J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 3

full recovery from the anesthesia. The animals were then housed in cages and provided with food and water *ad libitum* in a temperature-controlled room with a 12-h dark/light cycle. The animals were observed at 6-, 12-, and 24-h post-surgery for any sign of bleeding from the site of incision, as well as pain and discomfort and infection. The well-being of animals was followed daily throughout the experiment. Buprenorphine injections were repeated every 8 h if there was any sign of pain or discomfort.

Behavioral testing

Body weights of animals and their well-being were observed at 1, 2, 3, 5, 14, and 28 days after the surgery and prior to the time of sacrifice for histopathological studies. Similarly, behavioral testing, which indicated motor dysfunction, was performed on the days presented in Table I. Data of the normal rat group (no treatment, $n = 7$) were also collected.

Histopathological examination

At 1, 3, 7, and 28 days after the surgery, seven animals in each group were euthanized with ether in a gas chamber. The brain was isolated; its wet weight was measured. The brain was then fixed in a 10% formalin-phosphate buffer solution (Wako Pure Chemical Industries, Tokyo, Japan). The fixed brain was sliced to 1-mm thickness, anterior and posterior, vertical to the injection needle mark.

The sliced brain was examined histochemically using hematoxylin and eosin (HE) staining for any pathological changes, and Berlin blue staining to confirm the presence of hemosiderin. Immunohistochemical stains were performed for glial fibrillary acidic protein (GFAP) of astrocytes, inducible hemoxygenase-1 (HO-1), human Hb of HbV, and apoptotic cells.

For immunohistochemistry to observe astrocytes, the paraffin sections (4-μm thick) were mounted on 3-aminoprophyl triethoxysilane-coated glasses. After deparaffinization, the sections were subjected to microwave treatment for 10 min with 10 m*M* citrate buffer (pH 6.0) for antigen retrieval. They were incubated with methanol containing 0.3% hydrogen peroxide (H_2O_2) for 30 min at room temperature, and subsequently with 2.5% normal horse serum for 15 min at room temperature. The tissues were then reacted with mouse monoclonal antibody against GFAP (1/200 dilution; Dako, Glostrup, Denmark) for 3 h at room temperature. After washing in PBS, they were incubated in ImmPress Reagent Peroxidase anti-mouse Ig (ImmPress Reagent Kit; Vector Laboratories, CA) for 1 h at room temperature. The color was developed with 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.2 mg/mL; Dojindo Laboratories, Kumamoto, Japan) in 0.05 *M* Tris-HCl (pH 7.6) containing 0.003% H_2O_2; the tissues were then counterstained with hematoxylin.

The staining methods of anti-rat HO-1 and anti-human Hb antibody have been described previously.[13,14] Briefly, the sections were treated with proteinase K (Dako, Glostup, Denmark) for antigen retrieval. After blocking the nonspecific binding with DAKO Antibody Diluent Solu-

TABLE I
Daily Behavioral Testing After Direct Injection of the Sample Solutions into the Brain

	Circling Behavior[a] (%)			Piloerection[b] (%)			Hind Leg Weakness[c] (%)			Absence of Startle Response[d] (%)			Hanging Time[e] (s)		
Days	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC	Normal Rat	HbV	RBC
1	0	0	0	71	41	25	14	71	78	0	23	13	18 ± 3	10 ± 1*	9 ± 1*
2	0	0	0	29	30	43	14	63	86	0	11	15	15 ± 4	12 ± 1	10 ± 1
3	0	0	0	29	61	53	29	56	62	0	26	23	14 ± 3	15 ± 2	11 ± 1
5	0	0	0	43	60	70	14	44	36	0	7	20	16 ± 4	17 ± 2	11 ± 2
7	0	0	0	29	33	70	29	33	21	0	13	0	13 ± 2	16 ± 3	12 ± 3
14	0	0	0	14	36	57	14	50	57	0	0	0	13 ± 4	13 ± 2	8 ± 2
28	0	0	0	43	50	86	29	75	100	0	0	0	5 ± 1	9 ± 2	9 ± 2

The number of animals (n) at each day: HbV group, 1d (34), 2d (27), 3d (23), 5d(15), 7d (15), 14d (11), and 28d (8); RBC group, 1d (28), 2d (21), 3d (17), 5d (10), 7d (10), 14d (7), and 28d (7); the normal rat group (no treatment), 1d – 28d (7).

[a]Spontaneous body circling.
[b]Ruffling of the body hair.
[c]Hesitant to walk on the beam (1.5 cm wide), and misses steps often; drags the foot as a rat walks on the beam.
[d]Startles and jumps to the blow of air to the face.
[e]Length of time spent hanging in second on a round plastic rod, diameter 4 mm. Mean ± SE.
*$p < 0.05$ vs. the normal rat group.

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 4

tion, they were incubated with mouse monoclonal antibody against rat HO-1 (GTS-3; Takara, Tokyo, Japan). They were then incubated with the secondary antibody (Simple Stain Rat MAX-PO[M]); Nichirei Corp., Tokyo, Japan). The color was developed using DAB, and the sections were counterstained with hematoxylin. A comparative staining procedure was performed with non-immune mouse IgG to confirm that the staining was specific to HO-1 and not derived from the color of hemosiderin deposition (DAKO).

For detection of human Hb derived from HbV, the sections were treated with rabbit polyclonal antibodies against human Hb (Dako). They were further incubated with alkaline phosphatase-conjugated swine antibodies against rabbit immunoglobulins (Dako)). The color was then developed using a New Fuchsin Substrate Kit (Nichirei Corp., Tokyo, Japan); the sections were counterstained with hematoxylin.

Apoptotic cells were stained with terminal deoxynucleotidyl transferase dUTP Nic-end labeling (TUNEL) with ApopTag^R Peroxidase *In Situ* Apoptosis Detection Kit (Chemicon International, Temecula, CA). The detailed protocol is described in the kit operating instruction. After pretreatments for antigen retrieval and quenching of endogenous peroxidase, the sections were incubated with a terminal deoxynucleotidyl transferase (TdT) enzyme solution, followed by a solution of anti-digoxigenin conjugate. The color was developed with peroxidase substrate and counterstained with hematoxylin.

Transmission electron microscopic observation (TEM) was performed to visualize the presence of the HbV particles in the brain tissue (PCL Japan, Tokyo, Japan). One brain of the HbV group at 1, 3, 7, and 28 days was fixed with 2.5% glutaraldehyde solution, cut in ~2-mm^3 blocks, and stored in 8% sucrose solution (0.1 mol/L phosphate buffer, pH 7.4). The fixed blocks were then washed with 0.1 mL/L phosphate buffer and stained with 2% osmic acid solution at 4°C for 2 h. Next, the blocks were dehydrated with ethanol solution by a stepwise increase in ethanol concentrations (50, 60, 70, 80, 90, 95, and 100%), 10 min for each step, washed with propylene oxide; then polymerized using Quetol 812 at 60°C for 28 h. The obtained blocks were sliced into 60–70 nm thickness using an Ultracut S microtome. The sliced tissues were stained with 3% uranyl acetate solution for 16–20 min, then treated with Satoh's lead solution (lead acetate, lead nitrate, and lead citrate) in citrate for 5 min, washed, and dried. The sliced brain tissues were examined under a transmission electron microscope (TEM, JEM-100CX; JEOL, Tokyo, Japan) and photographed.

Data analysis

The *in vivo* data are presented as mean ± SE with the indicated number of animals tested. Unpaired *t*-tests were used to compare the HbV and RBC groups.

