

### Methemoglobin (metHb)

After plasma collection, a portion of the sample was ultracentrifuged and the HbV compartment recovered. The methemoglobin (metHb) component was measured after the liposomes were solubilized and increased with time, constituting 7% of total Hb immediately after resuscitation and 16.3% at the end of the experiment (240 min after resuscitation) (Figure 7). This phenomenon could have been caused by the oxidative stress of hemorrhagic shock.

### Oxygen Delivery and Oxygen Consumption

After resuscitation, oxygen delivery improved substantially in each group except for the LR group, in which the recovery of oxygen delivery was only about half that of the other groups (Figure 8a). The change in oxygen delivery in the albumin group was almost identical to that of the ASB and HbV groups. Although the Hb concentration was less than 7 g/dL (6.03–6.27 g/dL) after resuscitation (about 55–57% that of the ASB group), the increased cardiac output compensated the oxygen delivery. In the HbV group, 33.2% (30 min after resuscitation) to 25.8% (240 min after resuscitation) of the oxygen delivery was mediated by HbV itself.

Furthermore, in the HbV group the contribution of HbV-derived oxygen to the total oxygen consumed was calculated as 25.4% (30 min after resuscitation) to 20.1% (240 min after resuscitation) (Figure 8b).

### Serum Chemistry

We found a significantly increased albumin-to-globulin (A/G) ratio in the HSA and HbV groups. This is not surprising as we administered albumin as a resuscitation fluid in these groups (Table 1).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased in all groups, indicating

severe liver damage due to hemorrhagic shock. The increases in AST and ALT were significant in the ASB and HbV groups. Cholinesterase (ChE) is an important enzyme for indicating the liver functional reserve. In the HbV group, ChE decreased significantly compared to the baseline value, which may indicate that HbV influenced liver function.

The change in lactate dehydrogenase (LDH) was similar to those of AST and ALT. In the LR and HbV groups, the differences between baseline and 240 min post-resuscitation values were not statistically significant.

In the LR and HSA groups, the creatinine (Cr) level increased at the end of resuscitation. In the LR group, decreased organ perfusion seemed to account for the significant increase. In the HSA group, Cr increased to 120% of baseline but remained within normal limits.

Creatine phosphokinase (CPK) increased at the end of experiment in the ASB and HbV groups, but there were no significant inter-group or within-group pre- and post-resuscitation differences (ASB: baseline;  $419.8 \pm 250.3$  to 240 min;  $773.0 \pm 185.8$ ,  $p = 0.06$ ; HbV: baseline;  $459.3 \pm 132.8$  to 240 min;  $733.7 \pm 180.4$ ,  $p = 0.10$ ).

Hb vesicles contain a fair amount of lipid, about five times as much as red blood cells. Although a fair amount of lipid was infused into the animals, the serum lipid component decreased at the end of the experiment. In a rat study, serum cholesterol increased 24 hours after resuscitation [9]. HbV was thought to be still circulating in the blood, and metabolized HbV was small in volume in dogs.

The serum lipid component decreased at the end of experiment in all groups, presumably due to dilution by the resuscitation fluid.

### DISCUSSION

We have developed hemoglobin vesicles as an artificial oxygen carrier that is a candidate red blood cell substitute [10]. HbV are composed of concentrated Hb solution covered by a lipid bilayer [4–6]. The efficacy and safety of this material have been tested largely in small animals [11–14].

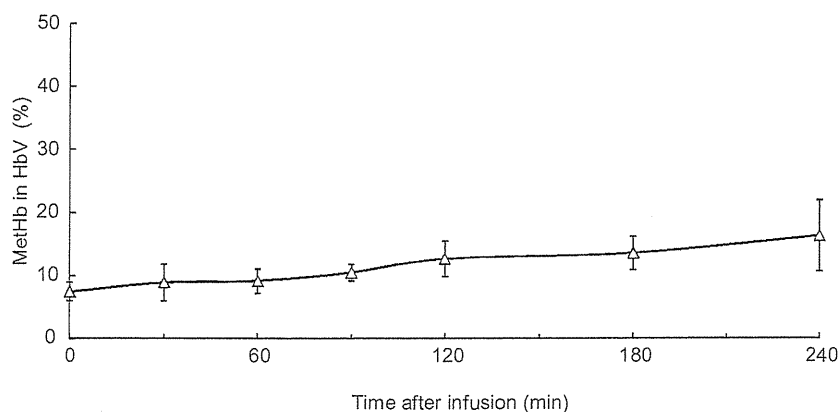
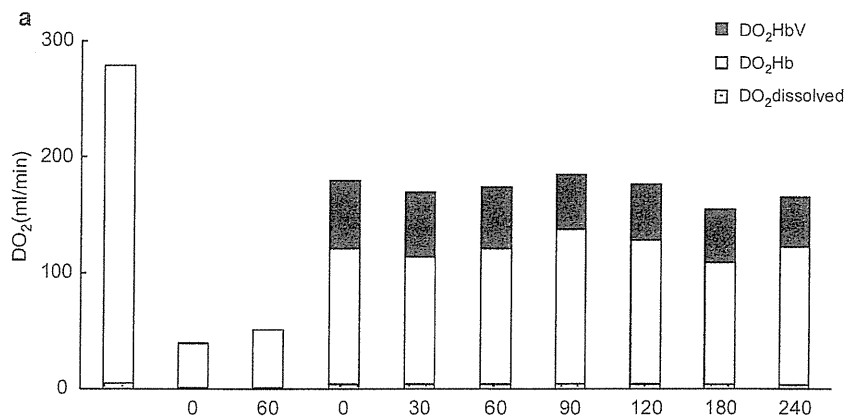


Figure 7. Proportion of MetHb in HbV hemoglobin. The concentration of methemoglobin gradually increased.



**Figure 8a.** Contribution of HbV to oxygen delivery. HbV delivered 33–26% of the tissue oxygen supply. (Black bar: oxygen delivered by HbV; blank bar: oxygen delivered by RBC).

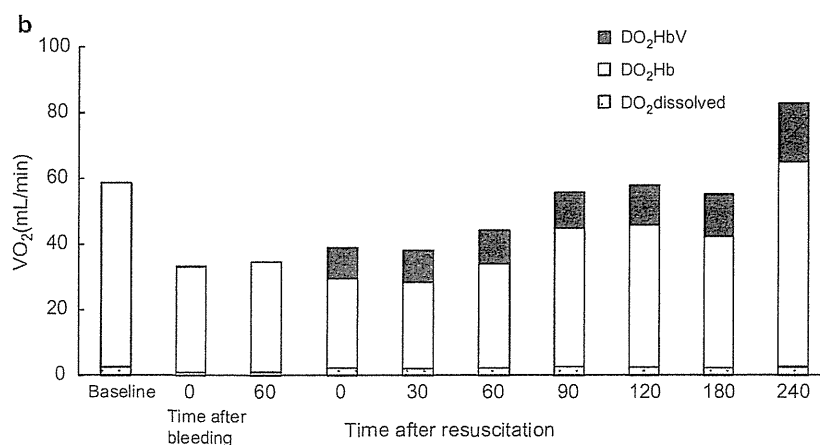
The results so far have been encouraging. However, we cannot evaluate the pulmonary circulation in small animal studies. Evaluation of the effect of HbV on the pulmonary circulation required that we use larger animals. We chose the canine model because the anatomy and metabolism are similar to those of humans.

We established a hemorrhagic shock model in Beagle dogs, examined the safety and efficacy of HbV as a resuscitation fluid, and evaluated the influence of fluid resuscitation on pulmonary circulation. It was difficult to establish a uniform hemorrhagic shock model in dogs because a dog's spleen contracts in response to blood loss, and splenic contraction supplies extra blood that contributes to the effect of the resuscitation fluid. Therefore, we surgically removed the spleen before the experiment, as previously reported, to produce a uniform hemorrhagic shock in Beagles [15,16].

In cases of hemorrhagic shock, proper hemostasis and restoration of circulation volume as well as transfusion to

maintain oxygen transport are the keys to saving life [1,17]. Traditionally, large volumes of crystalloid fluids have been used to maintain the circulation volume and blood pressure during hemorrhagic shock (Advanced Trauma Life Support; ATLS). This ATLS guideline has been criticized for its lack of evidence and poor outcomes, and recently the trend in fluid resuscitation has shifted to hypertonic saline plus a plasma expander [18–21], although several problems, such as coagulopathy and secondary insult to kidney function and head injury, have been noted [22–24]. In this study, isovolemic resuscitation was performed. This resuscitation method restores circulating volume, which we thought most appropriate for evaluating the efficacies of resuscitation fluids.

HbV are small particles, 250 nm in diameter [25]. HbV disperses in the plasma fraction and does not extravasate. As HbV is a particle in solution, HbV in saline does not exert colloid osmotic pressure. Therefore, we used 5% albumin in saline, which has the same colloid osmotic pressure as plasma, as a dispersion fluid.



**Figure 8b.** Contribution of HbV to oxygen consumed. HbV conveyed 25–20% of oxygen used. Black bar: calculated oxygen amount consumed from HbV; blank bar: calculated oxygen amount consumed from RBC.

Table 1. Results of serum chemistry analysis.

