが，種差がある．何らかの薬剤の併用によって対処できる？．米国のPhase IIIでは，DCLHbの死亡率（46%）が生理食塩水投与（17%）に比較して高くなったため，試験を中止した．しかし，因果関係は解らない．事実，欧州での試験では，死亡率は42% vs. 38%で，さほど違いはなかった．重篤な外傷患者の登録方法，管理方法に問題があったか．また，蘇生方法自体の問題（overloadなど），Halothaneとの相互作用，LPSとの相互作用，外傷の種類（鈍的外傷が貫通外傷よりDCLHb投与後の死亡率が高い），外傷性患者を対象とする臨床試験の難しさもあったか．重篤な患者の方がTreatment groupになり易い？その他，副作用として，DCLHb投与によってMOFになり易い傾向，膵臓炎，NO捕捉による酸素消費量の増大?今後HBOCsの開発に必要なことは，全組織への血流分布の計測，臓器の病理検査，非侵襲のモニタリングなど．問題は多いが，unmet medical needs の対処法としてHBOCsは有望である．

Development of PHP as an NO Scavenger in the Treatment of Distributive Shock.

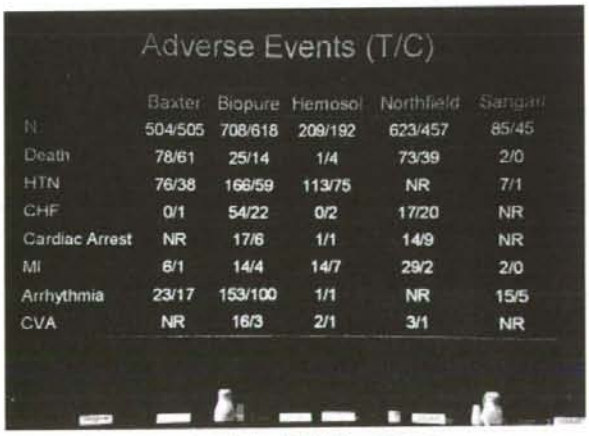
Joseph De Angelo (Apex Bioscience, Inc.)

PHP（pyridoxalated hemoglobin polyoxyethylene conjugated）は，血管外漏出し，細胞間質のNOと反応する．正常の場合には，NOは重要な働きを示すが，過剰量になると問題になる．この過剰産生されるNOの捕捉をPHPで行う．カテコールアミンが血液分布異常性ショックの治療に使用されるが，問題点もある．これに比較してPHPの方が，副作用が低減されるものと期待できる．臨床試験（SIRS患者，ショック患者に対する投与：20 mgHb/kg/hr）の結果，MAPの急激な上昇がみられたが，28日生存率は，PHP 57.6%に対してPlacebo 58.6%で殆ど違いは無い．心筋虚血の発症例も，高めであった（淡々と発表をしていたが，PHPの利点が殆ど解らない発表であった．）．カテコールアミン耐性の患者を対象としたPhase IIIを計画中．

Panel Discussion

Wednesday, April 30, 2008

Session III, Clinical Findings and Mechanisms



	Baxter	Biopure	Hemosol	Northfield	Sangart
N	504/505	708/618	209/192	623/457	85/45
Death	78/61	25/14	1/4	73/39	2/0
HTN	76/38	166/59	113/75	NR	7/1
CHF	0/1	54/22	0/2	17/20	NR
Cardiac Arrest	NR	17/6	1/1	14/9	NR
MI	6/1	14/4	14/7	29/2	2/0
Arrhythmia	23/17	153/100	1/1	NR	15/5
CVA	NR	16/3	2/1	3/1	NR

写真3．各社HBOCsの臨床試験の結果総括．T/C, Treatment versus Control; MI, myocardial infarct 心筋梗塞; CHF, chronic heart failure, 慢性心不全; CVA, cerebrovascular accident, 脳血管障害; HTN, hypertension, 高血圧; cardiac arrest, 心停止; arrithmia, 不整脈

Functional Aspects of the HBOCs as a Class
Panel Discussion

Moderator: Harvey Klein (Dept. of Transfusion Medicine, Clinical Center at NIH)
冒頭，臨床試験の結果全てが公表されている訳では無い事，FDAも知らされていない結果がある事を公言した．

Demetrios Demetriades (Univ. of South California)
臨床試験の難しさ．外傷の種類によって死亡率が異なる(penetrating trauma, blunt trauma)．輸血において大量輸血を必要とした場合，パラメータとして，血漿または血小板：赤血球の比が死亡率を決める重要な項目であることを主張．この比が1：8のとき，死亡率は65%．1：1.4のとき19%にまで低下．