In vitro stability of Hb-vesicles and RBC

Degradation rate of HbV would be influenced by the stability of HbV vesicles withstanding physical stimuli and enzymatic attack. To collect some information related to stability, the following three sets of *in vitro* experiments were performed:

1. Hypotonic hemolysis was induced by mixing HbV or RBC suspensions with distilled water at a 1:4 volume ratio ([Hb] = 1.72 g/dL). The mixture was then centrifuged and the supernatant Hb concentration was measured using a cyanomethemoglobin method[24] to determine the percentage of hemolysis.
2. Resistance of hemolysis to freeze-thawing was tested by diluting the sample of HbV or RBC 10 times with saline solution ([Hb] = 1.72 g/dL). One milliliter of the sample was put in a plastic tube and dipped in liquid-nitrogen for a few minutes. It was then thawed at room temperature. The samples were centrifuged and the supernatant Hb concentration was determined by the same method.
3. Hemolysis was induced by the enzymatic attack with phospholipase A_2 (PLA_2) to membrane phospholipids.[25] Four hundreds microliters of sample solutions of HbV or RBC in PBS ([Hb] = 1.6 g/L, pH 7.2) was added to a 400 μL stock solution of 10 m*M* $CaCl_2$ and 3 μg/mL PLA_2 (from Naja mossambica mossambica; Sigma, MO). The mixture was incubated for 0.5 and 2 h at 37°C; EDTA was then added to make a final concentration of 2 m*M*; the tube was cooled in ice for 5 min. The testing solutions were centrifuged or ultracentrifuged, and the supernatant was determined for Hb concentration to calculate the degree of hemolysis. The data are expressed as mean ± SE.

RESULTS

Body weight and behavioral testing

All animals in both HbV/rHSA and RBC/rHSA groups survived. After a slight weight reduction during the first 3 days, their body weights increased steadily to 327 ± 3 g at 28 days after the treatment (Fig. 1). Because of the initial loss, both groups showed lighter body weights in comparison to the normal rat group (untreated). The wet brain weight for both groups increased along with the increasing body weight (on the average HbV group, from 1.84 ± 0.04 to 1.94 ± 0.02 g; RBC group, from 1.86 ± 0.02 to 1.97 ± 0.02 g). No significant difference was apparent between the HbV and the RBC treated groups in brain weights. F1

The results of behavioral testing (Table I) indicate that animals in both HbV and RBC treated groups displayed no overt circling movement. Piloerection and startle responses were apparent for both groups; however, the startle responses reverted to normal by 7 days in both groups. The testing of the hind leg weakness on the beam showed signs of disability, that is, miss-steps and dragging of the left foot, in both treated groups; they were observed more often than in the untreated rat group. The testing of the

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 5

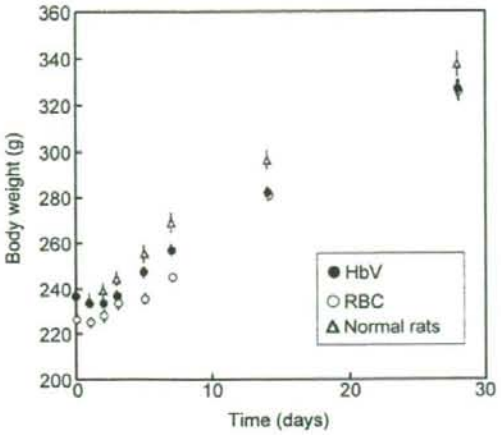

Figure 1. Changes in body weight after intracerebral injection of HbV or RBC. A slight reduction of body weight was apparent for 3 days at the beginning in both groups. However, the rats grew and gained weight rapidly to about 330 g at 28 days. Mean ± SE. Data of normal rats (no treatment) were also plotted as a reference.

ability to hang on a bar with front paws, which was the most sensitive test to detect the lack of motor coordination in our behavioral testing measures, showed some reduction in hanging time (HbV, 10 ± 1 s vs. RBC, 9 ± 1 s), with a significant difference from the untreated group (18 ± 3 s); however, no significant difference was found after 2 days until 28 days.

Histopathological examination of brain

The tip of the needle was positioned to the area of basal ganglia (Fig. 2). One day after the injection, HE staining showed the presence of hematoma in both groups. The HbV group showed a spread of red coloration in the perihematomal region, indicating that small HbV infiltrated into the surrounding parenchyma tissue. In the HbV group, the hematoma was composed of injected HbV and autologous blood because of bleeding caused by the needle insertion. A magnified photograph showed the presence of neutrophils surrounding the hematoma in both HbV and RBC groups because of the inflammatory reaction. The quantities of neutrophils were highest 1 day after injection in both groups; the numbers decreased significantly by 3 days. No notable difference in cellular responses was found between the two groups.

Staining with anti-human Hb antibody also supported the fact that HbV were diffused into the parenchyma. It was inferred from the micrographs taken 1, 3, and 28 days after the HbV injection (Fig. 3) that HbV was phagocytized by macrophages. The number of such macrophages decreased markedly by 28 days. However, the micrograph clearly showed the presence of human Hb in HbV, even after 28 days. Correspondingly, TEM clearly showed the presence of HbV in the phagosomes of macrophages at 1 and 3 days (Fig. 4). A macrophage phagocytizing both HbV and RBC is apparent as shown in the HbV group.

Staining with anti HO-1 antibody showed the induction of HO-1 at 3 and 7 days after injection for both HbV and RBC groups, particularly at the rim of the hematoma region (Fig. 5). However, the macrophages stained with HO-1 in the HbV group appeared more widely distributed in the perihematoma region compared with the RBC group at 28 days (data not shown). The DAB staining procedure with non-immune IgG instead of anti HO-1 antibody showed no brown staining (data not shown). It is concluded, therefore, that the brown stains are specific to HO-1 and not derived from hemosiderin.

Berlin blue method confirmed the hemosiderin deposition from 3 days after injection. A large amount of hemosiderin was deposited at a nearby hematoma site in both groups at 7 days (Fig. 6). The tissue area containing the HbV, however, showed less hemosiderin deposition. At 28 days, the hemosiderin remained in both groups.

Regarding immunochemical studies with GFAP, the reactive proliferation of astrocytes and gliosis was detectable at 3 days in both groups (Fig. 7). The hypertrophic and hyperplastic astrocytes were distributed in the area of the hematoma. The reconstruction processes were seen to occur by scar tissue formation, namely gliosis. Astrocytic cell reaction progressed. The glial fibers became stouter and more numerous, showing fibrillary gliosis at 28 days in both groups.

Furthermore, TUNEL-staining clarified the presence of apoptotic cells surrounding the hematoma in both groups. The HbV group showed 4.3 ± 3.6 cells (1 day), 2.0 ± 0.4 cells (3 days), 8.0 ± 1.9 cells (7 days), and 13.2 ± 4.1 cells (28 days). The RBC group showed 11.9 ± 7.2 cells (1 day), 1.5 ± 0.7 cells (3 days), 20.4 ± 9.8 cells (7 days), and 19.0 ± 6.1 cells (28 days). Both groups showed considerable variation in number of apoptotic cells. However, the RBC group displayed a greater number of apoptotic cells overall than in the HbV group, especially at 7 days.

In vitro stability of Hb-vesicles and RBC

Hypotonic challenge induced 94% hemolysis for rat RBC, although HbV showed essentially no hemolysis (Table II). Freezing of water, crystallization of bulk water molecules, facilitates dehydration of the surface of lipid membranes, and destroys the cellular structure. Freeze-thawing induced 78% hemolysis for