	LR(Baseline)	LR(240 min)	HSA(baseline)	HSA(240 min)	ASB(baseline)	ASB(240 min)	HbV(baseline)	HbV(240 min)
TP	4.45	3.70	4.70+/-0.30	4.30+/-0.36	4.90+/-0.87	4.65-/-0.31	4.87+/-0.65	4.47+/-0.47
Alb	2.50	2.05	2.73+/-0.15	3.33+/-0.32	2.63+/-0.51	2.53-/-0.10	2.70+/-0.20	3.23+/-0.29
A/G	1.28	1.24	1.41+/-0.22	3.50+/-0.62	1.15+/-0.17	1.19+/-0.10	1.27+/-0.18	2.77+/-0.88
AST	21.5	759.0	19.0+/-1.7	31.3+/-15.7	28.3+/-10.7	147.3+/-110.1*	24.3+/-2.1	822.3+/-837.3*
ALT	27.5	784.5	40.7+/-32.4	31.3+/-15.9	31.3+/-15.9	163.8-/-94.9*	29.7+/-5.8	678.3+/-619.5*
LDH	56.0	693.0	48.7+/-5.1	60.3+/-10.7	87.3+/-62.5	155.3-/-60.5	67.3+/-3.1	678.3+/-619.5*
ALP	244.0	353.0	213.0+/-40.9	121.7+/-30.6*	212.5+/-95.0	223.5-/-64.3	259.3+/-44.6	173.0+/-59.4*
GGTP	6.5	1.5	3.3+/-0.6	1.7+/-0.6*	2.8+/-1.3	3.0-/-1.6	1.3+/-1.5	0.3+/-0.6*
LAP	25.5	23.0	22.3+/-4.0	12.0+/-2.0	31.0+/-11.9	32.0-/-6.7	30.0+/-6.6	16.0+/-3.5
ChE	7.5	8.0	7.3+/-1.5	6.0+/-2.6	7.0+/-3.6	7.3-/-2.5	9.0+/-1.7	4.7+/-0.6*
Tbil	0.0	0.0	0.0+/-0.0	0.2+/-0.0	0.05+/-0.06	0.08-/-0.05	0.0+/-0.0	0.33+/-0.15
CRTNN	0.43	1.14	0.49+/-0.07	0.68+/-0.28	0.50+/-0.06	0.55-/-0.08	0.43+/-0.02	0.42+/-0.06
BUN	11.6	25.6	12.73+/-4.95	19.30+/-7.15	11.73+/-1.70	18.68-/-3.34*	11.07+/-0.75	18.20+/-3.41*
UA	0.20	0.85	0.13+/-0.06	0.17+/-0.12	0.23+/-0.10	0.58-/-0.26	0.23+/-0.12	0.37+/-0.15
Amylase	646.0	876.5	383.7+/-69.4	324.7+/-57.6	423.3+/-27.6	460.3-/-56.9	442.3+/-11.5	298.3+/-51.2*
Lipase	113.5	137.5	188.7+/-67.7	100.3-/-124.2	184.0+/-143.1	176.0-/-124.2	163.7+/-21.1	94.7+/-23.9*
CPK	401	1202	251.7+/-62.3	280.0+/-58.9	419.8+/-250.3	773.0-/-185.8	459.3+/-132.8	733.7+/-180.4
Tcho	97.0	80.0	111.3+/-22.4	55.3+/-9.7*	113.8+/-18.0	114.8-/-13.9	113.7+/-26.1	62.0+/-14.2*
Fcho	27.0	23.0	30.3+/-6.7	16.7+/-3.1*	30.0+/-6.1	31.3-/-6.5	26.0+/-5.2	17.0+/-6.9
Echo	70.0	57.0	81.0+/-15.7	38.7+/-6.7*	83.8+/-13.0	83.5-/-7.7	87.7+/-21.1	45.0+/-7.5*
β-LP	199.0	90.5	226.0+/-120.0	56.3+/-23.2*	247.3+/-120.2	172.5-/-74.1	160.7+/-42.0	107.3+/-69.0
HDL-C	86.4	70.3	94.4+/-11.3	46.6+/-9.8*	95.6+/-13.9	93.5-/-10.5	94.8+/-14.2	45.0+/-8.1*
TG	17.5	15.0	23.3+/-10.2	11.7+/-2.1	19.3+/-4.1	17.5-/-5.3	19.3+/-4.9	10.0+/-1.0*
T Lipid	249.5	207.5	290.0+/-63.4	144.0+/-25.2*	292.0+/-44.3	292.8-/-38.1	292.0+/-60.9	158.7+/-33.8*
FFA	0.30	0.35	0.90+/-0.65	0.63+/-0.18	0.67+/-0.29	0.80-/-0.29	0.49+/-0.23	0.84+/-0.08
PhL	218.5	182.5	241.7+/-28.9	138.0+/-20.2*	241.8+/-38.6	246.5-/-25.8	241.7+/-49.1	161.3+/-43.9*
K	3.55	8.05*	3.40+/-0.30	3.57+/-0.55	3.73+/-0.41	4.10-/-0.96	3.50+/-0.46	3.53+/-0.21
Ca	8.60	8.25	10.00+/-0.10	9.73+/-0.76	9.43+/-1.37	10.45-/-0.57	9.13+/-0.65	8.40+/-0.90
IP	8.25	14.90*	6.87+/-1.50	8.40+/-0.46	6.93+/-1.09	10.03-/-1.54*	7.07+/-1.70	8.40+/-1.08
Mg	1.75	2.30	1.93+/-0.15	1.97+/-0.06	1.90+/-0.24	2.13-/-0.21	1.90+/-0.17	2.00+/-0.17
Fe	105.0	169.5	125.0+/-14.0	93.0+/-26.1	98.3+/-29.7	142.8-/-37.2	133.7+/-48.1	105.7+/-44.8

\*significant difference between baseline and 240 minutes.

MAP increased after resuscitation in every group. In the LR group, the recovery of MAP was poor and unstable. One animal died during the experiment (150 minutes after resuscitation). For isotonic crystalloid resuscitation, large-volume resuscitation has been thought necessary. Our result indicated that the permeability of the microcirculation increased after hemorrhagic shock, allowing LR to leak into the interstitial space and thus decreasing the circulating blood volume. The other three groups (ASB, HSA, and HbV) received colloidal fluids that were able to maintain the circulation volume. After resuscitation, MAP was stable in the HbV, HSA, and ASB groups. The LR and HbV groups were significantly different, but we could find no significant differences between the HbV and HSA groups or between the HbV and ASB groups.

In contrast to the MAP, the MPAP increased in the HbV group after resuscitation. The MPAP immediately after resuscitation was also significantly higher in the HbV group than in the other groups. This increase was rapidly resolved, and there were no significant differences between the HbV group and the other groups at later time points. The increase in MPAP was very brief, lasting less than 10 minutes. The highest pressure measured was  $20 \text{ mm} \pm 5.6 \text{ mmHg}$ . Since pulmonary hypertension is diagnosed in humans when MPAP is above 25 mmHg in the resting state [26], we are inclined to describe this phenomenon as a transient rise in MPAP. This might be considered an effect of HbV infusion.

HbV were 250 nm in size and did not extravasate. Although HbV is able to bind NO, its binding kinetics are slow compared to those of the Hb molecule or a modified Hb-based oxygen carrier [27]. Therefore, it seems unlikely that the transient increase in MPAP could be due to NO scavenging by the HbV. In this study, we used deoxy-type HbV for resuscitation. Rapid infusion of deoxy-HbV might cause transient hypoxia. Although HbV is oxygenated immediately after passing through the lung, hypoxic pulmonary vasoconstriction could occur before the majority of the HbV is oxygenated. We cannot disprove that the significant increase in PAP was due to NO scavenging; however, if NO scavenging is the cause of the transient rise in MPAP, the increase in MPAP might have been expected to be of longer duration. To clarify this point, we will have to perform another study focusing on NO metabolism.

PCWP (pulmonary capillary wedge pressure) recovered after resuscitation in every group. In the HSA group, PCWP increased after resuscitation to significantly higher than the baseline. Cardiac output in the HSA group was high after resuscitation because of compensation for low hemoglobin concentration. High cardiac output was necessary to deliver adequate oxygen to the tissues. An increase in left ventricular endo-diastolic pressure (which is almost identical to PCWP) could be followed by increased cardiac output.

Cardiac output (CO) showed consistent changes in every group (Figure 2a). After resuscitation, CO recovery was high in the HSA group and remained significantly higher than in the HbV group. In the LR group, the recovery of CO decreased with time, indicating volume loss of circulating blood due to fluid shift to the interstitial space.

Vascular resistance after resuscitation decreased compared to the baseline level. After resuscitation, SVR was significantly lower in the HSA group than in the HbV group (Figure 2b). In contrast, SVR was also lower in the LR group than in the HbV group until 120 minutes after resuscitation. In the HSA and LR groups, the resuscitation fluid had no oxygen-carrying capacity. Therefore, it was physiologically appropriate to increase the cardiac output and decrease the SVR in order to facilitate oxygen delivery and maintain tissue oxygenation. This appears to be an auto-regulatory mechanism in this model.

As respiration was maintained by a ventilator,  $\text{PaO}_2$  and  $\text{PvO}_2$  were quite consistent (Figure 3a, 3b). This result indicated that administration of HbV itself did not influence oxygenation in the lung. The  $\text{PvO}_2$  was decreased in the LR group relative to the other groups, indicating increased oxygen extraction by the peripheral tissues.

We measured tissue oxygen tension at the renal cortex to evaluate the degree of hypoxia due to poor organ perfusion caused by hemorrhagic shock (Figure 4a). We also measured  $\text{rSO}_2$  (regional oxygen saturation) of the brain (Figure 4b). The kidney is one of the organs most vulnerable to hypoperfusion. The results showed that after exsanguination the  $\text{PtO}_2$  of the renal cortex decreased uniformly in every group but also recovered to be baseline after resuscitation in every group. HbV did not worsen the renal perfusion or oxygen metabolism.

The regional oxygen saturation ( $\text{rSO}_2$ ) of the brain recovered after resuscitation in all groups. These results indicated that HbV was an effective resuscitation fluid in terms of restoring the circulation of vital organs.

Hematocrit (Ht) changed in proportion to the hemorrhage and resuscitation (Figure 5a). The Ht level remained stable after resuscitation in every group. In the HbV group, the Hb concentration attributable to HbV was measured separately. The total hemoglobin concentration is shown in Figure 5. The Hb concentration derived from HbV was  $3.86 \pm 0.4 \text{ g/dL}$  immediately after resuscitation and decreased to  $3.24 \pm 0.14 \text{ g/dL}$  30 minutes after resuscitation. This decrease in Hb concentration was thought to be caused by phagocytotic elimination of HbV by the reticuloendothelial (RES) system, although the change in Hb concentration was not significant [9].

Blood pH and lactate are indicators of anoxic metabolism [28]. After resuscitation, the decrease of serum lactate was limited in the LR group because lactate was supplied in the resuscitation fluid (Figure 6b). Ninety minutes post-resuscitation, the LR group showed increased

lactate, and one animal later died, indicating hypoperfusion and anoxic metabolism. In the ASB, HSA, and HbV groups, lactate decreased over time to the baseline level, indicating that HbV fluid was as capable of restoring tissue oxygen metabolism as ASB and HSA.

Methemoglobin concentration (MetHb) in HbV was measured after ultracentrifugation to recover the HbV from the blood. The initial MetHb level in the HbV used for resuscitation was 5.0%. Immediately after resuscitation, MetHb constituted  $7.4 \pm 1.5\%$  of the Hb, and the proportion steadily increased to  $16.3 \pm 5.7\%$  240 minutes after resuscitation (Figure 7). The oxidization of Hb in HbV was attributed to oxidative stress during hemorrhagic shock and resuscitation. However, even in this oxidative environment, more than 80% of the injected HbV was functional as an oxygen carrier 4 hours after administration.

Oxygen delivery was calculated by measuring the Hb concentration of HbV after resuscitation and the  $O_2$  dissociation curve of HbV (Figure 8a). The proportion of the oxygen delivered to the tissues by HbV was 33% (immediately after resuscitation) to 26% (24 min post-resuscitation). When the impact of HbV was measured in terms of oxygen consumption, 25.0–20.1% of the oxygen consumed was delivered by HbV (Figure 8b). This result showed that HbV circulated during resuscitation and contributed to the oxygen delivered and consumed.

The influence on serum chemistry of HbV was evaluated. As HbV was dispersed in 5% albumin saline, the albumin increased after resuscitation similarly to that of the HSA group. The liver is one of the organs vulnerable to damage by hypoperfusion and hypoxia. From the results of hemodynamic observation, all groups experienced hemorrhagic shock. AST and ALT increased in the LR, HbV, and ASB groups. In the HbV and ASB groups, the end-experiment values were significantly increased from the baseline. In the HbV group, ChE decreased significantly after resuscitation. ChE is a short half-life serum enzyme that represents the protein synthesis function of the liver. This result showed that HbV suppresses hepatic protein synthesis after resuscitation of hemorrhagic shock. The liver functions as one of the RES organs, and HbV is phagocytosed by macrophages and further metabolized. This process might cause temporary suppression of liver function. ChE did not change after resuscitation in the rat, indicating a species difference in the effect of HbV [9]. Further investigation of the recovery of liver function will require a longer study.

