model. In these studies, a 5% albumin (Alb) solution containing Hb-vesicles was injected into the animals (15–17). The infusion of the vesicle suspension resulted in a sufficient resuscitative effect comparable to that of erythrocyte transfusion in terms of survival, hemodynamics, and the acid-base balance. However, we have not conducted resuscitation studies on uncontrolled hemorrhagic shock. In this study, we examined the ability of Hb-vesicles to achieve resuscitation in a rat model of continuous bleeding.

MATERIALS AND METHODS

Preparation of resuscitative fluid

The Hb-vesicles, manufactured according to the method invented by the Waseda group (2, 3), were provided by Oxygenix Co Ltd (Japan) and Nipro Corp (Japan). The 25% human recombinant Alb solution was provided by Nipro Corp.

For the HbV group, the Hb-vesicles were suspended in a 5% Alb solution: the vesicle suspension was mixed with 25% Alb solution at the proportion of 8.6 to 1.4 to be used as a colloidal resuscitative fluid containing 5% Alb. This mixture was filtered with a 0.45-μm filter unit (DISMIC-25 cs; Toyo Roshi Kaisha Ltd, Tokyo, Japan) to disperse large aggregates. The final Hb-vesicle solution contained Hb at a concentration of 8.6 g/dL. For the washed RBC (wRBC) group, the washed erythrocyte suspension was prepared as follows. After two donor rats were systemically heparinized with 500 U/body of heparin sodium, the homologous blood was withdrawn via the abdominal aorta. This blood was subsequently centrifuged (4°C; 2,000*g*, 15 min), and the RBCs were washed twice with hydroxyethyl starch (HES; Salinhes; Kyorin Pharmaceutical Co Ltd, Tokyo, Japan). After removing the plasma component, the RBCs were suspended in HES, and the volume was adjusted to twice that of the estimated circulating volume of the recipient rat (38–45 mL). For the Alb group, a 5% Alb solution was prepared by diluting 25% Alb solution with Sal. For the HES group, HES was applied without modification. Saline (Otsuka Pharmaceutical Factory, Inc, Tokushima, Japan) was used for the Sal group.

Animals and surgical preparation

Experiments were conducted using 35 male Wistar rats (8–9 weeks of age). The animals were kept on a bed of pulp paper in a ventilated, temperature-controlled (23°C ± 1°C), specific-pathogen–free environment with a 12-h light-dark cycle. They were given access to food and water *ad libitum*. All experimental protocols were reviewed by the Committee on the Ethics of Animal Experiments at our university and were conducted in accordance with the Guidelines for Animal Experiments issued by the Keio University School of Medicine and the Experimental Animal Centre and with law no. 105 and notification no. 6 issued by the Japanese Government. The ethical guidelines conformed to the American Physiological Society guiding principles for the care and use of animals.

The animals were anesthetized by inhalation of diethyl ether (Wako Pure Chemical Industries Ltd, Osaka, Japan) for induction. Subsequently, an air mixture containing 2.0% to 3.0% sevoflurane (Maruishi Pharmaceutical Co, Ltd, Osaka, Japan) was supplied to the animals throughout the experiment; spontaneous breathing was maintained.

For performing arterial pressure measurements, fluid injection, blood sampling, and continuous bleeding, four cannulas were inserted into each animal. A 24-gauge indwelling needle (Terumo Corp, Tokyo, Japan) was inserted into a ventral caudal artery. Another 24-gauge indwelling needle was inserted into the left dorsal metatarsal vein for fluid injection. For measuring arterial pressure, a polyethylene catheter (SP45; Natsume Seisakusho Co, Ltd, Tokyo, Japan) was inserted into the right carotid artery. For conducting frequent blood collections, a polyethylene catheter (SP31; Natsume Seisakusho Co, Ltd) was inserted into the left femoral artery. The cannulas were filled with heparinized Sal (40 U/mL) to prevent coagulation.

A polygraph system (PEG1000; Nihon Kohden Corp, Tokyo, Japan) was used to continuously record and monitor the mean arterial pressure (MAP) and the electrocardiograms. The indwelling needle on the dorsal metatarsal vein was connected to an infusion pump via a polyethylene catheter. For measuring the increase in the hemorrhage volume over the course of the experiment, a bottle was hung under the tube exit, which was connected to the indwelling needle of the caudal artery.

Grouping, establishment of hemorrhagic shock, and resuscitation

The male Wistar rats were assigned randomly to five groups: the HbV group (n = 6), the wRBC group (n = 6), the Alb group (n = 7), the HES group (n = 9), and the Sal group (n = 7). The average body weights were 364.5 ± 23.4 g in the HbV group, 358 ± 18.7 g in the wRBC group, 350.6 ± 22.8 g in the Alb group, 345.6 ± 19.7 g in the HES group, and 367.7 ± 36.9 g in the Sal group. No significant differences were found between groups.

The total circulated blood volume (tBV) was estimated to be 56 mL/kg body weight (18). After stabilization, the baseline data were measured. Hemorrhagic shock was subsequently developed by free bleeding from the caudal artery until the hemorrhage volume had reached 30% of the tBV. At this point, all animals showed shock status (mean pressure was near or below 40 mmHg). Immediately after the hemorrhage reached 30% of the tBV, the resuscitation was started, while the bleeding from the caudal artery was continued. The injection was continued at a speed of 56 mL/kg per hour until the animal died or the infusion volume reached 200% of the tBV. Resuscitation with 200% tBV fluid took 2 h.

In the HbV group, HES was first infused up to 50% of the tBV, and then the Hb-vesicle mixture was injected beginning at 50% of the tBV, whereas the respective fluids for the other experimental groups were used throughout the resuscitation. Because fluid resuscitation in clinical practice is usually initiated with crystalloid or colloid solutions, we chose to infuse the Hb-vesicles at 50% of the tBV to mimic clinical practice.

Experimental design

The animals were observed for a maximum of 2 h after the resuscitation started. For determining the hematocrit (Ht) levels, the blood lactate concentrations, and the pH, 0.2 mL of arterial blood was collected before hemorrhage and when the infusion volume reached 50%, 100%, 150%, and 200% of the tBV. An equal volume of heparinized Sal was injected to maintain the circulation volume of the animal. Afterward, the Ht was measured using the microhematocrit method (12,000 revolutions/min, 5 min). The arterial blood was collected in heparinized microtubes, and the serum lactate concentration and the pH were analyzed using a pH/blood gas analyzer (ABL725; Radiometer A/S, Copenhagen, Denmark).

Statistical analysis

All data sets were analyzed using Statcel2 (OMS Publishing Inc, Saitama, Japan, 2004) and were regarded as having a normal distribution. The survival curve was drawn using the Kaplan-Meier method, and the log-rank test was used to compare survival rates. For analyses involving three groups or more, the variance was evaluated using the Bartlett test. One-way analysis of variance (ANOVA) was used if the variance among groups was equal. Furthermore, these data sets were evaluated using the Tukey-Kramer test when significant differences were observed by ANOVA. If the variance was found to be unequal in comparisons involving three groups or more, the Kruskal-Wallis test was used for the analysis. Furthermore, these data sets were evaluated using the Scheffé F test when significant differences were observed by the Kruskal-Wallis test. Pairwise combinations were excluded from the calculations if the combinations of the data sets could not be analyzed. For analyses involving two groups, the variance was evaluated using an F test. These groups were analyzed using the Student *t* test and Welch *t* test if the variance was found to be equal and unequal, respectively. In addition, differences were considered significant at $P < 0.05$. Results were expressed as the mean ± SD.

RESULTS

Survival

Survival curves drawn by the Kaplan-Meier method and the cumulative survival rates for the five groups are shown in Figure 1, respectively. In the HbV and wRBC groups, all animals survived during continuous injection, whereas those in the Alb and HES groups died before the completion of infusion. In the Sal group, five of seven animals died (mortality of 71.4%). For the survival curves, significant differences were found between the HbV or wRBC group and the Alb, HES, and Sal groups by the log-rank test.

The cumulative survival rates were 100% in the HbV and wRBC groups (6/6 animals survived in each group), 0% in the Alb group (0/7), 0% in the HES group (0/9), and 28.6% in the Sal group (2/7). In the Sal group, one animal died near the very end of resuscitation, although three animals survived through the completion of the injection.