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 6

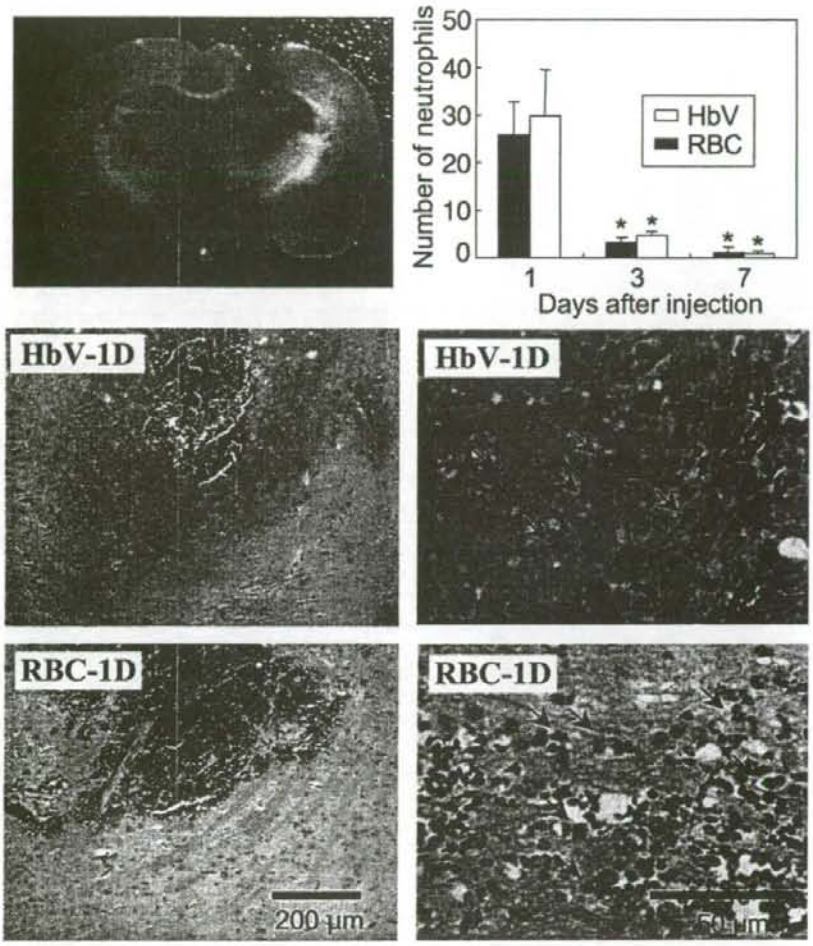

Figure 2. (Left Top) The needle tip for the intracerebral injection reached to the caudate nucleus. (Left middle, bottom) Hematoxylin/eosin staining of rat brain showing the presence of hematoma 1 day after injection. The HbV group showed the red area in the parenchymal region, indicating the presence of dispersed HbV. The RBC group showed that most of the RBCs remained in the hematoma. (Right, middle, and bottom) Hematoxylin/eosin staining of rat brain showing the presence of neutrophils (indicated with arrows) surrounding the hematoma in both HbV and RBC groups 1 day after injection because of the inflammatory reactions. (Right top) The quantities of neutrophils (in 0.26 mm^2) were greatest 1 day after injection. Then they decreased significantly 3 days after injection (* $p < 0.05$ vs. 1 day). However, no differences were found between any of the HbV with the RBC groups. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

AQ3

RBC whereas HbV showed only 20% hemolysis. HbV was resistant to the attack of PLA_2. Hemolysis was almost not induced in the HbV sample, whereas RBC showed 18% hemolysis.

DISCUSSION

The main finding of this study is that the intracerebral injection of 20 μL HbV showed normal progression of pathological responses to ICH. No difference was evident between HbV and RBC injections in the conventional rat ICH model, except that HbV distributed more widely in the perihematomal tissue and that a slight amount of HbV remained even after 28 days.

A clinical application of Hb-based oxygen carriers (HBOCs) can be a resuscitative fluid, which can be used for traumatic hemorrhaged patients.[26] However, most conventional experimental animal models have tested the effectiveness of HBOCs on artificially created hemorrhagic conditions that do not involve

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 7

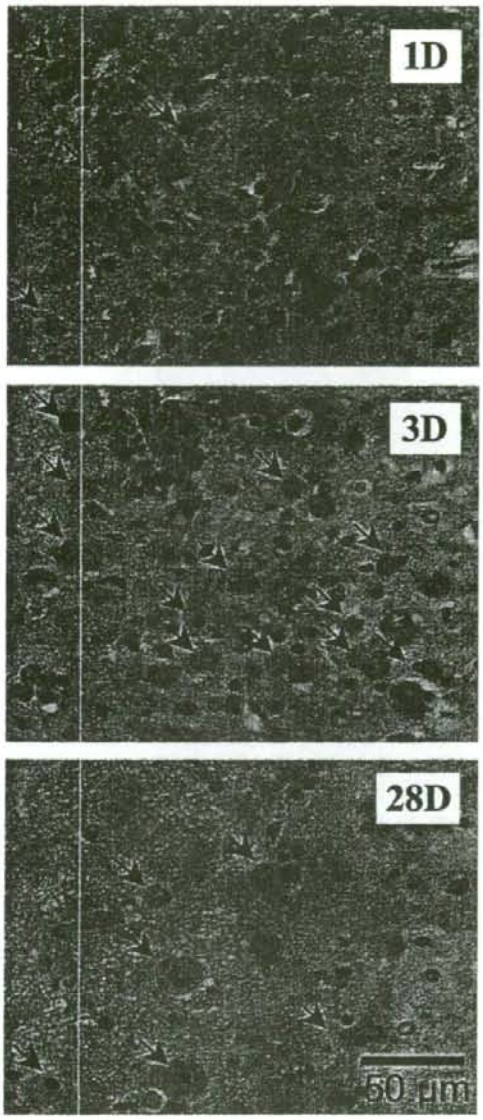

Figure 3. Immunochemical staining of rat brain tissue near the hematoma with anti-human Hb antibody. The round pink cells indicate the presence of HbV inside the cells. One day and three days after injection, the macrophages phagocytizing HbV are evident, as indicated with arrows. Even after 28 days, 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.]

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

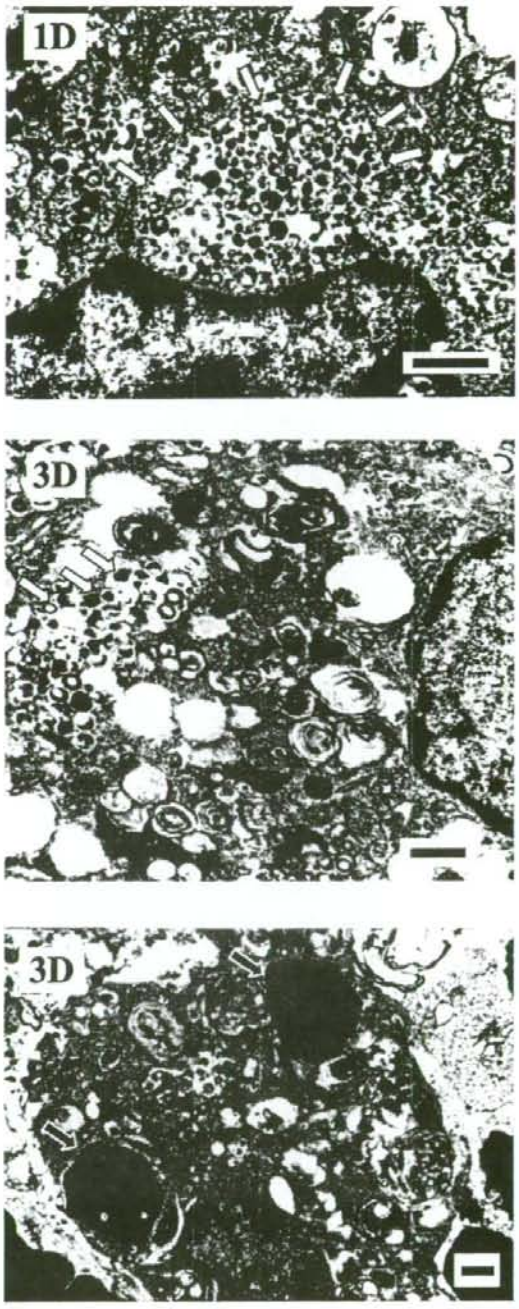

Figure 4. Transmittance electron micrographs of rat brain tissue in the HbV group 1 day and 3 days after intracerebral injection of HbV. The individual HbV particles are visible as black particles in the phagosomes of macrophages at 1 and 3 days, as indicated with white arrows. A macrophage phagocytizing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