The increase of LDH indicates systemic or organ cellular damage. The LDH results in the LR and HbV groups seem to indicate severe cellular damage. Hypoperfusion is thought to be the cause of the organ dysfunction in the LR group [29]. In the HbV group, the increased LDH might be a side effect. Further study is necessary to clarify the cause of the increased LDH and whether it is affected by dose and speed of administration.

Cardiac toxicity has been noted for a hemoglobin-based oxygen carrier [30]. Our HbV is encapsulated by a lipid bilayer, which we suspected would avoid this effect of free hemoglobin molecules. In this study, CPK did increase in the HbV group, although not significantly. It was noteworthy that the ASB group also showed increased CPK. Since we splenectomized the animals before the experiment, the abdominal wall, including muscles, was damaged during laparotomy and splenectomy. This damage might be the source of the increased CPK, and an isozyme study to test this hypothesis should be considered.

The decreased lipid component is thought to be a dilutional effect of the resuscitation fluid. This study was designed to examine the short-term circulatory effect on Beagle dogs; another study should be planned to investigate the long-term effects.

Organs were recovered at the end of the experiment and examined histologically; we observed no significant changes except in the LR group, in which slight congestion of the liver was noted at 240 min after resuscitation.

In this pilot study, we evaluated the short-term safety and efficacy of HbV as a resuscitation fluid for hemorrhagic shock in Beagle dogs. The long-term effects of administering a large volume of HbV should be tested in another study.

## CONCLUSIONS

HbV dispersed in a 5% albumin/saline solution was as effective as ASB or HSA in resuscitation of a 50% hemorrhagic shock resuscitation model in Beagle dogs. In this model, 50% of the circulation volume of resuscitation fluid was administered into the right femoral vein over 20 minutes. Rapid large-volume administration could be performed safely in Beagle dogs. We found a temporary increase in MPAP after resuscitation. The infused HbV was deoxy-form. Although oxygenation occurs immediately, hypoxic vasoconstriction could occur before most of the HbV was converted to the oxygenated form. We could not conclude that the significant increase in PAP was due to NO scavenging. MAP increased after resuscitation and remained stable in the HbV group. If the temporary increase in PAP is due to NO scavenging, it should have lasted longer and should have been reflected in the systemic pressure. We concluded that the transient increase of MPAP after infusion of HbV should be followed up in another study focused on NO metabolism. Long-term effects, including organ toxicity, should also be examined in follow-up studies.

**Declaration of interest:** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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# Cardiopulmonary hemodynamic responses to the small injection of hemoglobin vesicles (artificial oxygen carriers) in miniature pigs

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**Abstract:** Intravenous injection of liposomes into pigs reportedly induces anaphylactoid reactions at a small dose, resulting in circulatory disorder. Hemoglobin vesicles (HbVs) are artificial oxygen carriers encapsulating Hb solution in liposomes. It is not known how pigs respond to HbV injection. We aimed to analyze the cardiopulmonary responses to small injections of HbV and empty vesicle (EV) and compare them with a conventional liposome (CL) with a different lipid composition containing phosphatidylglycerol (PG). PG is known to induce an anaphylactoid reaction in pigs. Nine male miniature pigs were used for HbV, EV, and CL injections. The anesthetized pig received 0.05 and 0.5 mL/kg of a test fluid for the first and second injection with a 70 min interval. Results show that CL repeatedly induced significant increases in systemic and pulmonary arterial pressures and vascular resistances and decreases in heart rate and cardiac output (CO). HbV and EV at the first injection-induced pulmonary hypertension,

with significantly smaller changes in systemic arterial pressure and CO. No remarkable response was visible at the second injection in spite of a larger dosage. Only CL repeatedly induced thrombocytopenia, leukocytopenia, and plasma thromboxane B<sub>2</sub> increase resulting from complement activation, although HbV and EV showed smaller changes. Transmittance electron micrograph of pulmonary intravascular macrophages (PIMs) showed phagocytosis of HbV, indicating the possibility that nonspecific phagocytosis by PIMs relate to the responses observed after the first injection. HbV does not induce a significant anaphylactoid reaction in pigs compared with CL because of the different lipid composition. © 2012 Wiley Periodicals, Inc. *J Biomed Mater Res Part A*: 00A:000–000, 2012.

**Key Words:** liposome, blood substitutes, pulmonary hypertension, pulmonary intravascular macrophages, thromboxane

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## INTRODUCTION

Phospholipid vesicles or liposomes are widely used as a vehicle for drug delivery system aimed at cancer and antifungal therapies.<sup>1</sup> Stealth liposomes, of which the surfaces are modified with polyethylene glycol (PEG), improve biocompatibility and minimize the immunological recognition showing longer circulation persistence. Thereby, it enhances the targeting of incorporated drugs to the site of a disease. However, the so-called injection reaction is clarified as clinical experiences accumulate, such as dyspnea, tachypnea, tachycardia, hypotension and hypertension, chest pain, and back pain.<sup>2–5</sup> It is believed that these hypertensive reactions are a consequence of complement activation: so-called complement activation-related pseudoallergy.<sup>4</sup> Reportedly, pigs

respond reproducibly and significantly to the injection of liposomal products, especially those containing phosphatidylglycerol (PG), resulting in anaphylactoid reactions and cardiopulmonary disorders, manifested as systemic and pulmonary hypertension, increased vascular resistance, decreased cardiac output (CO), thrombocytopenia, tachycardia, and so forth. This model is useful for safety evaluation of liposomal drugs.<sup>4,5</sup>

Hemoglobin vesicle (HbV) is an artificial oxygen carrier encapsulating concentrated Hb solution (35–40 g/dL) in phospholipid vesicles (liposomes). It contains no pathogen or blood type antigen, and it can be stored at room temperature for years.<sup>6</sup> It is expected that HbV can be used as a transfusion alternative and for other clinical indications that

Of the authors, H.S. and K.S. are the holders of patents on Hb-vesicles.

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are not attainable with conventional blood transfusion. Safety and efficacy evaluations of its use, mainly using rodents, as a resuscitative fluid for hemorrhagic shock are conducted energetically,<sup>7,8</sup> as a priming fluid for extracorporeal membrane oxygenator,<sup>9</sup> and as a targeted oxygen carrier for ischemic tissues<sup>10-12</sup> and tumors.<sup>13</sup> Actually, Hb encapsulation shields the toxic effects of stroma-free Hbs, especially preventing unwanted reactions of Hb with endogenously produced NO,<sup>14,15</sup> thereby preventing NO-related vasoconstriction and hypertension.<sup>16</sup>

However, it is important to confirm the biocompatibility of "vesicles." The membrane components of the vesicles are phosphatidylcholine, cholesterol, PEG-conjugated lipid, and a negatively charged lipid. The negative charge on the surface of the vesicles is necessary to enhance the encapsulation efficiency by reducing the lamellarity of the vesicles.<sup>17</sup> In addition, it is a key molecule for determining biocompatibility in terms of complement activation. Our former formulation of HbV contained DPPG as a negatively charged lipid. It showed thrombocytopenia in a rat model.<sup>18</sup> The present formulation of HbV contains a different type of negatively charged lipid, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG).<sup>6</sup> It has been confirmed that DHSG significantly reduces the complement activation using a rat model,<sup>19,20</sup> and human plasma.<sup>21</sup> Nevertheless, it remains unknown how pigs respond to the injection of HbV. Pigs are known to respond sensitively to the injection of liposomes.<sup>4</sup> Moreover, they show different biodistribution of injected foreign particles because of the presence of pulmonary intravascular macrophages (PIMs), which are usually not seen in rodents and humans<sup>22</sup>; but in some pathological conditions PIMs appear.<sup>23,24</sup>

In this study, we aimed at examination of the response of pigs to the small injection of HbV and empty vesicles (EVs) that do not contain Hb. We analyzed the cardiopulmonary response, and compared it with that obtained through injection of PG-containing vesicles that are known to induce an anaphylactoid reaction.

## MATERIALS AND METHODS

### Preparation of HbVs and EVs

With only slight modifications, HbVs were prepared using a method reported previously.<sup>6</sup> Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Then, Hb was stabilized by carbonylation and concentrated using ultrafiltration to 38 g/dL. Subsequently, pyridoxal 5'-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the carbonylhemoglobin solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 2.5. The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, DHSG (Nippon Fine Chemical Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG<sub>5000</sub> (DSPE-PEG; NOF Corp., Tokyo, Japan) at the molar composition of 5/5/1/0.033. The particle diameter was regulated using the extrusion method. The encapsulated carbonylhemoglobin was converted to oxyhemoglobin by exposing the

liquid membranes of HbVs to visible light under an aerobic atmosphere. Finally, the Hb and whole lipid concentrations of the suspension were adjusted, respectively, to 10 and 6 g/dL. The particle diameter distribution was measured using a light-scattering method; it was 280 nm on average.

A suspension of EVs, with the same lipid composition as that of HbV but which does not contain Hb, was prepared using the same lipids by hydration with a saline solution. The particle diameter was regulated using the extrusion method. The lipid concentration (6 g/dL), the particle diameter (ca. 280 nm), and the viscosity (ca. 3 cP) were almost identical to those of HbV.

EVs containing DPPG as a conventional liposome (CL) were prepared by hydrating a freeze-dried lipid powder (DPPC/cholesterol/DPPG/DSPE-PEG = 5/4/0.9/0.03 by mol) with a saline solution. The particle diameter was regulated using the extrusion method. The lipid concentration (6 g/dL), the particle diameter (ca. 280 nm), and the viscosity (ca. 3 cP) were almost identical to those of HbV.

### Animal model preparation for injection and measurement of cardiopulmonary circulation

The Laboratory Animal Care and Use Committee of Nihon Bioresearch approved the entire experimental protocol. The protocol complies with the Basic Guidelines for the Use of Experimental Animals in Institutions under the Jurisdiction of the Ministry of Health, Labour and Welfare, Japan (Notification No. 0601001 of the Science Bureau, Japanese Ministry of Health, Labour and Welfare, Japan, June 1, 2006) in accordance with the Declaration of Helsinki, and the Regulations of the Committee for the Ethical Treatment of Animals (Nihon Bioresearch, April 2, 2007). Experiments were conducted using nine male NIBS pigs (33.6 ± 1.0 kg body weight, 11–18 months old). They were purchased from Nisseiken Co. (Yamanashi, Japan) and used after a quarantine period of 1 week and habituation for a few weeks.