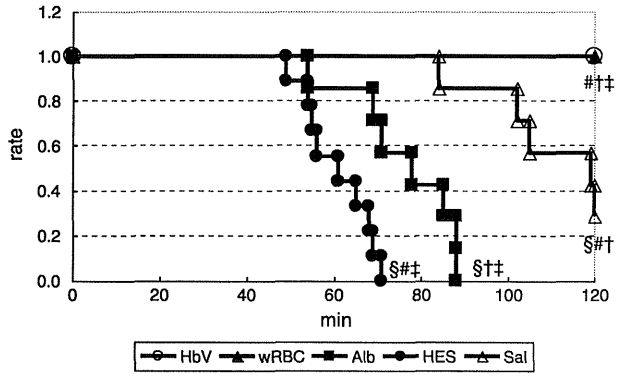

FIG. 1. **Survival curve according to the resuscitation fluid used.** $^{\S}P <$ 0.05 vs. the HbV and wRBC groups, $^{\#}P < 0.05$ vs. the Alb group, $^{\dagger}P < 0.05$ vs. the HES group, and $^{\ddagger}P < 0.05$ vs. the Sal group.

Hemorrhage volume

At the start of the resuscitation, all animals underwent 30% tBV bleeding (approximately 6 mL/body) to induce hemorrhagic shock. The hemorrhage volume values subsequently increased in a linear manner in the HbV, wRBC, Alb, and HES groups (Fig. 2). In the Sal group, the hemorrhagic volume increased at a slower rate relative to that of the other groups. All animals in the Alb and HES groups died before the 150% tBV infusion. At 200% tBV, the hemorrhage volumes reached 52.9 ± 4.0, 47.3 ± 4.7, and 21.8 ± 2.5 mL in the wRBC, Hbv, and Sal groups, respectively. A significant difference was found between the wRBC or HbV group and the Sal group. The HbV group showed no significant difference compared with the wRBC group at any time point.

Heart rate

Before the hemorrhage, the heart rate (HR) before hemorrhage was 395 ± 40 beats/min and decreased to 323 ± 54 beats/min when the hemorrhage volume reached 30% tBV (Fig. 3). At the 50% tBV infusion, the HRs were 340 ± 38 beats/min in the wRBC group, 290 ± 42 beats/min in the HbV group, 289 ± 46 beats/min in the Alb group, 245 ± 92 beats/min in the HES group, and 261 ± 25 beats/min in the Sal group. At the 100% tBV infusion, the HR decreased to 77 ± 107 beats/min in the HES group and was significantly different from that of the other groups. However, no other significant changes were observed. The values for the HbV, wRBC, and Sal groups did not change significantly at the 150% tBV infusion relative to those at the 100% tBV infusion. At the 200% tBV infusion, the HRs were 320 ± 30, 280 ± 31, and 170 ± 226 beats/min in the wRBC, HbV, and Sal groups, respectively. The Sal group showed a decrease in HR at the final measurement (n = 2), although no statistical significance was found.

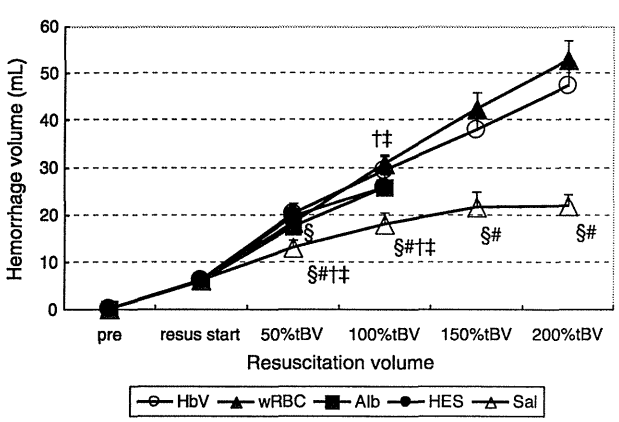

FIG. 2. **Change in the hemorrhage volume.** Differences between groups were analyzed using Tukey-Kramer test. $^{\S}P < 0.05$ vs. the HbV group, $^{\#}P <$ 0.05 vs. the wRBC group, $^{\dagger}P < 0.05$ vs. the Alb group, $^{\ddagger}P < 0.05$ vs. the HES group.

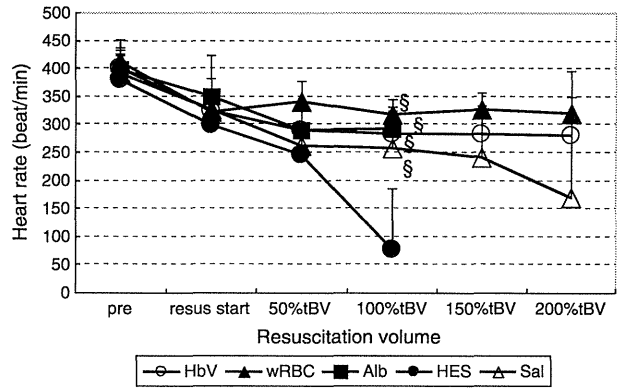

FIG. 3. **Changes in the HR.** For the 100% tBV infusion, statistical differences were detected using Tukey-Kramer test. For the 200% tBV infusion, HbV vs. wRBC was not significantly different ($P = 0.0982$, Student t test), but a comparison between the Sal group vs. the HbV and wRBC groups resulted in error. "Pre": before the hemorrhage. "Resus start": start of the hemorrhage. Error bars represent SD. $^{\S}P < 0.05$ vs. the HES group.

Mean arterial pressure

Before the hemorrhage, the MAP was 86.9 ± 10.3 mmHg in all groups (Fig. 4). The MAP subsequently decreased to 36.6 ± 7.6 mmHg when the hemorrhage volume reached 30% tBV. No significant difference was found among the groups. At 50% tBV infusion, the MAPs were 57.0 ± 6.6 mmHg in the wRBC group, 43.3 ± 15.9 mmHg in the HbV group (up until this

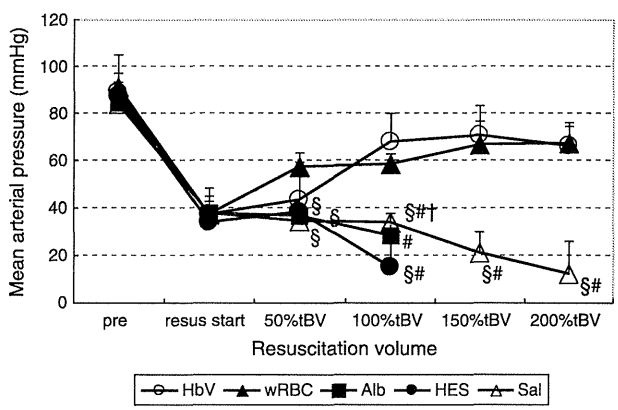

FIG. 4. **Change of MAP.** For the 50% tBV and 100% tBV infusions, statistical differences were detected with the Scheffé F test. For the 150% tBV and 200% tBV infusions, statistical differences were detected with Tukey-Kramer test. "Resus start": start of the hemorrhage. Error bars represent SD. $^{\S}P < 0.05$ vs. the wRBC group, $^{\#}P < 0.05$ vs. the HbV group, $^{\dagger}P < 0.05$ vs. the HES group.

point, the injection fluid did not contain Hb-vesicles), 36.4 ± 7.3 mmHg in the Alb group, 38.1 ± 5.3 mmHg in the HES group, and 34.4 ± 3.9 mmHg in the Sal group. A significant difference was detected between the wRBC groups and the Alb, HES, and Sal groups. At the 100% tBV infusion, the MAPs of the HbV and wRBC groups (67.7 ± 12.3 mmHg and 58.5 ± 4.5 mmHg, respectively) were significantly higher than those of the other groups (28.2 ± 5.1, 15.2 ± 14.4, and 33.7 ± 3.9 mmHg in the Alb, HES, and Sal groups, respectively). At the 150% tBV infusion, the MAPs were 70.8 ± 12.5 mmHg, 66.5 ± 10.1 mmHg, and 21.0 ± 9.1 mmHg in the HbV, wRBC, and Sal groups, respectively. The values for the HbV and wRBC groups were significantly different from those of the Sal group. At the 200% tBV infusion, the MAPs were 67.3 ± 6.9 mmHg in the wRBC group, 66.0 ± 10.1 mmHg in the HbV group, and 12.5 ± 13.4 mmHg in the Sal group. Statistical analysis again revealed a significant difference between the HbV and wRBC groups and the Sal group.