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 8

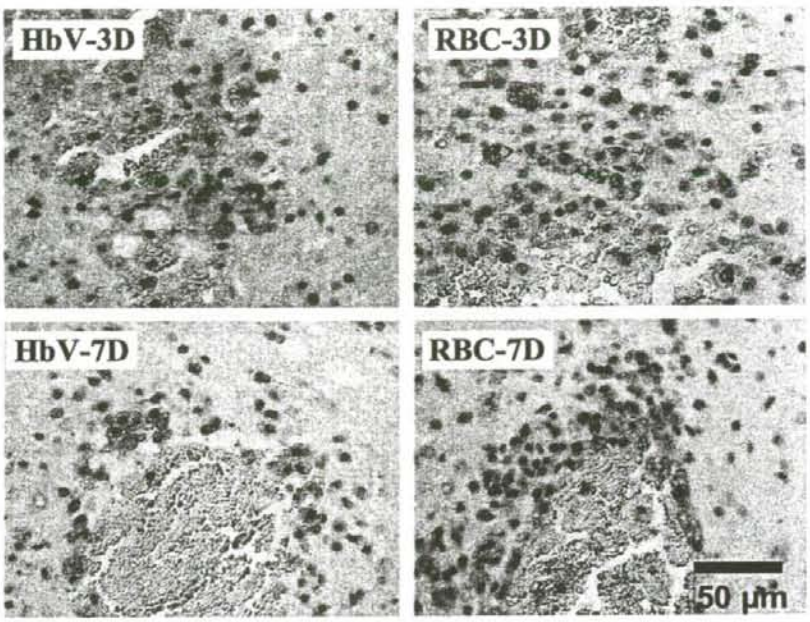

Figure 5. Immunochemical staining with anti-rat HO-1 antibody of rat brain 3 and 7 days after injection of HbV and RBC. Intense staining was confirmed especially at the rim of the perihematoma region. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

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

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 9

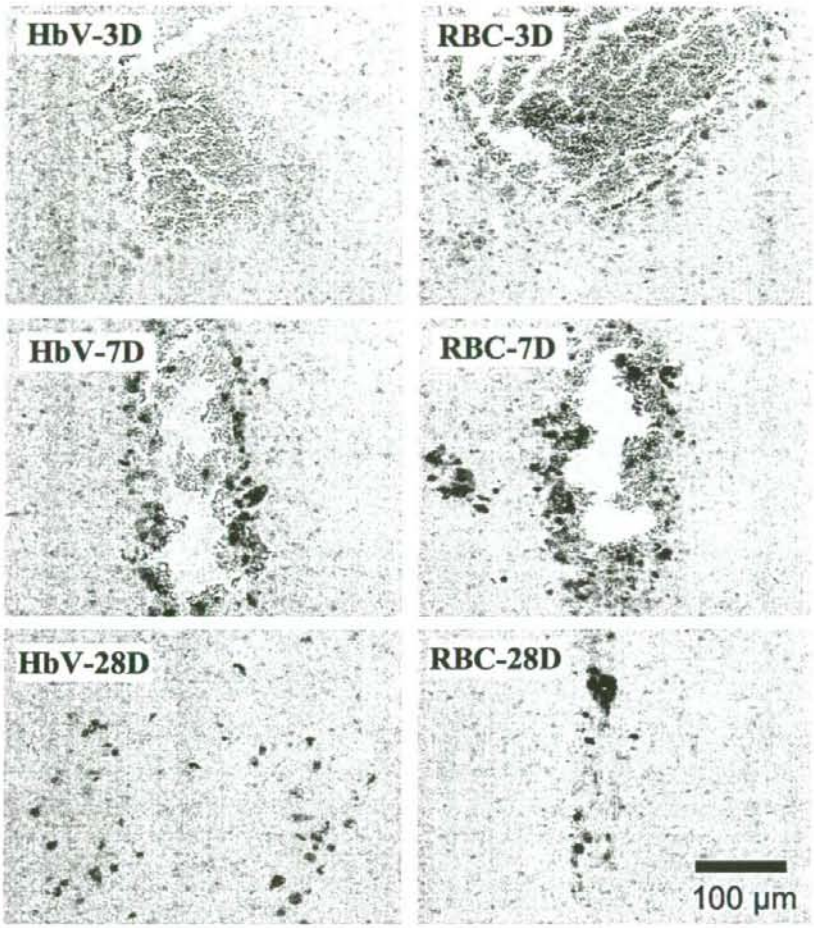

Figure 6. Berlin blue staining of rat brain 3, 7, and 28 days after injection of HbV and RBC. Large amount of hemosiderin deposition was confirmed just near the hematoma site for both groups. Hemosiderin deposition was confirmed at 28 days for both groups. [Color figure can be viewed in the online issue,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

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 10

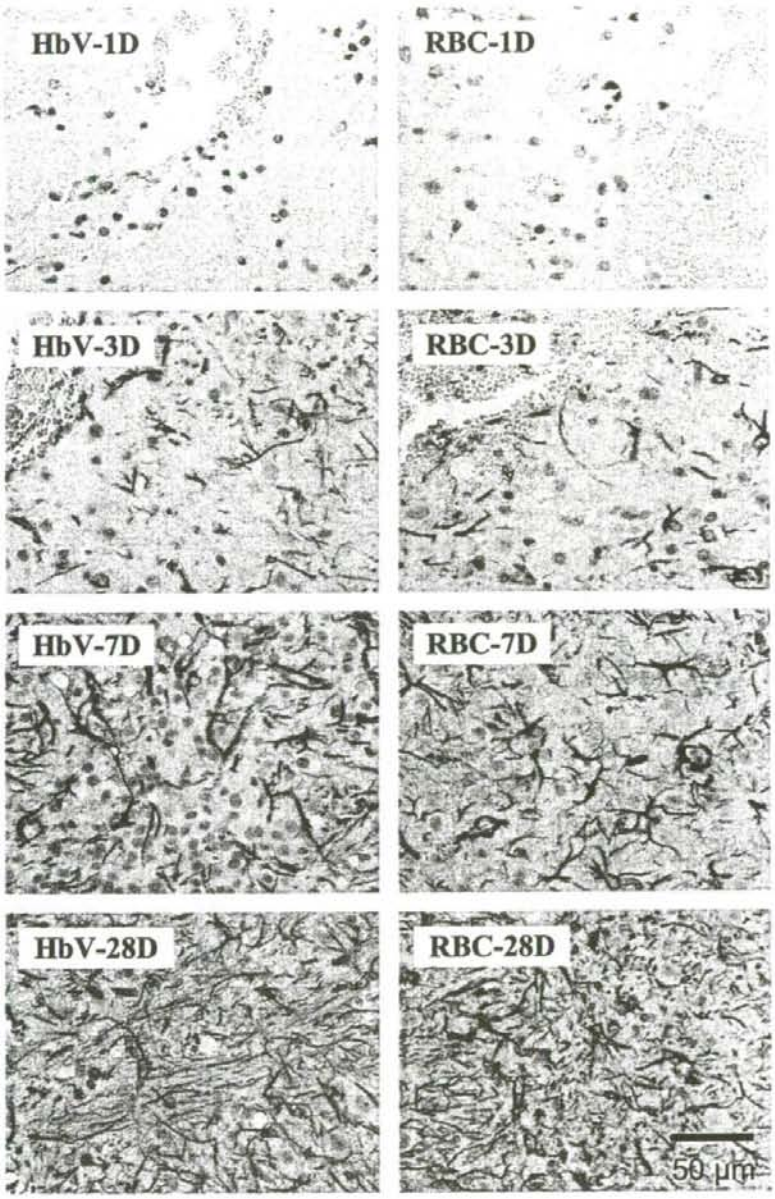

Figure 7. Immunochemical staining with anti-GFAP-staining 1, 3, 7, and 28 days after intracerebral injection of HbV and RBC. On day 1, the initial destruction of astrocytes in the perifocal zone for both groups, and from 3 days a dense network of hypertrophic processes reentered the perifocal zone. At 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

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 11

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.

The authors acknowledge Mr. H. Abe (Dept. of Pathology, Keio University), and Mr. M. Hashizume (Hist Science Laboratory Co., Tokyo) for assistance with immunochemical staining, and Dr. Keitaro Sou (Waseda University) for assistance with animal experiments. This work was supported in part by Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation), Ministry of Health, Labour and Welfare, Japan. The HbV and rHSA were provided respectively from Oxygenix Co., and Nipro Corp. Of the authors, HS, KK and ET were consultants to Oxygenix Co. Of the authors, HS and ET are holders of patents related to HbV production.