The pigs were anesthetized with an intramuscular injection of ketamine chloride (15 mg/kg) and then intubated and ventilated (10–15 mL/kg, 15–25 times/min; R-60; Anes, Tokyo) with 2–3% isoflurane in a mixed gas of N<sub>2</sub>O and O<sub>2</sub> (2:1 by vol.) using a vaporizer (Safer 100; Anes). A polyethylene tube (PE-90; Intramedic, Becton, Dickinson and Co., NJ) was inserted into a left femoral artery and connected to a transducer (TP-300T; Nihon Kohden Corp., Tokyo) and an amplifier (AP-641; Nihon Kohden Corp.) for mean arterial pressure (MAP) and heart rate (HR) monitoring. The electrocardiograph (ECG) of standard limb lead II was produced using an amplifier (AB-621; Nihon Kohden Corp.). A catheter-shaped transducer (5F; Millar Instruments, TX) was inserted through the left carotid artery to the left ventricle, which was connected to an amplifier (AP-601; Nihon Kohden Corp.) for measurement of left ventricular end-diastolic pressure (LVEDP). A Swan-Ganz catheter (T172HF7; Edwards Lifesciences LLC, Irvine, CA) was inserted through the right jugular vein to a pulmonary artery. It was connected to an amplifier (AP-641G; Nihon Kohden Corp., Tokyo) for measurement of pulmonary arterial pressure (PAP) and to a thermodilution monitor (MTC-6210; Nihon Kohden

Corp.) for CO measurement. CO was assessed using a thermodilution method with a single rapid injection of a cold glucose solution (5 mL, 0°C) at every measurement. Even though CO measurements usually require three times of injections for averaging, we avoided such multiple injections at every measurement because the changes of pulmonary circulation to be observed were so rapid (see "Results" section). Right atrial pressure (RAP) was measured using a PE-90 tube connected to an amplifier (AP-641G). For the intravenous injection of a test fluid, a PE-90 tube was inserted into the left femoral vein. For peripheral blood collection, a PE-90 tube was inserted into the right femoral vein. All PE-90 tubes and the catheter were filled with 20 U/mL heparin mixed saline solution. All data were recorded simultaneously using a recorder (WT-645G; Nihon Kohden Corp.); they were stored and analyzed using computer software (PowerLab; AD Instruments Pty.). Pulmonary vascular resistance (PVR) was calculated as  $(PAP/CO) \times 80$  (in  $\text{dyne}\cdot\text{s}\cdot\text{cm}^{-5}$ ), and systemic vascular resistance (SVR) was calculated as  $\{(MBP - RAP)/CO\} \times 80$  (in  $\text{dyne}\cdot\text{s}\cdot\text{cm}^{-5}$ ).

To confirm the anesthetic condition, arterial blood (2–3 mL) was collected from the femoral artery, and carbon dioxide tension was measured using a blood gas analyzer (AVL OPTI CCA cassette B; Sysmex Corp., Kobe, Japan). It was adjusted to 35–40 mmHg by changing the rate and amount of ventilation.

After confirming the stabilization of the anesthetic condition, a test fluid (0.05 mL/kg, HbV, EV, or CL) was injected intravenously as the first injection. After a 70 min interval, the same test fluid but increased dose (0.5 mL/kg) was injected as the second injection. According to a literature,<sup>5</sup> a bolus injection of liposome (5–100 mg lipids in 1 mL) into a pig (25–50 kg) induced a strong anaphylactoid reaction (0.2–4 mg/kg). In our experiment, the first and the second injections correspond to 3 and 30 mg/kg, respectively, which are, respectively, comparable and much higher doses than those used in a previous study. We intended to test the responses to both repeated injections (twice) and a dose dependence (0.05 and 0.5 mL/kg) in one pig. The volume of injections is less than 1% of total blood volume (77 mL/kg), and essentially it does not affect on blood oxygen content, blood volume, blood osmotic pressure, and so forth.

Arterial blood pressure, HR, left ventricular pressure, PAP, RAP, and ECG were recorded continuously from the preparation of the animal until 60 min after the second injection. MAP, HR, LVEDP, PAP, and RAP were measured at 10, 5, and 1 min before injection, and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, and 60 min after injection. CO was measured at 10 and 5 min before injection and 1, 2, 3, 5, 10, 20, 40, 60 min after injection. The time point of 60 min after the first injection was used as the time point of 10 min before the second injection.

#### Blood biochemistry and hematological and pathological studies

One milliliter of blood was withdrawn from the femoral vein into an EDTA-2K-treated syringe. Then the platelet (PLT) count and white blood cell (WBC) count were ascer-

tained using a blood cell counter (K-4500; Sysmex Corp.). Another 2 mL of blood was collected in a heparinized vacuumed tube. It was centrifuged at  $1600 \times g$  for 15 min to obtain blood plasma. It was then stored at  $-80^\circ\text{C}$  and was transferred to SRL (Tokyo) for quantitative measurements of thromboxane B<sub>2</sub> (TXB<sub>2</sub>) using a radioimmunoassay method provided by NEN-Life Science Products (Shelton, CT).

After the experiment, the pig was euthanized by exsanguination. Its lungs were examined macroscopically. Then a part of the lung tissue was dissected and fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries, Tokyo) for all the pigs. The paraffin sections were stained with hematoxylin and eosin. Transmission electron microscopic (TEM) observation was performed to visualize the presence of the HbV particles in the lung tissue (PCL Japan, Tokyo, Japan). For that purpose, tissues of the HbV group were fixed with 2.5% glutaraldehyde solution, cut into approximately 2-mm<sup>3</sup> 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 from 50% to 100%, washed with propylene oxide. They were 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 lung tissues were examined under a TEM (JEM-100CX; JEOL, Tokyo, Japan) and photographed.

#### *In vivo* data analysis

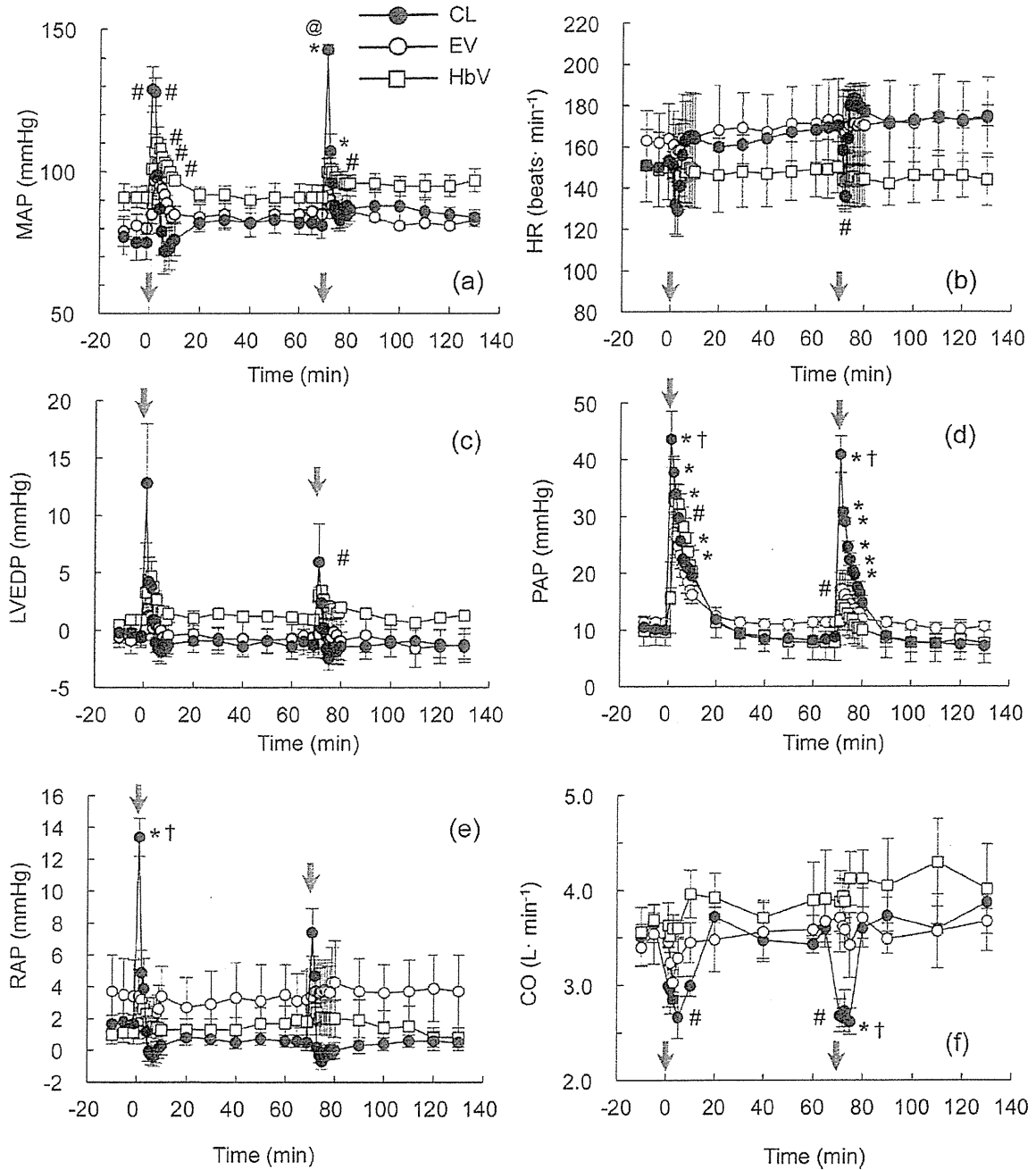
The *in vivo* data are given as the mean  $\pm$  standard error. Data were analyzed using Abel 3 (Gigawiz Co., Tulsa). Time-related differences compared with the baseline within each group were assessed using a paired *t* test. Differences among the groups at the same time point were assessed using analysis of variance, followed by Fisher protected least significant difference.

## RESULTS

### Cardiopulmonary responses to the injection of vesicles

MAP of the CL group increased significantly from  $75 \pm 6$  mmHg at the baseline to  $129 \pm 8$  mmHg at 1 min after the first injection ( $p < 0.05$ ) but returned to the baseline level in 5 min [Fig. 1(a)]. MAP repeatedly increased significantly to  $143 \pm 2$  mmHg at 1 min after the second injection ( $p < 0.01$ ). It reverted to the baseline level in 5 min. The EV and HbV groups showed the maximum MAP of  $98 \pm 6$  mmHg and  $110 \pm 6$  mmHg at 3 min ( $p < 0.05$ ), respectively, after the first injection, and returned to the original level in 10 min. The change was minimal (nonsignificant) for both groups after the second injection, which was contrasted to that of the CL group ( $p < 0.001$ ). The responses of the HbV and EV groups were delayed a few minutes in comparison to those of the CL group.

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**FIGURE 1.** Systemic cardiovascular responses to the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg): closed circles, CL; open circles, EV; open squares, HbV; (a) mean arterial pressure, MAP; (b) heart rate, HR; (c) left ventricular end-diastolic pressure, LVEDP; (d) mean pulmonary arterial pressure, PAP; (e) mean right atrial pressure, RAP; and (f) cardiac output, CO. For LVEDP, one pig for the HbV group was omitted because of unsuccessful measurement with an abnormal value at the baseline. The CL group showed stronger and repeated responses to the first and second injections. However, both HbV and EV showed lower responses. Arrows indicate the time points of the first and the second injections (0, 70 min). \* $p < 0.01$  vs. baseline, #  $p < 0.05$  vs. baseline, @  $p < 0.001$  vs. EV and HbV, and †  $p < 0.01$  vs. EV and HbV.

HR of the CL group tended to decrease repeatedly by about 20 beats/min and 40 beats/min, respectively, at the first and the second injection. However, the HbV and EV groups showed no such changes [Fig. 1(b)].