Respiration rate

The respiration rate (RR) before the hemorrhage was 61.3 ± 8.9 breaths/min in all five groups (Table 1). The RR of each group decreased gradually as the hemorrhage progressed. At the 200% tBV infusion, the RRs were 51.7 ± 5.6, 46.5 ± 6.9, and 16.5 ± 23.3 breaths/min in the wRBC, HbV, and Sal groups, respectively. Significant differences were observed between the HbV or wRBC group and the Sal group.

Hematocrit

Before the hemorrhage, the Ht was 41.9% ± 2.3% in all five groups (Fig. 5). The Ht values subsequently decreased during the hemorrhage because of hemodilution with the fluid infusions. The Ht values showed a similar decrease in the HbV, Alb, and HES groups over the course of the experiment. The Ht of the HbV group decreased remarkably to 1.5% ± 0.5% at the final measurement. The wRBC group showed the highest Ht values because of the homologous transfusion. At the 200% tBV infusion, the Ht value was 19.0% ± 2.1%, which was approximately equivalent to the Ht value of the erythrocyte suspension used for this group. In contrast, the Ht values of the Sal group decreased gradually. At the 50% tBV infusion, significant differences were found between the wRBC or Sal group and the HbV, Alb, and HES groups. At the 100% tBV infusion, the Ht values had decreased further for all groups. However, the presence of significant differences was the same,

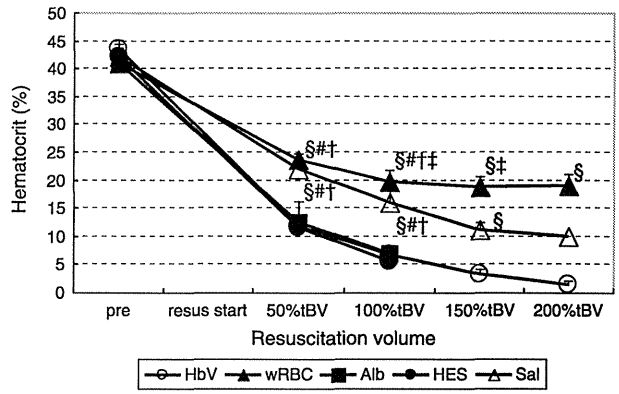

FIG. 5. **Change of Ht.** At 200% tBV, the Sal group vs. the wRBC and Hbv groups could not be analyzed statistically. For the 50% tBV, 100% tBV, and 150% tBV infusions, statistical differences were detected with Tukey-Kramer test. For the 200% tBV infusion, HbV vs. wRBC was significantly different (Welch *t* test), but a comparison between the Sal group vs. the HbV and wRBC groups resulted in error. Error bars represent SD. $^{\S}P < 0.05$ vs. the HbV group, $^{\#}P < 0.05$ vs. the Alb group, $^{\dagger}P < 0.05$ vs. the HES group, $^{\ddagger}P < 0.05$ vs. the Sal group.

with the exception of the wRBC and Sal groups, which now showed a significant difference. At the 150% tBV infusion, the Ht values were 11.1% ± 1.4%, 3.2% ± 1.0%, and 18.7% ± 2.0% in the Sal, HbV, and wRBC groups, respectively. Significant differences were found between these three groups. At the 200% tBV infusion, the Ht values were 10.0%, 1.5% ± 0.5%, and 19.0% ± 2.1% in the Sal, HbV, and wRBC groups, respectively. Significant differences were found between the HbV and wRBC groups.

Blood lactate levels

The average values of the blood lactate levels before hemorrhage were 0.8 ± 0.2 mmol/L in all groups (Fig. 6). At the 50% tBV infusion, the lactate levels were 3.8 ± 1.5 mmol/L in the HbV group, 5.1 ± 2.2 mmol/L in the Alb group, 4.5 ± 0.9 mmol/L in the HES group, and 4.6 ± 1.1 mmol/L in the Sal group. In the wRBC group, the lactate level was maintained at 1.7 ± 0.6 mmol/L. A significant difference was found between the wRBC group and the Alb, HES, and Sal groups. At the 100% tBV infusion, the HbV and wRBC groups showed significantly lower lactate concentrations (2.8 ± 1.0 and 1.8 ± 0.6 mmol/L, respectively) than did the HES, Alb, and Sal groups

TABLE 1. **Respiration rate**

	Pre		Resus start		50% tBV		100% tBV		150% tBV		200% tBV	
HbV	58.5 ± 10.9	(6)	62.7 ± 15.1	(6)	57.0 ± 14.6	(6)	46.7 ± 8.7	(6)	46.5 ± 5.8	(6)	46.5 ± 6.9	(6)
wRBC	63.8 ± 7.1	(6)	63.2 ± 10.7	(6)	62.5 ± 9.5	(6)	56.3 ± 6.5	(6)	55.3 ± 7.3	(6)	51.7 ± 5.6	(6)
Alb	60.0 ± 9.7	(7)	58.7 ± 7.9	(7)	56.4 ± 8.6	(7)	27.8 ± 16.1	(6)				
HES	60.7 ± 8.1	(9)	63.1 ± 9.0	(9)	61.4 ± 7.1	(9)	5.4 ± 10.5	(5)				
Sal	63.4 ± 10.0	(7)	66.9 ± 9.9	(7)	56.7 ± 9.9	(7)	44.4 ± 20.8	(7)	34.7 ± 17.8	(6)	16.5 ± 23.3	(2)

Values are mean ± SD (n) in breaths/min.
Results of statistical analysis, $P < 0.05$. pre: No significant difference ($P = 0.8144$, one-factor ANOVA).
resus start: No significant difference ($P = 0.7173$, one-factor ANOVA). 50% tBV: No significant difference ($P = 0.6674$, one-factor ANOVA). 150% tBV: Combination of groups with significant difference (method by Tukey-Kramer test) was as follows: wRBC vs. Sal. 200% tBV: Combinations of groups with significant difference (method by Tukey-Kramer test) were as follows: HbV vs. Sal, and wRBC vs. Sal. 100% tBV: Combinations of groups with significant difference (method by Tukey-Kramer test) were as follows: HbV vs. HES, wRBC vs. Alb, wRBC vs. HES, and HES vs. Sal.

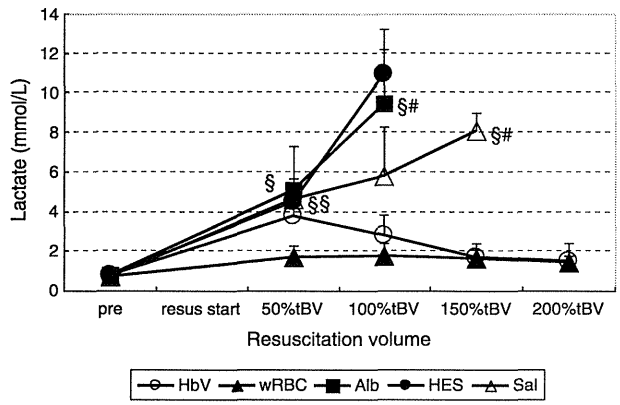

FIG. 6. **Change of blood lactate level.** At 100% tBV, HES group vs. others could not be analyzed statistically. For the 50% tBV and 150% tBV, statistical differences were detected with Tukey-Kramer test. For the 100% tBV group, statistical differences were detected with Scheffé F test, but the comparison of the HES group vs. the other groups resulted in error. Error bars represent SD. $^{\S}P < 0.05$ vs. wRBC group, $^{\#}P < 0.05$ vs. HbV group.

(11.0 ± 1.2, 9.4 ± 3.8, and 5.8 ± 2.5 mmol/L, respectively). At the 150% tBV infusion, the lactate concentrations were 1.6 ± 0.5, 1.7 ± 0.7, and 8.1 ± 0.9 mmol/L in the wRBC, HbV, and Sal groups, respectively. Significant differences were found between the Sal group and the HbV and wRBC groups.

Blood pH

Before the experiment, the mean pH was 7.400 ± 0.046 for all groups (Table 2). For the HES, Sal, and Alb groups, the pH decreased during the hemorrhage. However, in the wRBC and HbV groups, the pH remained within physiological range; at the end of experiment, the pH in the wRBC and HbV groups was 7.334 ± 0.032 and 7.316 ± 0.048, respectively.