References

1. Kobayashi K, Izumi Y, Yoshizu A, Horinouchi H, Park SI, Sakai H, Takeoka S, Nishide H, Tsuchida E. The oxygen carrying capability of hemoglobin vesicles evaluated in rat exchange transfusion models. Artif Cells Blood Substit Immobil Biotechnol 1997;25:357–366.
2. Yamaguchi M, Fujihara M, Wakamoto S, Sakai H, Takeoka S, Tsuchida E, Azuma H, Ikeda H. Influence of hemoglobin vesicles, cellular-type artificial oxygen carriers, on human umbilical cord blood hematopoietic progenitor cells in vitro. J Biomed Mater Res A. Forthcoming. (DOI, 10.1002/jbm.a. 31857) AQ1
3. Chang TM. Therapeutic applications of polymeric artificial cells. Nat Rev Drug Discov 2005;4:221–235.
4. Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. Crit Care Med 2004;32:539–545.

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 12

5. Sakai H, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Takaori M, Tsuchida E, Kobayashi K. Acute 40 percent exchange-transfusion with hemoglobin-vesicles (HbV) suspended in recombinant human serum albumin solution: Degradation of HbV and erythropoiesis in a rat spleen for 2 weeks. Transfusion 2006;46:339–347.
6. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E. Purification of concentrated hemoglobin using organic solvent and heat treatment. Protein Expr Purif 1993;4:563–569.
7. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E. Poly (ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. Bioconjug Chem 2000;11:425–432.
8. Naito Y, Fukutomi I, Masada Y, Sakai H, Takeoka S, Tsuchida E, Abe H, Hirayama J, Ikebuchi K, Ikeda H. Virus removal from hemoglobin solution using planova membrane. J Artif Organs 2002;5:141–145.
9. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M. Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. J Clin Invest 1998;101:604–612.
10. Sakai H, Hara H, Yuasa M, Tsai AG, Takeoka S, Tsuchida E, Intaglietta M. Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol Heart Circ Physiol 2000;279:H908–H915.
11. Sakai H, Sato A, Masuda K, Takeoka S, Tsuchida E. Hb encapsulation in vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. J Biol Chem 2008;283:1508–1517.
12. Sou K, Klipper R, Goins B, Tsuchida E, Phillips WT. Circulation kinetics and organ distribution of Hb-vesicles developed as a red blood cell substitute. J Pharmacol Exp Ther 2005;312: 702–709.
13. Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E. Hemoglobin-vesicles as oxygen carriers: Influence on phagocytic activity and histopathological changes in reticuloendothelial system. Am J Pathol 2001; 159:1079–1088.
14. Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, Suematsu M, Takaori M, Kobayashi K, Tsuchida E. Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. J Pharmacol Exp Ther 2004;311:874–884.
15. Krizbai IA, Lenzser G, Szatmari E, Farkas AE, Wilhelm I, Fekete Z, Erdos B, Bauer H, Bauer HC, Sandor P, Komjati K. Blood-brain barrier changes during compensated and decompensated hemorrhagic shock. Shock 2005;24:428–433.
16. Xue M, del Bigio MR. Intracerebral injection of autologous whole blood in rats: Time course of inflammation and cell death. Neurosci Lett 2000;283:230–232.
17. Xi G, Hua Y, Bhasin RR, Ennis SR, Keep RF, Hoff JT. Mechanisms of edema formation after intracerebral hemorrhage: Effects of extravasated red blood cells on blood flow and blood-brain barrier integrity. Stroke 2001;32:2932–2938.
18. Huang FP, Xi G, Keep RF, Hua Y, Nemoianu A, Hoff JT. Brain edema after experimental intracerebral hemorrhage: Role of hemoglobin degradation products. J Neurosurg 2002; 96:287–293.
19. Panter SS, Ellington BL, Regan RF. Hemoglobin and neurotoxicity. In: Winslow RM, editor. Blood Substitutes, Chapter 21. Elsevier; 2006. p 227–234.
20. Rosenthal G, Morabito D, Cohen M, Roeytenberg A, Derugin N, Panter SS, Knudson MM, Manley G. Use of hemoglobin-based oxygen-carrying solution-201 to improve resuscitation parameters and prevent secondary brain injury in a swine model of traumatic brain injury and hemorrhage: Laboratory investigation. J Neurosurg 2008;108:575–587.
21. Sou K, Naito Y, Endo T, Takeoka S, Tsuchida E. Effective encapsulation of proteins into size-controlled phospholipid vesicles using freeze-thawing and extrusion. Biotechnol Prog 2003;19:1547–1552.
22. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E. Physical properties of hemoglobin vesicles as red cell substitutes. Biotechnol Prog 1996;12:119–125.
23. Sakai H, Hisamoto S, Fukutomi I, Sou K, Takeoka S, Tsuchida E. Detection of lipopolysaccharide in hemoglobin-vesicles by limulus amebocyte lysate test with kinetic-turbidimetric gel clotting analysis and pretreatment of surfactant. J Pharm Sci 2004;93:310–321.
24. Matsubara T, Okuzono H, Senba U. A modification of van Kampen-Zijlstra's reagent for the hemiglobincyanide method. Clin Chim Acta 1979;93:163–164.
25. Hovav E, Halle D, Yedgar S. Viscous macromolecules inhibit erythrocyte hemolysis induced by snake venom phospholipase A_2. Biorheology 1987;23:377–384.
26. Sloan EP, Koenigsberg M, Gens D, Cipolle M, Runge J, Mallory MN, Rodman G Jr. Diaspirin cross-linked hemoglobin (DCLHb) in the treatment of severe traumatic hemorrhagic shock: A randomized controlled efficacy trial. JAMA 1999; 282:1857–1864.
27. Terajima K, Tsueshita T, Sakamoto A, Ogawa R. Fluid resuscitation with hemoglobin vesicles in a rabbit model of acute hemorrhagic shock. Shock 2006;25:184–189.
28. Yoshizu A, Izumi Y, Park S, Sakai H, Takeoka S, Horinouchi H, Ikeda E, Tsuchida E, Kobayashi K. Hemorrhagic shock resuscitation with an artificial oxygen carrier, hemoglobin vesicle, maintains intestinal perfusion, and suppresses the increase in plasma tumor necrosis factor-alpha. ASAIO J 2004;50:458–463.
29. Qureshi AI, Ling GS, Khan J, Suri MF, Miskolczi L, Guterman LR, Hopkins LN. Quantitative analysis of injured, necrotic, and apoptotic cells in a new experimental model of intracerebral hemorrhage. Crit Care Med 2001;29:152–157.
30. Kaufman HH, Pruessner JL, Bernstein DP, Borit A, Ostrow PT, Cahall DL. A rabbit model of intracerebral hematoma. Acta Neuropathol (Berl) 1985;65:318–321.
31. Kobari M, Gotoh F, Tomita M, Tanahashi N, Shinohara T, Terayama Y, Mihara B. Bilateral hemispheric reduction of cerebral blood volume and blood flow immediately after experimental cerebral hemorrhage in cats. Stroke 1988;19:991–996.
32. Wagner KR, Xi G, Hua Y, Kleinholz M, de Courten-Myers GM, Myers RE, Broderick JP, Brott TG. Lobar intracerebral hemorrhage model in pigs: Rapid edema development in perihematomal white matter. Stroke 1996;27:490–497.
33. Gong C, Boulis N, Qian J, Turner DE, Hoff JT, Keep RF. Intracerebral hemorrhage-induced neuronal death. Neurosurgery 2001;48:875–882.
34. Gong Y, Hua Y, Keep RF, Hoff JT, Xi G. Intracerebral hemorrhage: Effects of aging on brain edema and neurological deficits. Stroke 2004;35:2571–2575.
35. Hua Y, Schallert T, Keep RF, Wu J, Hoff JT, Xi G. Behavioral tests after intracerebral hemorrhage in the rat. Stroke 2002;33:2478–2484.
36. Nath FP, Jenkins A, Mendelow AD, Graham DI, Teasdale GM. Early hemodynamic changes in experimental intracerebral hemorrhage. J Neurosurg 1986;65:697–703.
37. Bhasin RR, Xi G, Hua Y, Keep RF, Hoff JT. Experimental intracerebral hemorrhage: Effect of lysed erythrocytes on brain edema and blood-brain barrier permeability. Acta Neurochir Suppl 2002;81:249–251.