LVEDP of the CL group tended to increase to  $12.8 \pm 5.2$  mmHg at 1 min after the first injection, and it repeatedly increased to  $5.9 \pm 3.4$  mmHg at 1 min after the second

injection. However, both HbV and EV showed minimal changes [Fig. 1(c)].

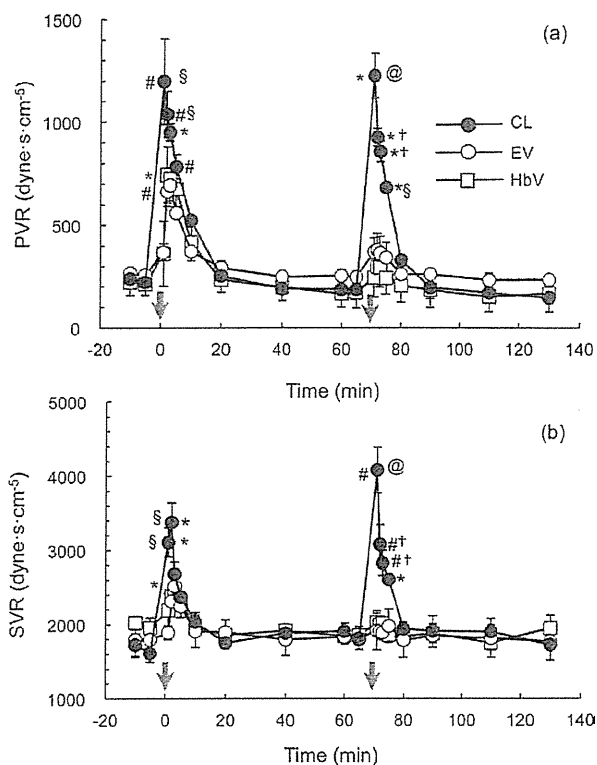
PAP of the CL group increased significantly from  $10 \pm 1$  mmHg at the baseline to  $44 \pm 5$  mmHg at 1 min after the first injection ( $p < 0.01$ ). It returned to the baseline level within 10 min [Fig. 1(d)]. Again, it increased repeatedly to  $41 \pm 3$  mmHg at 1 min after the second injection, and returned to the original level within 20 min. PAP of the EV group increased, respectively, to  $27 \pm 4$  and  $17 \pm 3$  mmHg at 3 min after the first and the second injections. PAP of the HbV group, respectively, increased to  $33 \pm 4$  and  $14 \pm 4$  mmHg at 3 min after the first and the second injections. The increased levels of the EV and HbV groups were significantly lower than that of the CL group ( $p < 0.01$ ). Furthermore, the EV and HbV groups showed a few minutes of delayed response in comparison to the CL group.

RAP of the CL group significantly increased from  $1.8 \pm 0.5$  mmHg at the baseline to  $13.4 \pm 1.2$  mmHg at 1 min after the first injection ( $p < 0.01$ ). It returned to the baseline level in 4 min [Fig. 1(e)]. It increased repeatedly to  $7.4 \pm 1.5$  mmHg at 1 min after the second injection ( $p < 0.05$ ), and returned to the baseline level within 2 min. RAP of both HbV and EV groups showed no such changes at either the first or second injections.

CO of the CL group at the baseline was  $3.5\text{--}3.7$  L·min<sup>-1</sup> on average. It started to decrease 1 min after the first injection and showed the minimum value of  $2.66 \pm 0.22$  L·min<sup>-1</sup> at 3 min after the injection ( $p < 0.05$ ) [Fig. 1(f)]. It reverted to the original level within 20 min after injection. At 1 min after the second injection, CO started to decrease significantly ( $p < 0.01$ ), reaching the minimum value of  $2.62 \pm 0.14$  L·min<sup>-1</sup> at 5 min after the second injection. It reverted to the original level within 10 min. The differences in CO between the CL group and the other two groups were significant ( $p < 0.01$ ).

PVR was calculated with PAP and CO. The CL group showed significant PVR increase from 220–240 dyne·s·cm<sup>-5</sup> on the average at the baseline to  $1197 \pm 208$  dyne·s·cm<sup>-5</sup> at 1 min after the first injection ( $p < 0.01$ ) [Fig. 2(a)], in parallel to the significant PAP increase and CO decrease. PVR returned to the original level in 20 min. Again it increased repeatedly to  $1228 \pm 109$  dyne·s·cm<sup>-5</sup> at 1 min after the second injection and subsequently returned to the original level within 20 min. The EV and HbV groups showed significant PVR increases, respectively, to  $694 \pm 74$  and  $726 \pm 53$  dyne·s·cm<sup>-5</sup> at 3 min after the first injection, but they were significantly lower than the level of the CL group ( $p < 0.05$ ). After the second injection, those groups showed much smaller changes than those of the CL group ( $p < 0.001$ ).

SVR was calculated with MAP, RAP, and CO. The CL group showed a significant increase in SVR from 1600–1700 dyne·s·cm<sup>-5</sup> on average, at the baseline to  $3378 \pm 262$  dyne·s·cm<sup>-5</sup> at 2 min after the first injection ( $p < 0.01$ ) [Fig. 2(b)]. It returned to the original level in 20 min. It repeatedly increased significantly to  $4081 \pm 309$  dyne·s·cm<sup>-5</sup> 1 min after the second injection ( $p < 0.001$ ); it returned to the original level in 10 min. Both EV and HbV



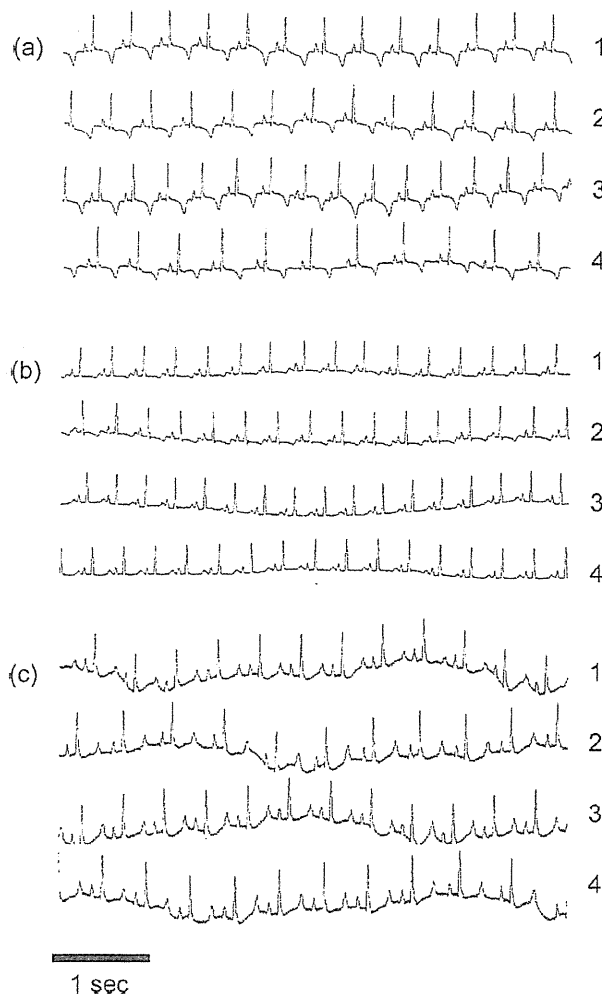
**FIGURE 2.** Changes in (a) pulmonary vascular resistance, PVR, and (b) systemic vascular resistance, SVR, at the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV; open squares, HbV. The CL group showed stronger and repeated changes in response to both the first and second injections. Arrows indicate the time points of the first and the second injections (0, 70 min). \* $p < 0.01$  vs. baseline, #  $p < 0.05$  vs. baseline, @  $p < 0.001$  vs. EV and HbV, †  $p < 0.01$  vs. EV and HbV, and §  $p < 0.05$  vs. EV and HbV.

groups showed smaller increases in SVR after the first injection, with no increase after the second injection.

In spite of the significant changes of cardiovascular responses to the injection of liposomal fluids, ECG of all the groups showed no notable change such as arrhythmia, ST depression, or T-wave changes (Fig. 3).

#### Hematological data and thromboxane B<sub>2</sub>

The number of PLTs of the CL group decreased significantly from  $44.7 \pm 9.3 \times 10^4$   $\mu\text{L}^{-1}$  at a baseline to  $13.5 \pm 4.9 \times 10^4$   $\mu\text{L}^{-1}$  1 min after the first injection ( $p < 0.05$ ) [Fig. 4(a)]. It returned to the baseline level at 30 min. After the second injection, it tended to decrease repeatedly to  $22.5 \pm 3.0 \times 10^4$   $\mu\text{L}^{-1}$ ; then it returned to the baseline level 30 min later. PLT for the HbV and EV groups showed no such dramatic change after either the first or second injection. The number of WBCs of the CL group tended to decrease from  $103 \pm 18 \times 10^2$   $\mu\text{L}^{-1}$  at the baseline to  $62 \pm 13 \times 10^2$   $\mu\text{L}^{-1}$  1 min after the first injection [Fig. 4(b)]. It returned to the baseline level at 30 min. After the second injection, it decreased repeatedly to  $42 \pm 10 \times 10^2$   $\mu\text{L}^{-1}$  1 min after the second injection ( $p < 0.05$ ). It returned to



**FIGURE 3.** Electrocardiographs obtained before and immediately after injection of the first and second injections of liposomes: (a) CL group, (b) EV group, and (c) HbV group. 1, before the first injection; 2, after the first injection; 3, before the second injection; 4, after the second injection.

$126 \pm 23 \times 10^2 \mu\text{L}^{-1}$  after 30 min. The changes of WBC for the HbV and EV were smaller than that of the CL group. No change was observed after the second injection.

The plasma level of TXB2, a hydrolyzed form of reactive TXA2, increased rapidly and significantly from  $52 \pm 12.2 \text{ pg}\cdot\text{mL}^{-1}$  at the baseline to  $20,000 \pm 4500 \text{ pg}\cdot\text{mL}^{-1}$  at 1 min after injection of CL ( $p < 0.001$ ) (Fig. 5). It decreased considerably to  $617 \pm 337 \text{ pg}\cdot\text{mL}^{-1}$  at 60 min after injection. After the second injection of CL, the TXB2 level repeatedly increased significantly to  $9830 \pm 1530 \text{ pg}\cdot\text{mL}^{-1}$ . However, both the EV and HbV groups showed significantly smaller increases. The EV group showed small increases of TXB2 to  $260 \pm 109 \text{ pg}\cdot\text{mL}^{-1}$  at 1 min after the first injection, and  $83 \pm 7 \text{ pg}\cdot\text{mL}^{-1}$  after the second injection. The HbV groups showed small increases to  $567 \pm 96 \text{ pg}\cdot\text{mL}^{-1}$  at 1 min after the first injection, and  $129 \pm 25 \text{ pg}\cdot\text{mL}^{-1}$  at 1 min after the second injection.