DISCUSSION

This study was conducted to evaluate the efficacy of the Hb-vesicle as a resuscitation fluid in a continuous hemorrhage-resuscitation model. In this model, we administered a large volume of resuscitation fluid with rapid infusion to maintain circulation. All animals in the Alb and HES groups died before the end of the experiment, whereas all animals in the HbV and wRBC groups survived. In the Sal group, two of the seven animals were able to survive for 120 min; however, the MAP was low, and the lactate levels were high in the two surviving animals, so that recovery seemed nearly impossible. Collectively, these findings indicate that the effectiveness of Hb-vesicles in resuscitation is comparable to that of RBCs in this model. Resuscitation with crystalloid solutions (e.g., Sal) may induce a decrease in the blood flow in the peripheral arteries and thereby result in a decrease in bleeding. In contrast, resuscitation with colloid solutions, such as an Alb solution or HES, may be harmful in trauma patients who are bleeding continuously. Perel and Roberts (19) reported the relative effectiveness of colloids compared with crystalloid fluids and described how none of the evidence from randomized controlled trials shows that resuscitation with colloids, when compared with resuscitation with crystalloids, reduces the risk of death in patients with trauma, burns, or following surgery. However, in another report, the restoration of blood pressure by vigorous infusion with crystalloid fluids increased the hemorrhage volume and resulted in a markedly higher mortality (13).

In our study, the hemorrhage volume increased continuously in all five groups. However, in the Sal group, the hemorrhage speed occurred at a slower rate than that of the other groups. This phenomenon may result in part from the infused Sal being lost through the vasculature to the interstitial space. In this study, the estimated circulation blood volume was around 20 mL/body. After the resuscitation was initiated, the resuscitation fluid was administered at a rate of 56 mL/kg per hour, a much more rapid injection rate than that of the usual intravenous resuscitation therapy. In the HbV and wRBC groups, the MAP was partially maintained, although the hemorrhage volume was greater than that of the resuscitation fluid (Figs. 2 and 4). However, in the other three groups, the MAP decreased below 40 mmHg, even

TABLE 2. **Blood pH**

	Pre		50% tBV		100% tBV		150% tBV		200% tBV	
HbV	7.398 ± 0.051	(6)	7.447 ± 0.100	(6)	7.343 ± 0.062	(6)	7.340 ± 0.051	(6)	7.316 ± 0.048	(6)
wRBC	7.434 ± 0.026	(6)	7.454 ± 0.051	(6)	7.421 ± 0.020	(6)	7.379 ± 0.020	(6)	7.334 ± 0.032	(6)
Alb	7.374 ± 0.044	(6)	7.360 ± 0.064	(5)	7.243 ± 0.103	(5)				
HES	7.388 ± 0.026	(6)	7.413 ± 0.028	(6)	7.179 ± 0.126	(2)				
Sal	7.407 ± 0.060	(6)	7.318 ± 0.049	(5)	7.258 ± 0.225	(6)	7.076 ± 0.074	(4)		

Values are mean ± SD (n). Results of statistical analysis, $P < 0.05$. pre: No significant difference ($P = 0.1984$, one-factor ANOVA). 100%tBV: No significant difference ($P = 0.0514$, Kruskal-Wallis test), but HES vs. others was error. 200%tBV: HbV vs. wRBC was no significant difference ($P = 0.4786$, Student t test). 50%tBV: Combinations of groups with significant difference (method by Tukey-Kramer test) were as follows: HbV vs. Sal, and wRBC vs. Sal. 150%tBV: Combinations of groups with significant difference (method by Tukey-Kramer test) were as follows: HbV vs. Sal, and wRBC vs. Sal.

after resuscitation had been initiated. The accumulated hemorrhage volume was similar in the HbV, wRBC, Alb, and HES groups. Therefore, no differences in the hemorrhage after resuscitation were apparent based on the resuscitation fluid, with the exception of the Sal. In Alb and HES groups, restoration of the circulating blood volume was possible, but the blood pressure could not be maintained within a physiological range because of the low Ht, which is the cause of organ hypoxia. In contrast, the infused crystalloid solution, such as the Sal, can escape to the interstitial space, followed by peripheral vasoconstriction. Although the hemorrhage rate and volume were lower than those of the colloid groups, hypoxia and organ hypoperfusion were unavoidable and eventually compromised the condition of the animals.

Before the animals received the 100% tBV resuscitation, no significant differences were found between the HbV, wRBC, Alb, and HES groups in terms of the hemorrhage volume and the speed at which the resuscitation fluids were administered. From these findings, we can speculate that the organ perfusion might have been the same in these groups. However, the MAP in Alb and HES groups did not recover, and the lactate levels were significantly high. These results indicate that the oxygen carrier is indispensable for resuscitation in this model. In terms of the oxygen supply, wRBC and HbV have the same oxygen-binding capacity *in vitro*. In this model, the lactate level was maintained at a low level in the HbV and wRBC groups and showed significant differences at the 100% tBV infusion between the Alb and Sal groups. In the Sal group, the increase in the lactate concentrations was suppressed. Moreover, in the Sal group, the Ht was maintained at a significantly higher level than in the Alb and HES groups. Therefore, the oxygen delivery to the peripheral tissues in the Sal group might be partially maintained with the subsequent suppression of the increase in the lactate levels.

The functional half-life of the Hb-vesicle is significantly shorter than that of the normal erythrocyte, although it takes about 14 days until HbV completely disappears *in vivo* (17, 20). However, the results of the present study suggest that animals in this model can survive for the long term if they are kept supplied with functional Hb-vesicles or blood. We infer that volume resuscitation along with oxygen supply is necessary for maintaining circulation in uncontrolled hemorrhage.

This study had some limitations. First, a group of rats resuscitated with a soluble Hb solution was not included. Second, the animals were examined for only 2 h in this study, so that no information was available regarding the effects on long-term survival. Finally, the effects of the various resuscitation regimens on coagulation parameters were not assessed.

CONCLUSIONS

For uncontrolled hemorrhage, we conclude that a large volume of colloid solution containing oxygen carriers such as Hb-vesicles is effective and comparable to wRBC, because the HbV suspension fluid maintained the circulating volume and oxygen metabolism and prevented acidosis. Continuous infusion of functional Hb-vesicles was able to extend survival in hemorrhagic shock caused by continuous hemorrhage. Hence, patients with uncontrollable hemorrhage may acquire sufficient time for transportation to the trauma center using this treatment.