Q2

J_ID: ZA1 Customer A_ID: 07-1037.R1 Cadmus Art: JBMA32164 Date: 10-JULY-08 Stage: I Page: 13

38. Wu J, Hua Y, Keep RF, Schallert T, Hoff JT, Xi G. Oxidative brain injury from extravasated erythrocytes after intracerebral hemorrhage. Brain Res 2002;953:45–52.
39. Strbian D, Karjalainen-Lindsberg ML, Tatlisumak T, Lindsberg PJ. Cerebral mast cells regulate early ischemic brain swelling and neutrophil accumulation. J Cereb Blood Flow Metab 2006;26:605–612.
40. Xi G, Keep RF, Hoff JT. Erythrocytes and delayed brain edema formation following intracerebral hemorrhage in rats. J Neurosurg 1998;89:991–996.
41. Nath FP, Jenkins A, Mendelow AD, Graham DI, Teasdale GM. Early hemodynamic changes in experimental intracerebral hemorrhage. J Neurosurg 1986;65:697–703.
42. Lennartz MR. Phospholipase and phagocytosis: The role of phospholipid-derived second messengers in phagocytosis. Int J Biochem Cell Biol 1999;31:415–430.
43. Yokohama H, Seino Y, Chung J, Sakai H, Takeoka S, Nishide H, Tsuchida E. Suppression of methemoglobin formation in Hb-vesicles by glutathione. Artif Organs Today 1994;4:107–116.
44. Szebeni J, Hauser H, Eskelson CD, Winterhalter KH. Factors influencing the in vitro stability of artificial red blood cells based on hemoglobin-containing liposomes. Biomater Artif Cells Artif Organs 1988;16:301–312.
45. Saxena R, Wijnhoud AD, Carton H, Hacke W, Kaste M, Przybelski RJ, Stern KN, Koudstaal PJ. Controlled safety study of a hemoglobin-based oxygen carrier. DCLHb, in acute ischemic stroke. Stroke 1999;30:993–996.
46. Sadrzadeh SM, Anderson DK, Panter SS, Hallaway PE, Eaton JW. Hemoglobin potentiates central nervous system damage. J Clin Invest 1987;79:662–664.
47. Goldstein L, Teng ZP, Zeserson E, Patel M, Regan RF. Hemin induces an iron-dependent, oxidative injury to human neuron-like cells. J Neurosci Res 2003;73:113–121.
48. Chen Y, Swanson RA. Astrocyte and brain injury. J Cerebral Blood Flow Metab 2003;23:137–149.
49. Ridet JL, Malhotra SK, Privat A, Gage FH. Reactive astrocytes: Cellular and molecular cues to biological function. Trends Neurosci 1997;20:570–577.
50. Karwacki Z, Kowiański P, Dziewiatkowski J, Domaradzka-Pytel B, Ludkiewicz B, Wójcik S, Narkiewicz O, Moryś J. The influence of sevoflurane on the reactivity of astrocytes in the course of the experimental intracerebral haemorrhage in rat. J Physiol Pharmacol 2005;56:455–469.
51. Koeppen AH, Dickson AC, McEvoy JA. The cellular reactions to experimental intracerebral hemorrhage. J Neurol Sci 1995;134:102–112.
52. Torchilin VP. Recent advances with liposomes as pharmaceutical carriers. Nat Rev Drug Discov 2005;4:145–160.
53. Begley DJ. Delivery of therapeutic agents to the central nervous system: The problems and the possibilities. Pharmacol Ther 2004;104:29–45.
54. Firth GB, Firth M, McKeran RO, Rees J, Walter P, Uttley D, Marks V. Application of radioimmunoassay to monitor treatment of human cerebral gliomas with bleomycin entrapped within liposomes. J Clin Pathol 1988;41:38–43.
55. Firth G, Oliver AS, McKeran RO. Studies on the intracerebral injection of bleomycin free and entrapped within liposomes in the rat. J Neurol Neurosurg Psychiatry 1984;47:585–589.

SHOCK, Vol. 00, No. 00, pp. 00–00, 2008

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

Q1

Hiromi Sakai,* Hirohisa Horinouchi,† Eishun Tsuchida,* and Koichi Kobayashi†

**Research Institute for Science and Engineering, Waseda University; and †Department of General Thoracic Surgery, School of Medicine, Keio University, Tokyo, Japan*

Received 27 May 2008; first review completed 18 Jun 2008; accepted in final form 17 Jul 2008

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).

Address reprint requests to Koichi Kobayashi, Department of General Thoracic Surgery, School of Medicine, Keio University, Tokyo 160-8582, Japan. E-mail: hiromi@waseda.jp; kobayash@sc.itc.keio.ac.jp.

This work is supported by the Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation), the Ministry of Health, Labour and Welfare, Japan (H.S. H.H, E.T, K.K.), and the Grants in Aid for Scientific Research from the Japan Society for the Promotion of Science (B19300164) (H.S.). The rHSA and some of the HbV samples were provided by the Nipro Corp (Osaka) and the Oxygenix Co Ltd (Tokyo), respectively. H.S., E.T., and K.K. were the consultants of Oxygenix. Co Ltd. H.S. and E.T. are the holders of patents on Hb vesicles.

DOI: 10.1097/SHK.0b013e318188f815

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,

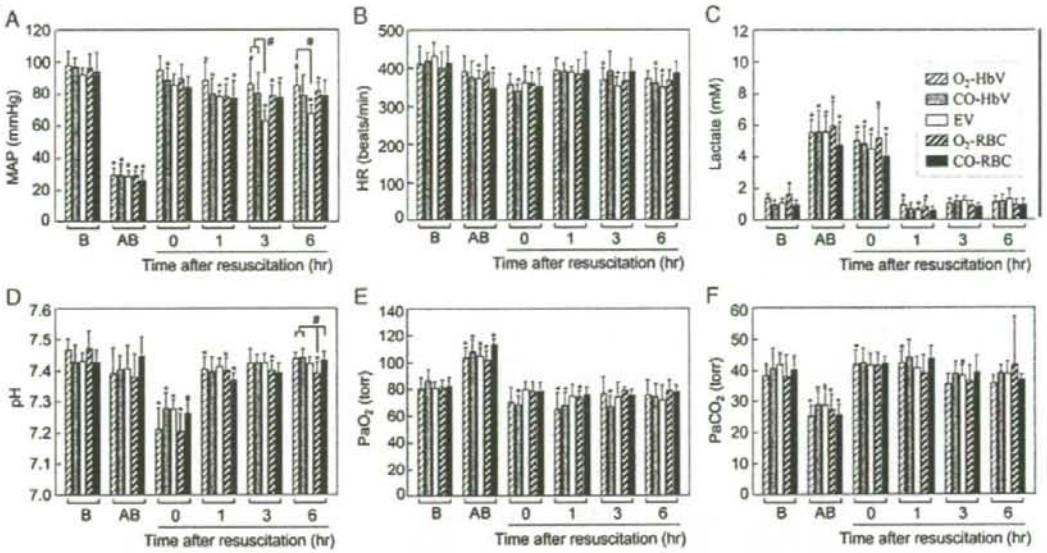

FIG. 1. **Changes in systemic parameters in rats before and after hemorrhage and resuscitation by injection of O_2-HbV, CO-HbV, EV, O_2-RBC, or CO-RBC suspended in 5% rHSA: (A) MAP, (B) HR, (C) lactate, (D) pH, (E) PaO_2, and (F) $PaCO_2$.** B indicates baseline; AB, after bleeding. $^*P < 0.01$ (Bonferroni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

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

In vitro *CO exchange reaction from HbV to RBC*

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

RESULTS

Systemic responses to hemorrhagic shock and resuscitation

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

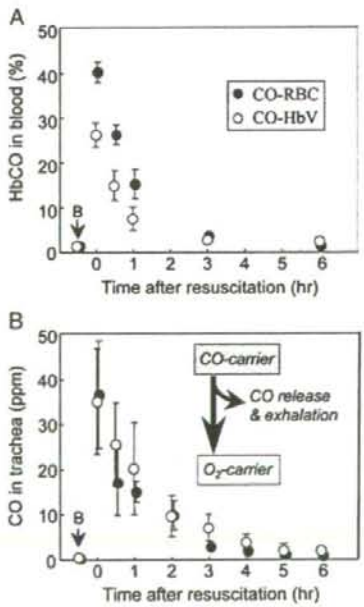

FIG. 2. **Time course of HbCO levels in blood (A) and CO levels in the trachea (B) before and after injection of CO-HbV or CO-RBC suspended in 5% rHSA as a resuscitative fluid for hemorrhagic shock in rats.** B indicates baseline. Both CO-HbV and CO-RBC released CO and gradually became O_2 carriers.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