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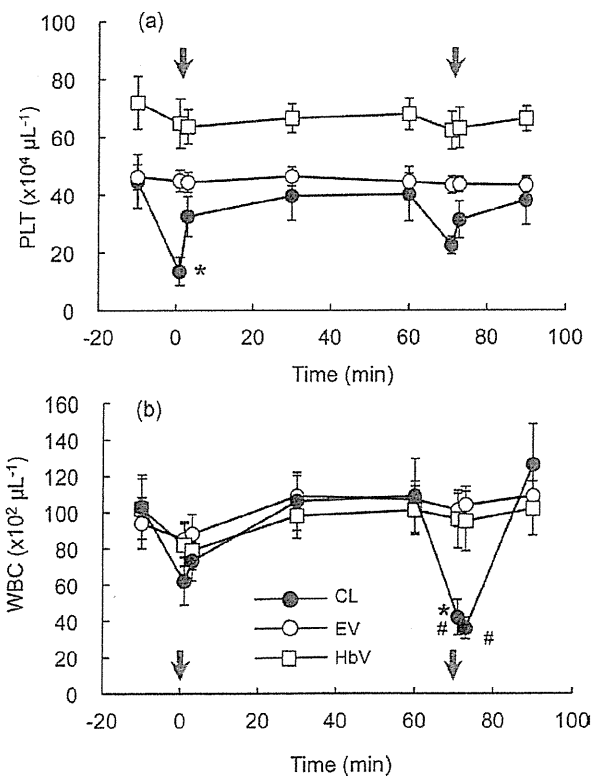
**Histopathological examination and TEM**

No notable abnormality was observed by the examination of the lung during autopsies. Because of acute studies, histological examination of sections of lung after hematoxylin and eosin staining did not show any abnormalities, such as edema or capillary embolism for all the groups (data not shown). TEM observations (Fig. 6) clearly revealed the presence of HbV particles in PIMs of pigs that were sacrificed immediately after the experiment. The HbV particles were visible in the phagosomes of PIMs. The HbV diameter is about 280 nm, which is about one-thirtieth of that of red blood cells. Because one Hb contains four irons and consequently has higher electron density, the HbV particles and red blood cells are black.

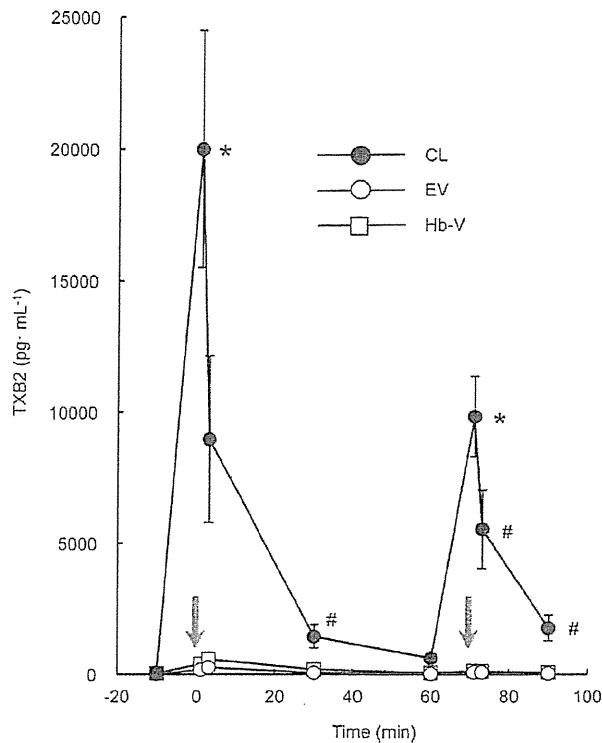
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**DISCUSSION**

The intravenous injection of the DPPG-containing CLs in pigs repeatedly induces pulmonary and systemic hypertension, decreases CO, and increases pulmonary and peripheral resistance at the first and second injections. Our results suggest that such significant cardiovascular responses are explainable by not only complement activation with liposomes but also phagocytosis of liposomes by PIMs.



**FIGURE 4.** Changes in (a) platelet count, PLT and (b) white blood cell count, WBC, in response to the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV, open squares, HbV. For PLT, one pig in the HbV group was omitted because of an abnormally high baseline value. Arrows indicate the time points of the first and the second injections (0, 70 min). \*  $p < 0.05$  vs. baseline and #  $p < 0.05$  vs. EV and HbV.



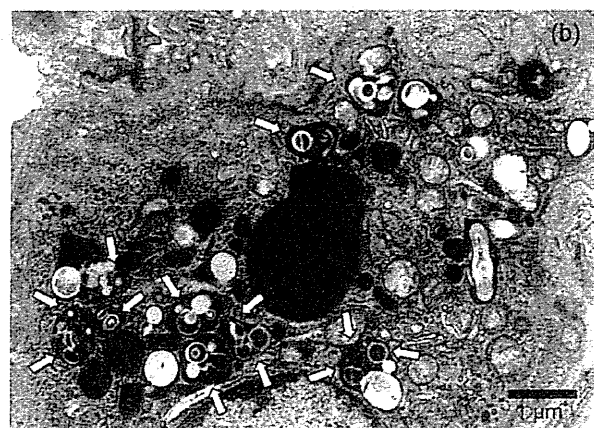
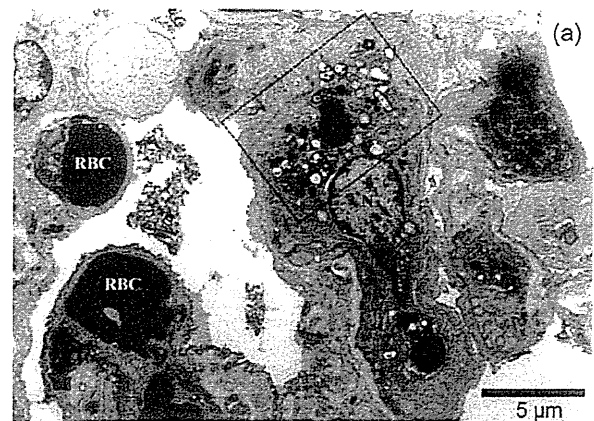
**FIGURE 5.** Changes in the plasma level of thromboxane B<sub>2</sub> (TXB<sub>2</sub>) in response to repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV, open squares, HbV. Arrows indicate the time points of the first and the second injections (0, 70 min). \*  $p < 0.001$  vs. EV and HbV; #  $p < 0.05$  vs. EV and HbV.

Improved surface biocompatibility of HbV and EV can prevent complement activation, but they are nonspecifically phagocytized by PIMs and induce mild pulmonary hypertension only at the first injection.

Many groups have tested so-called liposome-encapsulated Hb (LEH) of various kinds. Generally, the lipid composition of liposomes includes a phospholipid, cholesterol, a negatively charged lipid, and a surface modifier.<sup>6</sup> A small amount of a negatively charged lipid is required as one component of a lipid membrane to minimize the lamellarity (the number of bilayer membranes in a vesicle) to produce large unilamellar vesicles (LUVs) with a larger inner aqueous volume, which is important to encapsulate a functional material efficiently, a concentrated (35 g/dL) hemoglobin solution.<sup>17,25</sup> Commonly used negatively charged lipid includes fatty acids and PG. However, rat experiments clarified that PG induces complement activation.<sup>5,20,21,26–28</sup> Pape et al.<sup>29</sup> described in their report that a top-load infusion of LEH containing stearic acid in pigs induced “fatal” pulmonary hypertension and right ventricular failure. A subdomain of complement component 1 (C1q) has a cationic region that presumably interacts with negatively charged vesicles<sup>30</sup> that initiate the cascade of the classical pathway of complement activation, producing anaphylatoxins such as C3a and C5a, and which initiate a wide array of responses

through their effect on mast cells, polymorphonuclear cells, monocytes, and PLTs.<sup>31</sup> Actually, in the present experiment, we observed leukocytopenia and thrombocytopenia in the CL group only. Arachidonic acid cascade is activated and thromboxane A<sub>2</sub> (TXA<sub>2</sub>) is produced to induce strong systemic vasoconstriction. This scheme of responses is evident from the significant increases of plasma TXB<sub>2</sub>, a metabolite of TXA<sub>2</sub>, and simultaneous hypertension, reduction of CO, and increase of total peripheral resistance. All these systemic cardiovascular and hematological responses repeatedly observed in the CL group at the first and second injection seem to be related to the complement activation.

In fact, we tried to measure serum complement titer in pig plasma by the conventional reactive lysis method using hemolytic assay of sensitized sheep RBCs because it would be the direct evidence of the presence or the absence of complement activation. However, that method was



**FIGURE 6.** Transmittance electron micrographs of a lung tissue in the HbV group, which was sacrificed immediately after the experiment of two injections of HbV. (a) Whole view of a pulmonary intravascular macrophage, PIM, and the surrounding tissues. “N” denotes the nucleus of the PIM. RBCs are the red blood cells in the pulmonary capillaries. (b) A magnified view of the section in (a) surrounded by a broken line. Individual HbV particles are apparent in the phagosomes of the PIM, as indicated with white arrows. The large blackened parts in the center and smaller ones are phagocytized senescent red blood cells.

ineffective in the case of pig plasma. Even so, we were able to reproduce the experimental condition of Szebeni et al.<sup>5</sup> showing repeated anaphylactoid reaction at repeated injections in pigs. In fact, the surface of CL containing DPPG is modified with PEG (0.3 mol %). It does not seem to prevent complement activation in human plasma,<sup>21</sup> in rodents,<sup>20</sup> and pigs, as shown in this study, even though a previous report shows that dense PEGylation, like 5 mol % PEG<sub>2000</sub>-DSPE, prevented complement activation.<sup>32</sup> However, we have already clarified, from experiments using rodents, that our HbV and EV containing DHSG of a different negatively charged lipid do not induce complement activation and thrombocytopenia and do not induce significant cardiovascular changes. Nevertheless, we did not know how pigs would respond to the injection of HbV and EV.

This report is the first of a trial of HbV and EV injection into pigs. At the first injection, they showed significant increases in PAP and PVR but significantly smaller changes in MAP and CO. Even though the CL group showed the maximum MAP and PAP 1 min after the first injection, the HbV and EV groups showed slightly delayed responses; the maximum was visible 3 min after the first injection. At the second injection, the HbV and EV showed minimal changes in spite of 10 times' larger dosage. The levels of TXB2 of the HbV and EV were much lower than that of the CL group. The responses to the injections of HbV and EV were apparently different from those of CL, which showed repeated responses at the second injection.