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REFERENCES

1. Holcomb JB, Jenkins D, Rhee P, Johannigman J, Mahoney P, Mehta S, Cox ED, Gehrke MJ, Beilman GJ, Schreiber M, et al.: Damage control resuscitation: directly addressing the early coagulopathy of trauma. *J Trauma* 62(2):307–310, 2007.
2. Sakai H, Hamada K, Takeoka S, Nishide H, Tsuchida E: Physical properties of hemoglobin vesicles as red cell substitutes. *Biotechnol Prog* 12(1):119–125, 1996.
3. Sakai H, Masada Y, Takeoka S, Tsuchida E: Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. *J Biochem* 131(4):611–617, 2002.
4. Solomonov E, Hirsh M, Yahiya A, Krausz MM: The effect of vigorous fluid resuscitation in uncontrolled hemorrhagic shock after massive splenic injury. *Crit Care Med* 28(3):749–754, 2000.
5. Hatoum O, Bashenko Y, Hirsh M, Krausz MM: Continuous fluid resuscitation for treatment of uncontrolled hemorrhagic shock following massive splenic injury in rats. *Shock* 18(6):574–579, 2002.
6. Matsuoka T, Hildreth J, Wisner DH: Uncontrolled hemorrhage from parenchymal injury: is resuscitation helpful? *J Trauma* 40(6):915–921, 1996.
7. Matsuoka T, Wisner DH: Resuscitation of uncontrolled liver hemorrhage: effects on bleeding, oxygen delivery, and oxygen consumption. *J Trauma* 41(3):439–445, 1996.
8. Krausz MM, Kablan M, Rabinovici R, Klin B, Sherman Y, Gross D: Effect of injured vessel size on bleeding following hypertonic saline infusion in "uncontrolled" hemorrhagic shock in anaesthetized rats. *Circ Shock* 35(1):9–13, 1991.
9. Krausz MM, Horn Y, Gross D: The combined effect of small volume hypertonic saline and normal saline solutions in uncontrolled hemorrhagic shock. *Surg Gynecol Obstet* 174(5):363–368, 1992.
10. Capone AC, Safar P, Stezoski W, Tisherman S, Peitzman AB: Improved outcome with fluid restriction in treatment of uncontrolled hemorrhagic shock. *J Am Coll Surg* 180(1):49–56, 1995.
11. Marshall HP Jr, Capone A, Courcoulas AP, Harbrecht BG, Billiar TR, Udekwu AO, Peitzman AB: Effects of hemodilution on long-term survival in an uncontrolled hemorrhagic shock model in rats. *J Trauma* 43(4):673–679, 1997.
12. Amathieu R, Racine S, Triba M, Poloujadoff MP, Borron SW, Bouchemal N, Lapostolle F, Merouani M, Le Moyec L, Adnet F: Use of nuclear magnetic resonance spectroscopy to assess renal dysfunction after hypertonic-hyperoncotic resuscitation in rats. *J Trauma* 63(2):379–387, 2007.
13. Stern SA, Dronen SC, Birrer P, Wang X: Effect of blood pressure on hemorrhage volume and survival in a near-fatal hemorrhage model incorporating a vascular injury. *Ann Emerg Med* 22(2):155–163, 1993.
14. Leppäniemi A, Soltero R, Burris D, Pikoulis E, Ratigan J, Waasdorp C, Hufnagel H, Malcolm D: Early resuscitation with low-volume PolyDCLHb is effective in the treatment of shock induced by penetrating vascular injury. *J Trauma* 40(2):242–248, 1996.
15. Izumi Y, Sakai H, Kose T, Hamada K, Takeoka S, Yoshizu A, Horinouchi H, Kato R, Nishide H, Tsuchida E, et al.: Evaluation of the capabilities of a hemoglobin vesicle as an artificial oxygen carrier in a rat exchange transfusion model. *ASAIO J* 43(4):289–297, 1997.
16. 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(2):539–545, 2004.
17. Sakai H, Seishi Y, Obata Y, Takeoka S, Horinouichi 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* 31(2):192–200, 2009.
18. Izumi Y, Sakai H, Hamada K, Takeoka S, Yamahata T, Kato R, Nishide H, Tsuchida E, Kobayashi K: Physiologic responses to exchange transfusion with hemoglobin vesicles as an artificial oxygen carrier in anesthetized rats: changes in mean arterial pressure and renal cortical tissue oxygen tension. *Crit Care Med* 24(11):1869–1873, 1996.
19. Perel P, Roberts I. Colloids versus crystalloids for fluid resuscitation in critically ill patients. *Cochrane Database Syst Rev* (4):CD000567, Oct. 17, 2007.
20. Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Tsuchida E, Kai T, et al.: Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. *Drug Metab Dispos* 37(7):1456–1463, 2009.

Microcirculation and NO-CO Studies of a Natural Extracellular Hemoglobin Developed for an Oxygen Therapeutic Carrier

Amy G. Tsai[a], Marcos Intaglietta[a], Hiromi Sakai[b], Eric Delpy[c], Christophe Drieu La Rochelle[c], Morgane Rousselot[d], Franck Zal*[,d]

[a]*Department of Bioengineering, University of California, San Diego, La Jolla, California, USA;* [b]*Research Institute for Science and Engineering, Waseda University, Tokyo 169-8555, Japan;* [c]*Biotrial Pharmacology, 7-9 rue Jean-Louis Bertrand, 35000 Rennes France;* [d]*Hemarina, Aéropôle Centre, Biotechnopôle, 29600 Morlaix, France*

Abstract: Extracellular soluble hemoglobins (Hbs) have long been studied for their possible use as safe and effective alternatives to blood transfusion. While remarkable progress has been made in the use of cell-free Hb as artificial oxygen carrier, significant problems remain, including susceptibility to oxidative inactivation and propensity to induce vasoconstriction. Hemarina-M101 is a natural giant extracellular hemoglobin (3600 kDa) derived from marine invertebrate (polychaete annelid). Hemarina-M101 is a biopolymer composed of 156 globins and 44 non-globin linker chains and formulated in a product called HEMOXYCarrier®. Prior work has shown Hemarina-M101 to possess unique anti-oxidant activity and a high oxygen affinity. Topload experiment with this product into rats did not revealed any effect on heart rate (HR) and mean arterial pressure (MAP). A pilot study with the hamster dorsal skinfold window chamber model showed absence of microvascular vasoconstriction and no significant impact on mean arterial blood pressure. *In vitro* nitric oxide (NO) and carbon monoxide (CO) reaction kinetics measurements show that Hemarina-M101 has different binding rates as compared to human Hb. These results revealed for the first time that the presence of this marine hemoglobin appears to have no vasoactivity at the microvascular level in comparison to others hemoglobin based oxygen carriers (HBOCs) developed so far and merits further investigation.

Keywords: Annelid, blood substitute, extracellular hemoglobin, hemodynamic activity.

INTRODUCTION

Blood substitute development has mainly centered on fluids that transport oxygen as this is a principal function of the circulation. This focus on oxygen carrying capacity has led to the development of two main groups of blood substitutes: perfluorocarbon emulsions and hemoglobin (Hb)-based oxygen carriers (HBOCs) [1]. HBOCs derived from human, bovine or recombinant Hb have been found to present different forms of toxicity [2]. This toxicity appears to be circumvented by polyethylene glycol conjugation and phospholipid encapsulation [3-5]. However, these approaches introduce significant quantities of additional material in the circulation whose effect and clearance are not well established. Molecular Hb in solution is an elegant method for implementing an oxygen carrier because it can reach and deliver oxygen to tissue regions inaccessible to red blood cells. However the natural tendency of molecular Hb solutions is to cause vasoconstriction and thus attainment of the goal to transport oxygen to tissue is hindered by most molecular Hb solutions. Vasoconstriction while not necessarily a negative effect *per se,* molecular Hb solutions are associated with significant incidence of adverse effects [4, 6].

*Address correspondence to this author at Hemarina, Aéropôle Centre, Biotechnopôle, 29600 Morlaix, France; Tel: + 33 (0)2 98 88 1402; Fax: + 33 (0)2 98 88 3894; E-mail: franck.zal@hemarina.com

A fundamental observation that defines how the circulation reacts to molecular Hb in blood was made by Sakai *et al.* [7] who showed that vasoactivity is an inverse function of molecular dimension. The validity of this tenent is supported by results obtained with the zero-link polymer bovine Hb and ultra-high molecular weight Hb polymers [8, 9]. These mechanistic aspects have been explored by studies with macromolecular plasma expanders that do not carry oxygen [10].

A molecular Hb that fulfills the dimensional paradigm of Sakai *et al.* [7] is the oxygen transporter of the marine invertebrate *Arenicola marina* [11, 12]. This is a natural extracellular respiratory pigment of high molecular weight (~3600 kDa) that does not require chemical modification. This giant extracellular O_2 carrier consists of globin and non-globin linker chain complexes that have a large Hb oxygen binding capacity, carrying up to 156 O_2 molecules when saturated [11, 13]. This material named Hemarina-M101, is purified from extracellular hemoglobin of *Arenicola marina* and used to produce HEMOXYCarrier® (Hemarina S.A., France), a product being developed as an oxygen carrying therapeutic. HEMOXYcarrier® could be used in a large range of concentration in terms of biorheological considerations since its viscosity is similar to that of plasma. Furthermore, recent works showed that a different formulation of Hemarina-M101 ($HEMO_2life$®, Hemarina S.A., France) considerably improves kidney preservation waiting to be transplanted [14].

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An important physiological aspect of red blood cell (RBC) function is the management of endogenous gaseous messenger molecule transport such as nitric oxide (NO) and carbon monoxide (CO). These molecules are potent vasodilators and are effective at very small concentrations. NO bioavailability in the vessel wall is determined by very complex enzymatic mechanism [15] and also the balance between NO production by shear stress on the endothelium and its scavenging by hemoglobin [16]. CO bioavailability appears to be of significance in the hepatic microcirculation [17].