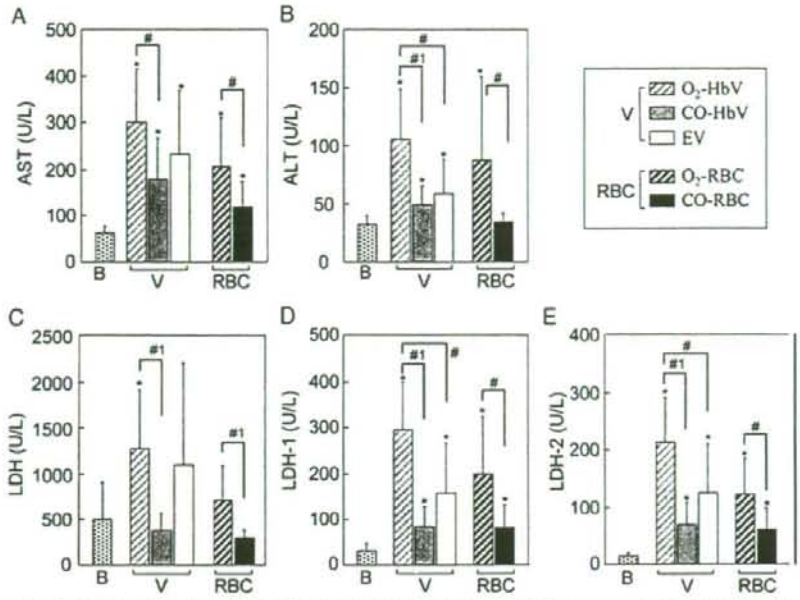

FIG. 3. **Plasma enzyme levels 6 h after injection of O_2-HbV, CO-HbV, EV, O_2-RBC, or CO-RBC suspended in 5% rHSA: (A) AST, (B) ALT, (C) LDH, (D) isozyme LDH-1, and (E) isozyme LDH-2.** B indicates baseline. *$P < 0.05$ versus baseline group; #$P < 0.05$ versus the indicated group; #1$P < 0.01$ versus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

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

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

HbCO in blood and CO in exhaled air

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

Clinical laboratory tests of blood serum

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

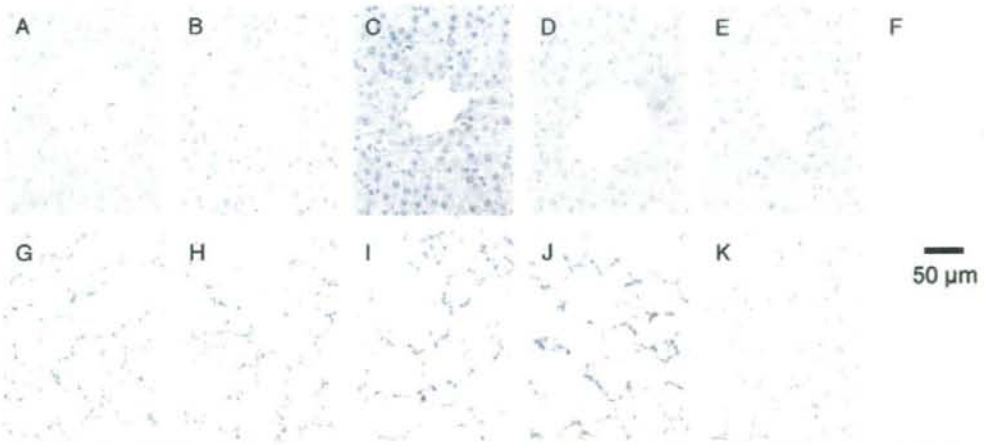

Fig. 4. 4/C

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.

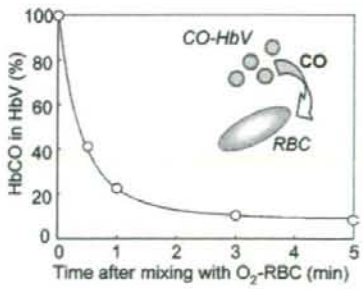

Fig. 5. ***In vitro* CO release of CO-HbV after rapid mixing with O_2-RBC.** A CO-HbV ([Hb] = 10 g/dL, 0.5 mL) and an O_2-RBC suspension (10 g/dL, 4.5 mL) were mixed immediately. The level of HbCO of HbV fraction after centrifugation was monitored. Results indicate that the CO release rate is unexpectedly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

The authors gratefully acknowledge Prof M. Intaglietta and Dr P. Cabrales (University of California, San Diego) for the suggestion of initiating this research, Prof M. Suematsu (Keio University) for experimental suggestions, and Dr Y. Nishiwaki (Keio University) for a suggestion related to statistical analyses.