PIMs have been found only in pulmonary capillaries and only in selected animal species such as ruminants and pigs but not in humans.<sup>22</sup> PIMs are actively phagocytic for circulating particles including foreign materials such as liposomes and colloids, and senescent erythrocytes (erythrophagocytosis). Such nonspecific phagocytosis by PIMs is much greater than those of Kupffer cells and spleen macrophages, which contrast against the systems found for rodents or humans.<sup>23,33</sup> Injection of foreign particles into sheep sometimes induces pulmonary hypertension, suggesting that the PIMs play a role in lung physiology and pathophysiologic changes through release of vasoactive and inflammatory mediators.<sup>34</sup> Actually, in our experiment, we confirmed that HbV particles were phagocytized by PIMs, as shown in TEM (Fig. 6). TEM was an effective tool for detecting the HbV particles in tissues.<sup>35</sup> Usually detection of liposomes is difficult and requires a marker such as gold particles. The high electron density of HbV caused by the highly concentrated Hb solution in the inner aqueous phase of HbV as well as in RBC provided sufficient contrast of the particle. It is speculated that not only HbV but also EV and CL are phagocytized by PIMs because of the same lipid composition and the same surface properties of HbV and EV, and because we confirmed that both HbV and Hb-encapsulated CL are similarly phagocytized by rodent spleen macrophages and Kupffer cells.<sup>35,36</sup> Reportedly, injection of liposomes into sheep also induces pulmonary hypertension because of phagocytosis by PIMs and release of TXB2.<sup>37-39</sup> Even though Szebeni et al.<sup>4,5</sup> concluded that liposome-induced pulmonary hypertension in pigs is related to complement activation,

the involvement of phagocytosis by PIMs was not considered. Our results suggest that the pulmonary hypertension and increase in peripheral and systemic resistances is induced by the release of vasoactive TXA2 not only from activated PLTs through complement activation but also from liposome-phagocytizing PIMs. In our experiment, both HbV and EV groups showed slight increases in TXB2 even though it is much lower than that of the CL group. In the case of TXA2 from PIMs, the amount would be small and the affected range would tend to be localized in lung tissues. The CL group showed the maximum changes in MAP and PAP at 1 min after injection. However, other groups showed maxima at 3 min. This slight delay would be explained by the time course of reactions. Complement activation would be initiated at the contact of blood and vesicles instantaneously from the injection site. In contrast, phagocytosis of vesicles is initiated when the intravenously injected vesicles come through the lung capillaries.

Reportedly, naturally occurring autoantibodies to cholesterol might initiate complement activation, which associates with anaphylactoid reactions.<sup>40</sup> However, neither HbV nor EV induces complement activation even though they contain 44 mol % of cholesterol in the lipid membrane, which is an important component to reduce the curvature of vesicles and to produce LUVs. Szebeni et al.<sup>5</sup> reported that injection of their liposome induced arrhythmia, tachycardia, ST depression, and T-wave changes in the ECG, implying cardiac ischemia. In stark contrast, in our experiment, the ECG showed no abnormality even in the CL group. This difference might result from the PEGylation of CL, well-regulated particle size of our CL, or the lower endotoxin level of our samples prepared in a sterilized condition.<sup>41,42</sup> A report describes that liposomes without a negatively charged lipid reduce pulmonary hypertension,<sup>39</sup> probably because the liposomes are less recognized by PIMs in sheep. As described above, a negatively charged lipid, DHSG, cannot be excluded for preparation of HbV because it is necessary to encapsulate a large amount of Hb molecules in the inner aqueous phase of liposomes.

The presence of PIMs is species dependent.<sup>23,33</sup> A morphometric study of human lung tissues showed no macrophages or macrophage-like cells in the pulmonary capillaries.<sup>43</sup> Therefore, such pulmonary side effects would not usually appear with injection into humans. Most foreign particles injected intravenously into humans are taken up by Kupffer cells and spleen macrophages. However, they are rarely taken up by the lung when liver function is damaged.<sup>23,24</sup> It might be necessary to consider patients' hepatic condition before injection of liposomes.<sup>44</sup>

## CONCLUSIONS

Collectively, HbV does not induce significant anaphylactoid reactions in pigs compared with CLs because of the different lipid composition. The changes that are apparent after the first injection relate mainly to the phagocytosis by PIMs, and it would not relate to complement activation. Our previous report of rodent experiments showed better biocompatibility with no complement activation, in comparison to the

CLs. In this study of pigs, we were able to reconfirm the biocompatibility of HbV. Pigs are often used for preclinical studies because of their similarity to humans in terms of their body size, anatomical structure and physiological function. However, due attention must be given when a pig receives a liposomal suspension because of the different biodistribution and cardiovascular responses.

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# Gas Bioengineering Using Hemoglobin-Vesicles For Versatile Clinical Applications

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**Abstract:** Blood transfusion systems have greatly benefited human health and welfare. Nevertheless, some problems remain: possibility of infection, blood type mismatching, immunological response, and a short shelf life that is insufficient for stockpiling for emergency situations. Realization of artificial O<sub>2</sub> carriers is anticipated to solve such problems. During the long development of hemoglobin (Hb)-based O<sub>2</sub> carriers, many side effects of cell-free Hb molecules have arisen, and have implied the physiological importance of the cellular structure of red blood cells (RBCs). Therefore, Hb-vesicles (HbVs) have been developed as artificial red cells that encapsulate a concentrated Hb solution in thin lipid bilayer vesicles. This Hb-encapsulation can shield various toxic effects of molecular Hbs, especially reactions with endogenous NO and CO as vasorelaxation factors. Physicochemical analyses have clarified that Hb encapsulation retards these gaseous reactions significantly. "Gas Bioengineering" is intended to create systems using bioengineering and chemical engineering techniques to facilitate the transport of or regulate the concentration of endogenous or exogenous gaseous molecules (such as O<sub>2</sub>, NO, and CO) that are sometimes vital and sometimes toxic to humans. Gas bioengineering using HbVs underscores the potential of HbVs as a transfusion alternative and promises its use for other clinical applications that remain unattainable using RBC transfusion.

**Keywords:** Blood substitutes, liposome, hemodynamics, microcirculation.

## 1. INTRODUCTION

Hemoglobin (Hb), the most abundant protein in blood (12–15 g/dL), is the most important protein for maintaining the oxygen metabolism in a living body. It is compartmentalized in red blood cells (RBCs) with intracellular concentration of about 35 g/dL. However, Hb becomes toxic once released from RBCs, which is evident in some pathological hemolytic diseases. Because of blood borne infectious diseases such as hepatitis and HIV, occurrence of blood type mismatching, and the short storage period of packed RBCs, the realization of artificial blood has remained a dream for a long time [1]. Historically, a purified Hb solution was tested as an artificial oxygen carrier after it became clear that Hb is an oxygen-binding protein and that the blood type antigen is present on the RBC surface [2]. However, that solution was not successful because of the various side effects of molecular Hb.

Then chemically modified cell-free Hb based oxygen carriers (HBOCs), such as intramolecularly crosslinked, polymerized, and polymer-conjugated Hbs were synthesized to prevent toxic effects of cell-free Hbs (such as renal failure due to extravasation of dissociated Hb and deposition in glomerular capillaries, neurotoxicity, esophageal motor function disorder, vasoconstriction, pro-oxidant activity, myocardial lesions, etc.). However, no product is commercially available yet. Some safety issues arose during the final stage of clinical trials [3]. It seems difficult to eliminate the side effects of cell-free Hbs completely through chemical modification. It is necessary to reconsider why Hb is compartmentalized in RBCs with such a complicated corpuscular structure for designing an appropriate structure of HBOCs. Hb-vesicles (HbVs) are artificial oxygen carriers encapsulating concentrated Hb solution (> 35 g/dL) with a phospholipid bilayer membrane [4]. In fact, HbVs are designed to mimic or overcome the function of RBCs. This review paper presents specific examination of the concept of Hb-encapsulation and explanation of some recent topics related to HbVs, especially with respect to the physicochemical analyses of the reactions with gaseous molecules (O<sub>2</sub>, NO, CO) that are closely related to its safe hemodynamics and its new applications.

## 2. CONCEPT OF HB-ENCAPSULATION AND CHARACTERISTICS OF HB-VESICLES

Using a polymer membrane, Chang first performed Hb encapsulation in microcapsules in the 1950s [5]. Some Japanese groups also tested Hb encapsulation using membranes of gelatin, gum Arabic, silicone, etc. Nevertheless, it was extremely difficult to regulate the particle size to make them appropriate for blood flow in capillaries and to attain sufficient biocompatibility. After Bangham and Horne reported in 1964 [6] that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevic and Miller [7] prepared a liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acid, etc. Since that time, many groups have tested encapsulated Hbs using liposomes [7–11]. Some failed initially, and some are progressing with the aim of clinical usage. The Naval Research Laboratory presented remarkable progress of LEH [12], but it suspended development about 10 years ago, probably because of the insufficient Hb encapsulation efficiency and biocompatibility of complement activation, which induced important circulatory disorders (such as pulmonary hypertension, arrhythmia, increased systemic vascular resistance, reduced cardiac output) [13]. What we call Hb-vesicles (HbVs), with high-efficiency production processes and improved properties, have been established by our group based on nanotechnologies of molecular assembly and pharmacological and physiological aspects [14] (Fig. 1). Cellular type HbVs have not yet been tested clinically because of their difficulty of production. Chemically modified cell-free HBOCs are much easier to produce because purified Hb is simply mixed with a crosslinker, activated polymer; then the unreacted agents are removed. Because of that relative ease of production, more researchers have tested the cell-free types. For that reason, they have advanced further than the cellular type and have entered clinical trials. However, during the long history of R&D, some unexpected problems have arisen for cell-free HBOCs, probably because of the direct exposure of Hb to vasculature and reactions with gaseous molecules.

The HbVs encapsulate concentrated Hb solution (> 35 g/dL) with a phospholipid bilayer membrane [11]. Concentration of the HbV suspension is extremely high ([Hb] = 10 g/dL, [lipids] = 6 g/dL, volume fraction, ca. 40 vol%), and it has an oxygen carrying

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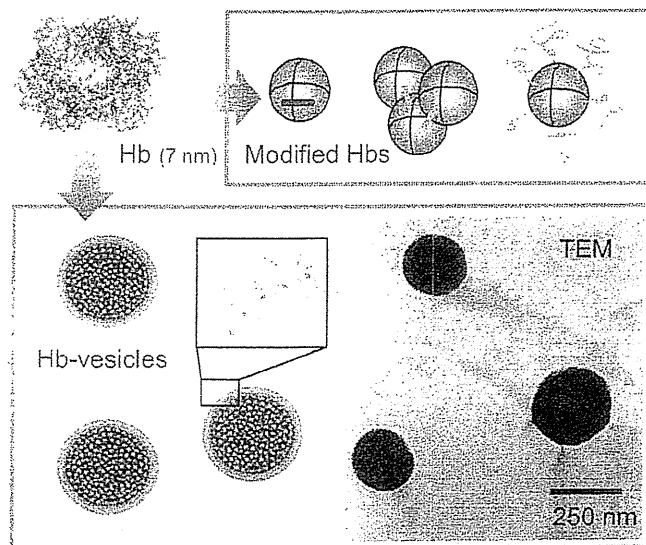


Fig. (1). Hb-based oxygen carriers (HBOCs). Hb solution is purified from outdated Hb; a concentrated Hb solution is encapsulated in phospholipid vesicles to produce Hb-vesicles. Chemically modified cell-free HBOCs such as crosslinked Hb, polymerized Hb, and PEG-conjugated Hb have been tested clinically in the US and Europe.

capacity that is comparable to that of blood. Moreover, HbVs are much smaller than RBCs (250 nm vs. 8000 nm), but they recreate the functions of RBC that have been confirmed through numerous animal experiments to test their effectiveness as a resuscitative fluid for massive hemorrhage, hemodilution, and as a prime candidate for cardiopulmonary bypass [15–17] (Table 1). Other characteristics resembling those of RBCs are the following: (i) the colloid osmotic pressure of the HbV suspension is zero at  $[Hb] = 10$  g/dL, and it must be co-injected with or suspended in a plasma substitute such as albumin and hydroxyethyl starch [29,30]; (ii) the viscosity of an HbV suspension is adjustable to that of blood (3–4 cP) [31]; (iii) HbVs are finally captured by the reticuloendothelial system (RES), and the components are degraded and excreted promptly [18,32,33]. The HbV particle itself is not eliminated through glomeruli [34]; (iv) co-encapsulation of PLP as an allosteric effector, instead of 2,3-diphosphoglyceric acid, to regulate oxygen affinity (Fig. 2) [23,35]; and (v) no hemolysis occurs during circulation and the lipid bilayer membrane prevents direct contact of Hb and vasculature. Moreover, (vi) the gas reactions of Hb are adjusted through compartmentalization in RBCs or HbVs, which we address in the following sections.