The present study reports a pilot microvascular investigation of the effect of a topload intravenous infusion of HEMOXYCarrier® on a) microhemodynamics in the hamster dorsal skinfold window chamber model, and b) systemic parameters in the rat. The *in vitro* biochemical activity in terms of NO and CO reaction kinetics was also explored.

MATERIAL AND METHODS

HEMOXY Carrier® Production and Sample Preparation

HEMOXYCarrier® (Hemarina SA, France) was manufactured using GMP standards governing medicinal products [18]. HEMOXYcarrier® is formulated in an injectable saline solution and its properties are presented in Table **1**.

Table 1. Functional Properties of HEMOXYCarrier®

P50 (mmHg)	**7.05 ± 0.93[a] (*n*=9)**
n50	2.54 ± 0.23 [a] (*n*=9)
Bohr coefficient [b]	-0.5 [a]
ΔH (KJ mol-1) [b]	-19 [a]
COP (mmHg) [c]	1.0
Viscosity (cP) [c]	1.23
SOD activity (U/mg Hb) [d]	3.53 ± 0.02 (*n*=3)
CN inhibition	100% [e]
Fe (atom/molecule)	156 [f]
Cu (atom/molecule)	3.58 ± 1.17 (*n*=5) [g]
Zn (atom/molecule)	5.13 ±0.75 (*n*=5) [g]

a) The oxygen equilibrium binding data were collected in dissolved lyophilized plasma (Sigma) in 10 mM HEPES, pH 7.35 at 37°C for a final heme concentration of 40 mg/mL. The Bohr coefficient was measured over the pH range 7.2-7.6, and the temperature effect from 33°C to 41°C, which corresponds to the range of values encountered in human, including pathological cases.
b) The Bohr coefficient and temperature sensitivity of hemoglobin are calculated as previously described [24].
c) COP and viscosity were determined on a M101 sample of 58 mg/mL at room temperature on an Onkometer BMT 293 (BMT MESSTECHNIK GMB, Germany) according the company protocol and on a Brookfield viscometer (Brookfield Engineering Laboratories Vertriebs, Germany) according to the manufactures protocol.
d) The SOD activity was determined using the Flohé and Ötting method [25] and value come from [12].
e) HEMOXYCarrier® was incubated for 10 min with 50 mM KCN prior to the addition of xantine oxidase.
f) Fe was determined by mass spectrometry [11].
g) The Cu and Zn contents were determined by inductively coupled plasma-MS [26].

Hamster and In Vitro Gas Studies

HEMOXYCarrier® (68 mg/mL) was defrosted slowly by exposing the vial to room temperature. The sample was then diluted with 0.9% normal saline solution to produce 40 mg/mL solution for the study (4%). This diluted solution was then aliquoted and returned to the -80°C freezer. In preparation for infusion into the animal, samples were removed from the -80°C freezer and warmed to room temperature. HEMOXYCarrier® concentration was reduced in order to compare the results to a previous study with several molecular Hbs also tested at the same concentration [19]. The dose for these animals was approximately 280 mg/kg.

Rat Studies

For this study, HEMOXYCarrier® was administered by i.v. infusion at the dose level of 600 mg/kg, a dose that has shown a tissular oxygenation potential on rodent model (unpublished data). Concentration adjustment was performed using an injectable saline solution developed by Hemarina as previously mentioned. Prior to infusion, HEMOXYCarrier® was removed from the -80°C freezer and warmed to room temperature.

Animal Models and Preparation

I. Microcirculation Study with the Hamster Dorsal Skinfold Window Model

Studies were performed in golden Syrian male hamsters (Charles River Laboratories, Boston, MA), weight range of 50-70g. Animals were handled according to the Guide for the Care and Use of Laboratory Animals (US National Research Council, 1996) and experiments were approved by the University of California, San Diego Institutional Animal Care and Use Committee. The hamster dorsal skinfold window chamber model has been widely used for microvascular studies and allows for the study of intact tissue in an awake animal, thus reducing complications due tissue exposure and anesthesia. Chamber implantation and vascular catheterization were performed under general anesthesia (pentobarbital 50 mg/kg i.p.) as previously described [20, 21]. Mean arterial blood pressure (MAP) and heart rate (HR) were continuously monitored using a data acquisition system (MP 150; Biopac Systems, Inc., Santa Barbara, CA). Blood was sampled from the arterial catheter into heparized microcapillary tubes and centrifuged to determine systemic hematocrit (Hct). Arterial blood chemistry (pH, pO2, pCO) was measured using a blood gas analyzer (RAPIDLab 248, Siemens, Deerfield IL). Animals were included into the study if their MAP > 80 mmHg, and systemic Hct > 45% which are within the normal physiological range [22]. After baseline systemic and microvascular measurements were made, animals were given an i.v. infusion of the study material (100-150 µL/min), volume equal to 10% of its blood volume which was estimated as 7% of the body weight. MAP and HR were assessed at 10, 30 and 60 min after completion of the infusion. Hematocrit and arterial blood gases were reassessed at the end of the experiment (60 min after topload).

The unanesthetized animal was placed into a restraining tube, which was then affixed to the stage of an intravital microscope (BX51WI, 40 x objective, NA 0.7 SW; Olympus, Central Valley, PA). The tissue image was projected onto a CCD camera (4815-2000; COHU, San Diego, CA) connected to a timer and viewed on a monitor. Animals had at least 30 minutes to adjust to the tube environment prior to

measuring baseline parameters (MAP, HR, blood gases and Hct). Arterioles and venules, chosen by their visual acuity (4-6 each type), were characterized by their blood flow velocity and diameter. Vessels were chosen from baseline observations and the same vessels were followed throughout the experiment. Microvessel diameters were measured with an image-shearing system (Digital Video Image Shearing Monitor 908, Vista Electronics, San Diego, CA), while arteriolar and venular blood flow velocities were measured with photodiodes using the cross-correlation technique (Velocity Tracker Mod-102 B, Vista Electronics., San Diego, CA). Blood flow rates (Q) were calculated as: $Q = \pi$(RBC velocity/R_v)(diameter/2)^2 where $R_v = 1.6$ is the ratio of the centerline velocity to the bulk velocity.

II. In Vivo Cardiovascular Effects in the Anaesthetized Rat

These experiments were carried out on male Wistar rats (Elevage R. Janvier, France) weighing 250-300g (7 weeks old). Rats were anesthetized with sodium pentobarbital (60 mg/kg i.p.). A catheter was placed into the left carotid artery to measure arterial blood pressure and heart rate. A Millar-type pressure probe was placed into the left ventricular cavity via the right carotid artery to measure left ventricular pressure (LVP). Lastly, a catheter was placed into the pudendal vein for infusion of the test sample. The animal's body temperature was maintained at 37°C with a water circulating warming blanket.

After stabilization of hemodynamic parameters, baseline measurements were performed and HEMOXYCarrier® (600 mg/kg) or saline solution 0.9% (used as a control) was infused intravenously (5 min, 1.2 mL/kg/min). Experiments were carried out in 18 rats. Six animals received HEMOXYCarrier® (600 mg/kg) and twelve animals received saline solution as control. Hemodynamic parameters were monitored for a period of 45 min post infusion. Heart rate was derived from the phasic arterial blood pressure signal. Left ventricular dP/dt_{max}, an index of myocardial contractility, was calculated as the maximal rate of rise of left ventricular pressure signal.

III. In Vitro Measurement of NO and CO Binding

The time course of the ligand binding was analyzed during rapid mixing of the deoxygenated HbV (human Hb vesicles) and Hb solutions and a NO- or CO-containing solution using a stopped-flow rapid scan spectrophotometer (RSP-1000; Unisoku Co. Ltd., Osaka, Japan). Briefly, 3 mL of a deoxygenated PBS solution containing HbV [23], human HbA, Oxyglobin (Biopure) and HEMOXYCarrier® at [heme] = 3 μM was rapidly mixed with NO or CO containing PBS solution. The change of absorption at 430 nm was monitored. The methods are described in detail in a previous report [23].

Data Analysis

Statistics were performed using Prism version 4.0 for Windows (GraphPad, San Diego, CA). Data are presented as mean ± sem.