REFERENCES

1. Ryter SW, Alam J, Choi AM: Heme oxygenase-1/carbon monoxide: from basic science to therapeutic applications. *Physiol Rev* 86:583–650, 2006.
2. Morita T, Perrella MA, Lee ME, Kourembanas S: Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. *Proc Natl Acad Sci U S A* 92:1475–1479, 1995.
3. Goda N, Suzuki K, Naito M, Takeoka S, Tsuchida E, Ishimura Y, Tamatani T, Suematsu M: Distribution of heme oxygenase isoforms in rat liver. Topographic basis for carbon monoxide-mediated microvascular relaxation. *J Clin Invest* 101:604–612, 1998.
4. Motterlini R, Clark JE, Foresti R, Sarathchandra P, Mann BE, Green CJ: Carbon monoxide-releasing molecules: characterization of biochemical and vascular activities. *Circ Res* 90:e17–e24, 2002.
5. Morse D, Pischke SE, Zhou Z, Davis RJ, Flavell RA, Loop T, Otterbein SL, Otterbein LE, Choi AM: Suppression of inflammatory cytokine production by carbon monoxide involves the JNK pathway and AP-1. *J Biol Chem* 278: 36993–36998, 2003.
6. Zuckerbraun BS, McCloskey CA, Gallo D, Liu F, Ifedigbo E, Otterbein LE, Billiar TR: Carbon monoxide prevents multiple organ injury in a model of hemorrhagic shock and resuscitation. *Shock* 23:527–532, 2005.
7. Cabrales P, Tsai AG, Intaglietta M: Hemorrhagic shock resuscitation with carbon monoxide saturated blood. *Resuscitation* 72:306–318, 2007.
8. Sakai H, Takeoka S, Yokohama H, Seino Y, Nishide H, Tsuchida E: Purification of concentrated hemoglobin using organic solvent and heat treatment. *Protein Expr Purif* 4:563–569, 1993.
9. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E: Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol Prog* 12:119–125, 1996.
10. Chung J, Hamada K, Sakai H, Takeoka S, Nishide H, Tsuchida E: Ligand-exchange reaction of carbonylhemoglobin to oxyhemoglobin in a hemoglobin liquid membrane. *Nippon Kagaku Kaishi* 2:123–127, 1995.
11. Sakai H, Masada Y, Horinouchi H, Yamamoto M, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E: Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit Care Med* 32:539–545, 2004.
12. Sakai H, Seishi Y, Obata Y, Takeoka S, Horinouchi H, Tsuchida E, Kobayashi K: Fluid resuscitation with artificial oxygen carriers in hemorrhaged rats: profiles of hemoglobin-vesicle degradation and hematopoiesis for 14 days. *Shock* 2008. In press.
13. Bosman RJ, Minten J, Lu HR, Van Aken H, Flameng W: Free polymerized hemoglobin versus hydroxyethyl starch in resuscitation of hypovolemic dogs. *Anesth Analg* 75:811–817, 1992.
14. Lehnert M, Arteel GE, Smutney OM, Conzelmann LO, Zhong Z, Thurman RG, Lemasters JJ: Dependence of liver injury after hemorrhage/resuscitation in mice on NADPH oxidase-derived superoxide. *Shock* 19:345–351, 2003.
15. Hierholzer C, Billiar TR: Molecular mechanisms in the early phase of hemorrhagic shock. *Langenbecks Arch Surg* 386:302–308, 2001.
16. Bitensky MW: Method using CO for extending the useful shelf-life of refrigerated red blood cells. US Patent No. 5,476,764. September 16, 1994.
17. Sakai H, Tomiyama KI, Sou K, Takeoka S, Tsuchida E: Poly(ethylene glycol)-conjugation and deoxygenation enable long-term preservation of hemoglobin-vesicles as oxygen carriers in a liquid state. *Bioconjug Chem* 11:425–432, 2000.
18. Morimatsu H, Takahashi T, Maeshima K, Inoue K, Kawakami T, Shimizu H, Takeuchi M, Yokoyama M, Katayama H, Morita K: Increased heme catabolism in critically ill patients: correlation among exhaled carbon monoxide, arterial carboxyhemoglobin, and serum bilirubin IXalpha concentrations. *Am J Physiol Lung Cell Mol Physiol* 290:L114–L119, 2006.
19. Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, Tsuchida E: Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. *Clin Chem Lab Med* 41:222–231, 2003.
20. Preus M, Bhargava AS, Khater AE, Gunzel P: Diagnostic value of serum creatine kinase and lactate dehydrogenase isoenzyme determinations for monitoring early cardiac damage in rats. *Toxicol Lett* 42:225–233, 1988.
21. Ohno F: Changes in plasma LDH-isozyme in rats during hypoxia and its recovery stage. *Masui* 41:1455–1460, 1992.
22. Mota-Filipe H, McDonald MC, Cuzzocrea S, Thiemermann C: A membrane-permeable radical scavenger reduces the organ injury in hemorrhagic shock. *Shock* 12:255–261, 1999.
23. McDonald MC, Izumi M, Cuzzocrea S, Thiemermann C: A novel, potent and selective inhibitor of the activity of inducible nitric oxide synthase (GW274150) reduces the organ injury in hemorrhagic shock. *J Physiol Pharmacol* 53:555–569, 2002.
24. Sakai H, Horinouchi H, Tomiyama K, Ikeda E, Takeoka S, Kobayashi K, Tsuchida E: Hemoglobin-vesicles as oxygen carriers: influence on phagocytic activity and histopathological changes in reticuloendothelial system. *Am J Pathol* 159:1079–1088, 2001.
25. Vallejo JG, Nemoto S, Ishiyama M, Yu B, Knuefermann P, Diwan A, Baker JS, Defreitas G, Tweardy DJ, Mann DL: Functional significance of inflammatory mediators in a murine model of resuscitated hemorrhagic shock. *Am J Physiol Heart Circ Physiol* 288:H1272–H1277, 2005.
26. Cabrales P, Intaglietta M, Tsai AG: Increase plasma viscosity sustains microcirculation after resuscitation from hemorrhagic shock and continuous bleeding. *Shock* 23:549–555, 2005.
27. Raub JA, Mathieu-Nolf M, Hampson NB, Thom SR: Carbon monoxide poisoning–a public health perspective. *Toxicology* 145:1–14, 2000.
28. Gorman D, Drewry A, Huang YL, Sames C: The clinical toxicology of carbon monoxide. *Toxicology* 187:25–38, 2003.
29. Piantadosi CA: Carbon monoxide, oxygen transport, and oxygen metabolism. *J Hyperbaric Med* 2:27–44, 1987.
30. Glabe A, Chung Y, Xu D, Jue T: Carbon monoxide inhibition of regulatory pathways in myocardium. *Am J Physiol Heart Circ Physiol* 274: H2143–H2151, 1998.
31. Savolainen H, Kurppa K, Tenhunen R, Kivisto H: Biochemical effects of carbon monoxide poisoning in rat brain with special reference to blood carboxyhemoglobin and cerebral cytochrome oxidase activity. *Neurosci Lett* 19:319–323, 1980.
32. Sakai H, Tsai AG, Rohlfs RJ, Hara H, Takeoka S, Tsuchida E, Intaglietta M: Microvascular responses to hemodilution with Hb vesicles as red blood cell substitutes: influence of O_2 affinity. *Am J Physiol Heart Circ Physiol* 276: H553–H562, 1999.
33. Kanten WE, Penny DG, Francisco K, Thill JE: Hemodynamic responses to acute carboxyhemoglobinemia in the rat. *Am J Physiol Heart Circ Physiol* 244:H320–H327, 1983.
34. Hangai-Hoger N, Tsai AG, Cabrales P, Suematsu M, Intaglietta M: Microvascular and systemic effects following top load administration of saturated carbon monoxide-saline solution. *Crit Care Med* 35:1123–1132, 2007.
35. Kavdia M, Popel AS: Wall shear stress differentially affects NO level in arterioles for volume expanders and Hb-based O_2 carriers. *Microvasc Res* 66: 49–58, 2003.
36. Matsumoto H, Hirai R, Uemura T, Ota T, Urakami A, Shimizu N: Experimental evaluation of the effects of the intraportal administration of cyclic guanosine monophosphate on ischemia/reperfusion in the porcine liver. *Surg Today* 29:1158–1163, 1999.

37. Chin BY, Jiang G, Wegiel B, Wang HJ, Macdonald T, Zhang XC, Gallo D, Cszimadia E, Bach FH, Lee PJ, et al.: Hypoxia-inducible factor 1a stabilization by carbon monoxide results in cytoprotective preconditioning. *Proc Natl Acad Sci U S A* 104:5109–5114, 2007.
38. Jaeschke H, Farhood A: Neutrophil and Kupffer cell-induced oxidant stress and ischemia-reperfusion injury in rat liver. *Am J Physiol Gastrointest Liver Physiol* 260:G355–G362, 1991.
39. Babior BM, Lambeth JD, Nauseef W: The neutrophil NADPH oxidase. *Arch Biochem Biophys* 397:342–344, 2002.
40. Taillé C, El-Benna J, Lanone S, Boczkowski J, Motterlini R: Mitochondrial respiratory chain and NAD(P)H oxidase are targets for the antiproliferative effect of carbon monoxide in human airway smooth muscle. *J Biol Chem* 280:25350–25360, 2005.
41. Gunther MR, Sampath V, Caughey WS: Potential roles of myoglobin autoxidation in myocardial ischemia-reperfusion injury. *Free Radic Biol Med* 26:1388–1395, 1999.
42. Zhou JL, Jin GH, Yi YL, Zhang JL, Huang XL: Role of nitric oxide and peroxynitrite anion in lung injury induced by intestinal ischemia-reperfusion in rats. *World J Gastroenterol* 9:1318–1322, 2003.
43. White KA, Marletta MA: Nitric oxide synthase is a cytochrome P-450 type hemoprotein. *Biochemistry* 31:6627–6631, 1992.
44. Ishikawa M, Kajimura M, Adachi T, Maruyama K, Makino N, Goda N, Yamaguchi T, Sekizuka E, Suematsu M: Carbon monoxide from heme oxygenase-2 is a tonic regulator against NO-dependent vasodilatation in the adult rat cerebral microcirculation. *Circ Res* 97:e104–e114, 2005.
45. Kooy NW, Royall JA, Ye YZ, Kelly DR, Beckman JS: Evidence for *in vivo* peroxynitrite production in human acute lung injury. *Am J Respir Crit Care Med* 151:1250–1254, 1995.
46. Thom SR, Bhopale VM, Fisher D, Zhang J, Gimotty P: Delayed neuropathology after carbon monoxide poisoning is immune-mediated. *Proc Natl Acad Sci U S A* 101:13660–13665, 2004.
47. Uchiyama T, Harafuji K: Life-saving effect of pyridoxalated hemoglobin-polyoxyethylene conjugate on carbon monoxide intoxication of rabbits. *Artif Organs* 18:576–587, 1994.

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．

(1) 早稲田大学理工学術院　理工学研究所　〒169-8555　東京都新宿区大久保3-4-1　早稲田大学理工学研究所　Research Institute for Science and Engineering, Waseda University

(2) 慶應義塾大学医学部 呼吸器外科　Department of General Thoracic Surgery, School of Medicine, Keio University

論文受付 2008年７月18日