**Table 1. Preclinical Studies of HbVs as a Transfusion Alternative and for Other Therapeutics for Potential Clinical Application**

	Application	Ref.
a.	Resuscitative fluid for massive hemorrhage	[16-19]
b.	Priming fluid for extracorporeal membrane oxygenator (ECMO) for cardiopulmonary bypass	[15]
c.	Perfusate for resected organs	[20,21]
d.	Oxygenation of ischemic tissues (brain, skin flap)	[22-25]
e.	Solid tumor oxygenation for irradiation sensitization	[26]
f.	CO carrier	[27]
g.	Monitoring of brain oxygen consumption by positron emission tomography	[28]

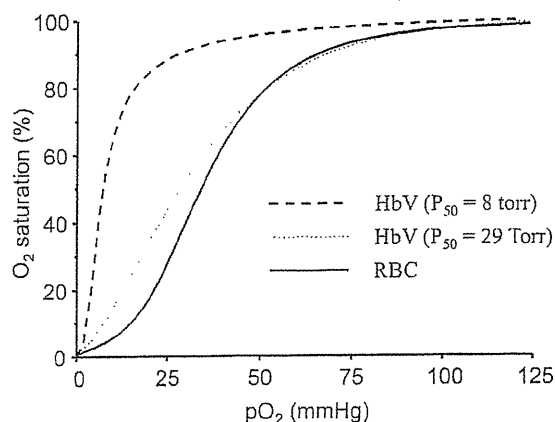


Fig. (2). Oxygen equilibrium curves of HbV ( $P_{50} = 8$  and 29 torr) measured with a Hemox Analyzer (TCS Medical Products) at 37 °C in comparison with hamster blood ( $P_{50}$  is partial pressure of oxygen at which Hb is half saturated with oxygen) [35]. Hill number of HbV is around 2.1, which is lower than that of RBC (2.7 - 3.0) because of a trace amount of metHb and different intracellular electrolyte concentration. (Reproduced with permission from *Am J Physiol Heart Circ Physiol* 2005; 288: H2897-H2903).

### 3. GAS REACTIONS OF HBV MEASURED USING STOPPED-FLOW RAPID SCAN SPECTROPHOTOMETRY

The major remaining hurdles before clinical approval of the earliest generation of HBOCs are vasoconstriction and resulting hypertension, which are suggested to be attributable to the high reactivity of Hb with endothelium-derived nitric oxide (NO) [3]. It has been suggested that small molecular Hbs permeate across the endothelial cell layer to the space near the smooth muscle, and inactivate NO. In contrast, cellular HbVs induce neither vasoconstriction nor hypertension [36].

In 1996, using stopped-flow rapid scan spectrophotometry, we observed for the first time that Hb-encapsulation in vesicles retards NO-binding in comparison to molecular Hb [37], which might con-

tribute to some degree to the absence of vasoconstriction. However, it remains unclear how Hb encapsulation retards the NO-binding. It is unknown whether the lipid bilayer membrane has a barrier function, an unstirred layer is formed on the surface of a particle that becomes a diffusion barrier, or the intracellular Hb causes a diffusion barrier [38]. To clarify the mechanism, we prepared HbVs with different intracellular Hb concentrations ( $[\text{Hb}]_{\text{in}}$ , 1–35 g/dL) and different particle sizes (mean diameter, 178–452 nm), and analyzed the gas reactions using stopped-flow spectrophotometry [39]. The apparent NO binding rate constant,  $k'_{\text{on}}(\text{NO})$ , of HbVs at  $[\text{Hb}]_{\text{in}} = 1$  g/dL was  $2.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , which was almost equal to  $k'_{\text{on}}(\text{NO})$  of molecular Hb, indicating that the lipid membrane presents no obstacle for NO binding (Fig. 3). With increasing  $[\text{Hb}]_{\text{in}}$  to 35 g/dL,  $k'_{\text{on}}(\text{NO})$  decreased to  $0.9 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , which was further decreased to  $0.5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$  with particle diameter enlargement from  $265 \pm 57$  to  $452 \pm 184$  nm. For CO binding, which is intrinsically much slower than NO binding,  $k'_{\text{on}}(\text{CO})$  did not change greatly with  $[\text{Hb}]_{\text{in}}$  and the particle diameter. Results obtained using diffusion simulations coupled with elementary binding reactions concur with these tendencies and clarify that NO is trapped rapidly by Hb which exist at the interior surface region at a high  $[\text{Hb}]_{\text{in}}$ , retarding NO diffusion toward the core of an HbV. In contrast, slow CO diffusion allows time for additional CO-diffusion to the core. Simulations extrapolated to larger particles (8  $\mu\text{m}$ ) showed retardation even for CO binding. The obtained  $k'_{\text{on}}(\text{NO})$  and  $k'_{\text{on}}(\text{CO})$  yield values similar to those reported for RBCs. In summary, the intracellular, not extracellular, diffusion barrier is caused predominantly by rapid NO binding, which induces a rapid sink of NO from the interior surface to the core, thereby retarding further NO diffusion and binding.

Results of this study imply that the requisites for this diffusion barrier are i) a more concentrated intracellular Hb solution, and ii) a larger particle size [39,40]. Although liposome-encapsulated Hb of

various kinds have been studied by many groups [11], our HbVs encapsulate a highly concentrated Hb solution ( $> 35$  g/dL) with a regulated large particle diameter (250–280 nm). They attain 10 g/dL Hb concentration in the suspension. Actually, Rudolph et al. were unable to detect the retardation of NO binding with their liposome encapsulated Hb (LEH), probably because LEH encapsulates diluted (only 14 g/dl) Hb solution [41].

In an artery where oxygen tension is high, the main reaction would be NO + oxyhemoglobin ( $\text{HbO}_2$ ), not NO + deoxyHb. We confirmed the reaction profiles of oxygenated HbV and NO using stopped flow rapid scan spectrophotometry, and compared the profiles with those of cell-free Hb solutions [40]. The reaction with NO oxidizes oxyHb to convert to metHb. Results confirmed that the cell-free human  $\text{HbO}_2$  solutions with or without PLP showed  $k'_{\text{ox}}(\text{NO})$  of  $7.4$ – $8.7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , and polymerized bovine Hb (Poly $\beta$ Hb) showed  $6.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , which were faster than that of the binding reaction of NO to deoxyHb ( $k'_{\text{on}}(\text{NO}) = 2.4$ – $2.6 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ ). Encapsulation of  $\text{HbO}_2$  in vesicles retarded the reaction with NO considerably;  $k'_{\text{ox}}(\text{NO})$  was  $0.88 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ . Accordingly, we can conclude that Hb encapsulation retards the reaction with NO in either aerobic or anaerobic conditions.

The absence of vasoconstriction in the case of intravenous HbV injection might be related to the lowered NO-reaction rate constants, although they are much larger than those of RBCs, as shown in Fig. 3. It implies the presence of other mechanisms to prevent NO-related vasoconstriction in the case of HbVs.

#### 4. GAS REACTIONS OF HBV FLUID IN A FLOWING CONDITION

The proposed mechanism of vasoconstriction induced by HBOCs in relation to gaseous molecules is not limited to NO scav-

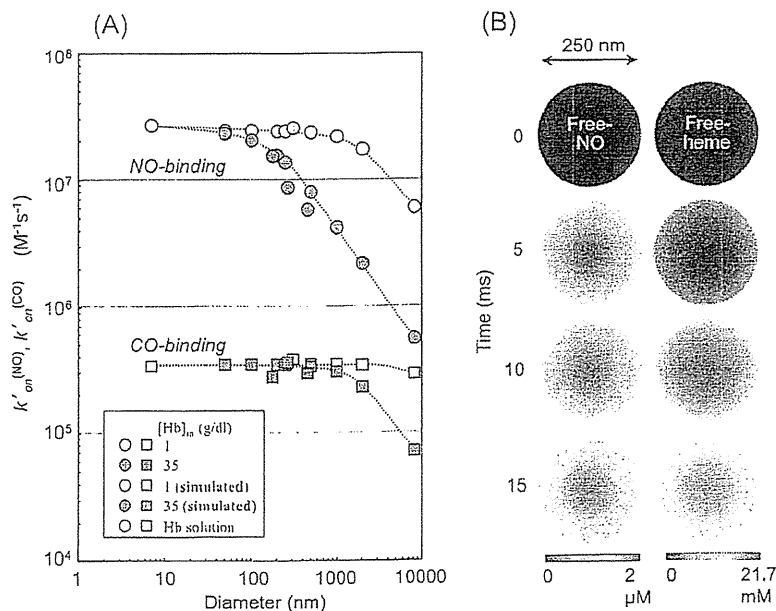


Fig. (3). (A) Encapsulation of Hb in vesicles retards NO-binding and CO-binding depending on the intracellular Hb concentration and particle size, as measured using stopped-flow rapid scan spectrophotometry and simulated based on the diffusion process. At  $[\text{Hb}]_{\text{in}} = 35$  g/dL, the apparent CO binding rate constant,  $k'_{\text{on}}(\text{CO})$ , is almost identical up to 1000 nm in diameter; then it is reduced considerably, concomitantly with increased diameter. In contrast,  $k'_{\text{on}}(\text{NO})$  decreases slightly up to 100 nm diameter; it then decreases steeply with increasing particle diameter. For  $[\text{Hb}]_{\text{in}} = 1$  g/dL, both NO-binding and CO-binding showed less change in the binding rate constants. Experimental values for the Hb solution and HbV closely approximate the simulated values. The apparent binding rate constants,  $k'_{\text{on}}(\text{NO})$  and  $k'_{\text{on}}(\text{CO})$ , of a spherical particle with diameter of 8000 nm and  $[\text{Hb}]_{\text{in}} = 35$  g/dL are estimated, respectively, to be reduced to  $5.6 \times 10^5$  and  $7.3 \times 10^4 \text{ M}^{-1} \text{ s}^{-1}$ . The reported  $k'_{\text{on}}(\text{NO})$  values of a series of chemically modified HBOCs (diameter, 6–28 nm), about  $3.0 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ , were identical to that of an unmodified Hb solution [45], which coincided well with our simulation. (B) Schematic two-dimensional representation of the simulated time courses of distributions of unbound free-NO and unbound free-heme (deoxy form) in one HbV (250 nm) after immediate mixing of NO and HbV using stopped-flow method. Computer simulation shows that both free NO and unbound hemes are distributed heterogeneously, indicating the presence of an intracellular diffusion barrier.