In the hamster studies, N and n denote the number of animals and vessels studied, respectively. Differences within groups were first tested with one-way analysis of variance (ANOVA) for repeated measures and for multiple comparisons between groups. Bonferroni post hoc test was used if significance was obtained. In haemodynamic studies performed on anaesthetized rat, homogeneity of baseline values (T0) between the two groups was tested for each parameter using a two-tailed Student's t test for independent samples. For each parameter, changes from baseline value (delta %) at each measurement time were compared between the two groups using a two-way ANOVA (group, time) with repeated measurements over time. If group x time interaction was significant, comparison between the two groups was performed at each time by a student t test. Changes were considered statistically significant if $P < 0.05$.

RESULTS AND DISCUSSION

I. Hamster Studies

The study was performed in 5 animals of 60 ± 5 g. All animals tolerated and completed the protocol without any adverse events. Table **2** presents the changes in hematocrit and arterial blood gas parameters at baseline and 60 min after topload infusion of HEMOXYCarrier®. Blood gases parameters were not significantly affected by the administration of HEMOXYCarrier® while Hct was slightly reduced ($P < 0.05$). This slight change in Hct, while being statistically significant, is not considered physiologically significant. These data are consistent with previous experiments done on mice and rats with this product [12]. Figs. (**1A**) and (**1B**) show the effect of HEMOXYCarrier® on MAP and HR. HEMOXYCarrier® induced slight and transient changes in MAP and HR after completion of topload compared to baseline. No significant changes were observed 10 minutes after the infusion until the end of the observation period after 1 hr. Arteriolar (n=31, n: number of vessels) and venular (n=25) diameters, microvascular flow velocity and flow were not significantly modified by HEMOXYCarrier® (Figs. **2A** and **2B**). We speculate that HEMOXYCarrier® in circulation may not react with NO as found with others cell free Hbs or that other properties such as its ability to transport CO may lead to the arteriolar vaso-inactivity. Thus, it is important to extend these microvascular studies of HEMOXYCarrier® to measure perivascular NO to validate this hypothesis.

Table 2. Hematocrit and Arterial Blood Gases before and after i.v. Topload Infusion of HEMOXYCarrier®

	Baseline	60 min
Hct	49.8 ± 1.6	48.3 ± 0.9*
Ph	7.37 ± 0.02	7.37 ± 0.05
PO2	55.9 ± 4.1	57.5 ± 2.4
PCO2	56.3 ± 1.2	55.7 ± 6.5
BE	7.0 ± 1.3	5.8 ± 1.4

Figs. (**3**) and (**4**) present a summary of our previous findings concerning MAP and microvascular response for several molecular Hbs (sourced from human and bovine) when infused with the same protocol as the current study [19]. The previous study used (1) αα cross-linked Hb (ααHb, HemAssist®, Baxter); (2) polymerized bovine Hb (PBH4, Oxyglo-

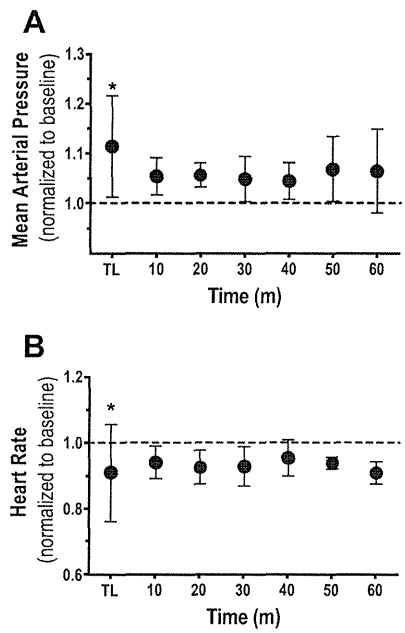

Fig. (1). Time course of MAP and HR after 10% blood volume topload of HEMOXYCarrier® in hamster model. The slight increase in MAP and decrease in HR initially observed after topload (TL) completion were statistically significant. The minor changes observed during the rest of the observation period were no significatively different from baseline. Parameters are presented relative to baseline, thus no change from baseline would be denoted as 1, while 1.05 would represent a 5% increase from baseline. Data are presented as mean ± sem.

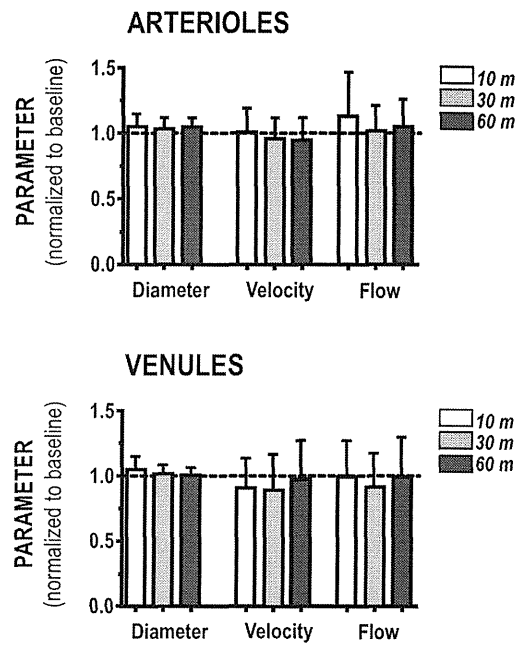

Fig. (2). Microhemodynamic changes as a function of time after a 10% blood volume topload with HEMOXYCarrier® in hamster model. Arteriolar and venular vessels responded with a weak and non-significant increase in diameter by 5.2 ± 0.9% and 4.7 ± 0.9%, respectively ($P < 0.05$, relative to baseline) at 10 min. Panel A: Arterioles. Panel B: Venules. Parameters are presented relative to baseline, thus no change from baseline would be denoted as 1, while 1.05 would mean a 5% increase from baseline. Data are shown 10 (white bar), 30 (light grey bar) and 60 (dark grey bar) minutes after completion of topload. All changes were not statistically different from their baseline levels. Data are presented as mean ± sem.

bin®, Biopure Corp.), (3) polyethylene glycol decorated Hb (PEG-Hb, Hemospan®, Sangart) or (4) 0.9% NaCl (saline). Saline infusion was the no-treatment group/volume control used to assess the effect of the experimental protocol. It was a more extensive study where changes in microvessel diameter, blood flow, and perivascular NO concentration along with systemic hemodynamic parameters were followed. The results obtained in the current study with HEMOXYCarrier® were introduced into these figures for comparison.

The smaller molecular Hbs PBH4 and αα Hb cause arteriolar constriction and reduced microvascular perfusion, while both HEMOXYCarrier® and PEG-Hb (higher molecular size of human and bovine Hbs, 95 kDa) maintained arteriolar diameter. However, PEG-Hb induced blood volume expansion in comparison with HEMOXYCarrier® that maintained microvascular flow.

All these solutions resulted in an increased mean arterial pressure due to vasoconstriction for PBH4 and αα Hb and to blood volume expansion and maintenance of cardiac output for PEG-Hb. This previous work with included perivascular NO measurement using microelectrodes concluded that perivascular NO was reduced to the same level for all Hb solutions because NO binding affinities are similar. However, effects on vascular resistance are related to the type of molecular modification, molecular volume and oxygen affinity.

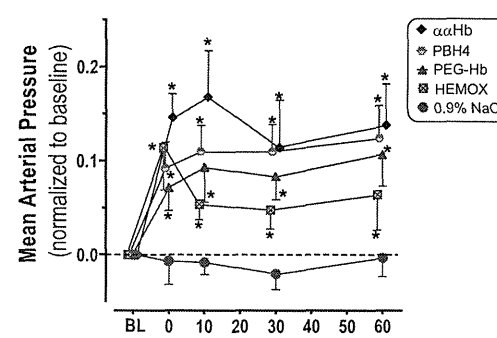

Fig. (3). Changes in MAP after topoading with different molecular Hbs in hamster model. MAP was statistically increased by all molecular hemoglobins compared to saline ($P < 0.05$) but no statistical differences were obtained between the groups at each time point. All data are presented as mean ± sem. Parameter is presented as change relative to baseline, thus no change from baseline would be denoted as 0, while 0.1 would mean a 10% increase from baseline.