Daniel Freilich (Naval Medical Research Center)
軍服姿で発表．非臨床試験の結果から，外傷を対象とする臨床試験のプロトコルを決定しなければならない．効果を最大限に引き出すため，死亡率が高い重篤な出血性ショック患者を対象とする．輸血ができない状況を選択する．Hb濃度の高いHBOCsを用いる．損害を最小限にするため，高齢よりも若年層を対象とする．血圧の回復だけを指標としない，他の汎用輸液も併用する，担当者の危機管理意識，副作用が出たときには直ぐにHBOCsの投与を止める．副作用の報告は迅速に．血管活性の少ないHBOCsを選ぶ，止血ができたらニトログリセリンを投与して，HBOCsの血管活性の影響を低減させる．制御不能出血に使用する状況を考えている様子であった．

John Holcomb (Univ. of Texas Health Science Center at San Antonio)
大量出血患者がHb濃度が11 g/dLとすれば，Hbは十分にある．Bleeding problemとperfusion problemをどう考えるか．各社とも臨床第1相，第2相試験で，利点が得られなかったが，出血性ショック患者について現場で，試験対象患者基準と，除外基準をどうすべきであったかが，最も重要であった．

Charles Natanson (Critical Care Medicine, NIH)
現在のHBOCsの臨床試験の結果について，JAMAに発表の内容を説明（記述の通り）[1]．メタ解析の結果から，現行のHBOCsは死亡率が平均で30%上昇，心筋梗塞の発症率ともに高い．違うものを一色単にして解析して良いのかという議論もあるが，どれか一つの製品についてのデータを除去しても，同じ結果になった．P_{50}値との関係，公表データと未公表データの関係なども検討したが，結論からいうと，臨床試験プロトコル，製造者，物質の特性，結果の出典に拘わらず，死亡率，心筋梗塞の発症率はHBOCs群で高い．

Edward J. Norris (Johns Hopkins Univ. School of Medicine)
発表スライド無し．エホバの証人の患者100人に対し，総量200unitも投与してきた．HBOCsがunmet medical needsに対応できることを評価するべき．

Edward P. Sloan (Univ. of Illinois at Chicago)
Baxter社のDCLHbの臨床第三相試験：重傷出血性ショック患者に対する投与で，投与28日後の死亡率の評価では，Control群で32%に対し，DCLHb群で45%と高い値あった[2]．しかし，DCLHb投与に起因する血圧亢進は殆ど無かった．また，塩基欠乏，乳酸値についてもDCLHbとの因果関係は無かった．ショック指数について（SI = HR/BBPが ≧1は，非代償性ショック，SI ＜1は代償性ショック），DCLHbを投与することによるSIの変化は無かった．DCLHbの検討の結果，i) HBOC は重要である，ii) 論理的に想定されるDCLHbの影響が，*in vivo* でいつも観察される訳ではなかった，iii) 臨床的に有用なHBOC蘇生液を見つける必要あり．今後のHBOCs開発に対する忠告として，i) HBOCsの基礎研究と臨床試験は継続すべき．ii) 論理的に想定できる現象が臨床的に認められるか見極める，iii) 効能に関する質問に的確に答えるられるよう，臨床試験の方法の最適化を行う．

Gus J. Vlahakas (Harvard Medical School)
Biopure社のHBOC-201の臨床第二相試験について．心臓手術後の血液希釈された状態に使用し，ヘマトクリットが回復するまでの一時的な酸素運搬体として使用し，同種血輸血を回避することが目的．特に心筋梗塞は認められなかった．しかし，HBOCsは血管収縮を起こす事が良く知られている．NOの結合，体動脈圧，肺動脈圧の上昇が報告されているが，NO結合と血管抵抗の上昇が，代謝性自動調節機能を覆すような作用を及ぼす事は無い．HBOCsの特性が有用である場面もありうる．血管収縮の他に，HBOCsはどのような不安定要素を持っているか，消化器系において，膵臓疾患（lipase, amylase 上昇），肝臓疾患（AST, ALT上昇），胸痛（食道の蠕動運動への影響）が報告されている．心筋梗塞について，DCLHbのサルを使った実験で最初に報告され，L-NAME投与のばあいと同様の症状であった．NOの結合が低減されたrHb2.0で低減された．

Stephen Cohn (Univ. of Texas Health Science Center at San Antonio)
米国では，Trauma Centerに1時間以内に到着出来るのは40%．従って田舎で外傷に遭うと，輸血が出来ない場合がありうる．

Organ Specific Aspects of Safety
Panel Discussion

Moderator : Richard Weiskopf (Univ. of California, San Francisco)