Thus, in the current study, the slight rise in MAP could be explained by changes to blood volume expansion. However

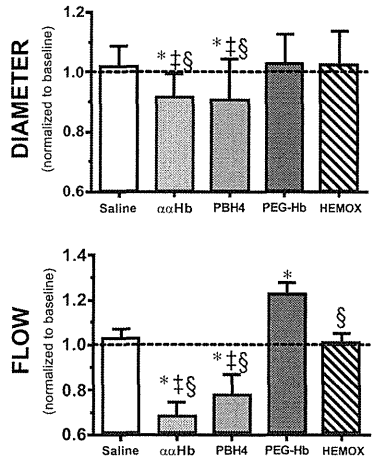

Fig. (4). Summary of diameter and flow (Q) response at the microvascular level with molecular hemoglobins in hamster model. The smaller molecular Hbs (ααHb and PBH4) caused arteriolar constriction leading to reduced microvascular perfusion compared to PEG-Hb and HEMOXYCarrier®. Both HEMOXYCarrier® and PEG-Hb did not change arteriolar diameter compared to baseline and saline. However, increased arteriolar blood flow levels were achieved with PEG-Hb, while HEMOXYCarrier® had unchanged levels. ααHb and PBH4 induced reduced arteriolar blood flow levels. Blood flow data (Q) is presented as mean ± sem. Parameters are presented relative to baseline, thus no change from baseline would be denoted as 1, while 1.05 would mean a 5% increase from baseline. *, $p < 0.05$ relative to saline; ‡ , $p < 0.05$ relative to HEMOX; § , $p < 0.05$ relative to PEG-Hb.

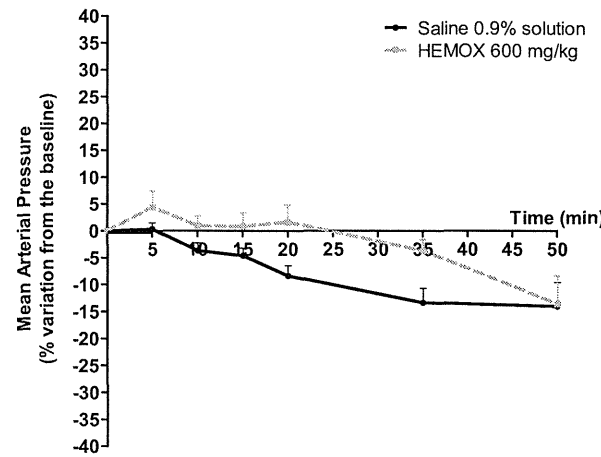

Fig. (5). Comparison of the changes in MAP after i.v. infusion of 600 mg/kg of HEMOXYcarrier® or saline solution in the anaesthetized rat model. Parameter is presented relative to baseline. No statistical difference was obtained between HEMOXYCarrier® and saline.

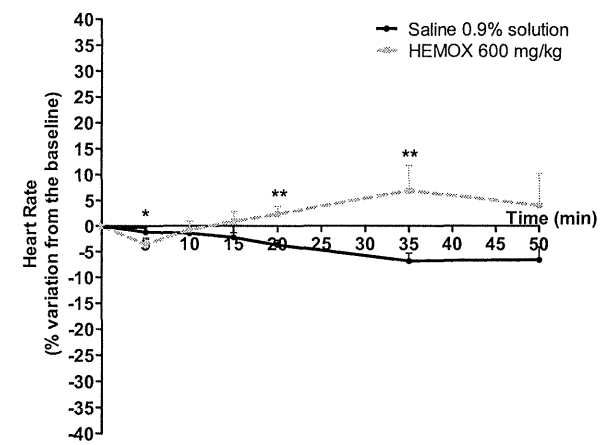

Fig. (6). Comparison of the changes in HR after i.v. infusion of 600 mg/kg of HEMOXYcarrier® or saline solution in the anaesthetized rat model. Parameter is presented relative to baseline. Statistical differences observed between HEMOXYCarrier® and saline are indicated for each time point (*, **, $p < 0.05$).

without significant changes to vessel diameter compared to baseline, one needs to consider expanding the current study with HEMOXYCarrier® to make perivascular NO and cardiac output measurements in order to better compare to the other molecular Hbs. It is important to note that these findings that indicate the absence of vasoconstriction in the dorsal window chamber which consists of a resting skeletal muscle and connective tissue may not be reflective of the absence of vasoconstriction in organs. We have shown in parallel experiments which measure organ blood flow distribution that results deduced from findings in the hamster chamber window model, which allow the observation and quantitative characterization of the microcirculation in the intact and unanesthetized state, are representative of even in some of the major organs that are not accessible by microvascular techniques [10].

II. *In Vivo* Cardiovascular Effects in the Anaesthetized Rat

All animals completed the experimental protocol and are included in these results.

Compared to saline-treated animals, injection of HEMOXYCarrier® at 600 mg/kg (5-min i.v. infusion) showed only minor effects on mean arterial pressure (differences not statistically significant compared to saline), heart rate and left ventricular dP/dt_{max} (an index of myocardial contractility), as shown on Figs. (**5, 6** and **7**), respectively.

The maximal effect measured on left ventricular dP/dt_{max} was a slight increase (9%, $P<0.05$) observed 15 min after the end of the infusion and was followed by a return to baseline value. Similarly, the effect of HEMOXYCarrier® on heart rate was minor as heart rate increased slightly within 30 min following the treatment ($P<0.05$) and tended to return to baseline value thereafter. Since heart rate and myocardial contractility are almost not affected by HEMOXYCarrier® and are the major components of cardiac output, this parameter may not be affected by HEMOXYCarrier®. The lack of effects of HEMOXYCarrier® on arterial blood pressure in the anaesthetized rats suggests that HEMOXYCarrier® may also not have a vasoconstrictor effect in the rat. These results are consistent with those obtained in the hamster dorsal skin-

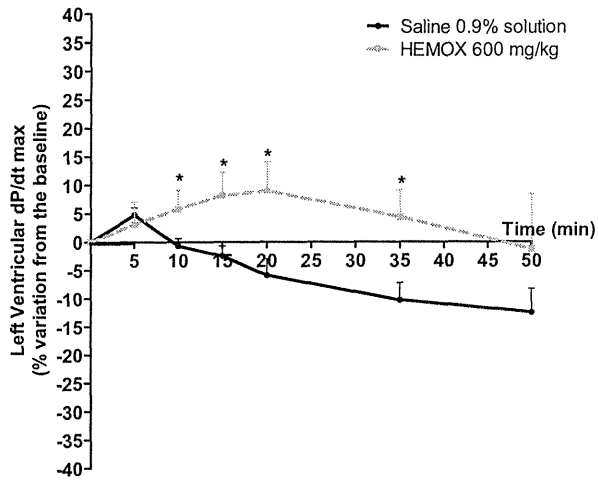

Fig. (7). Comparison of the changes in myocardial contractility (Left ventricular dP/dt_{max}) after infusion of 600 mg/kg of HEMOXYcarrier® or saline solution in the anaesthetized rat model. Parameter is presented relative to baseline. Statistical differences observed between HEMOXYCarrier® and saline are indicated for each time point (*, $p < 0.05$).

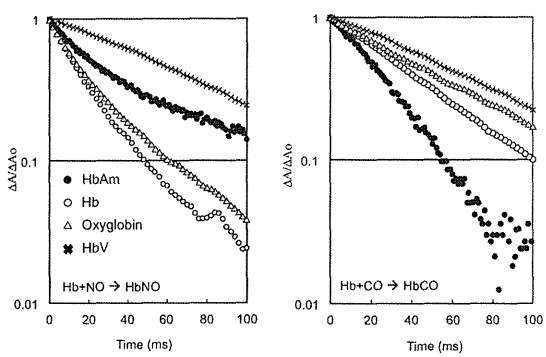

Fig. (8). Nitric oxide (NO) and carbon monoxide (CO) binding rates. HbV, hemoglobin vesicles (Waseda University, Tokyo, Japan), polymerized bovine Hb (Oxyglobin, Biopure Corp, Boston, MA), human HbA and HEMOXYcarrier® (Hemarina SA, France). All measurements were performed three times. Data were accumulated and averaged automatically by using a stopped-flow rapid scan spectrophotometer (model RSP-1000, Unisoku Co.) [23].

fold window studies demonstrating the lack of microhemodynamic changes and no significant modification in heart rate and arterial pressure after a 10% blood volume topload.

Vasoactivity has hampered progress of HBOCs due to concern for adverse blood pressure responses and secondary complications. Taken together, these preliminary investigations promote the large reduction of systemic side effects with HEMOXYCarrier® and its use of as oxygen transporter.

III. Nitric Oxide and Carbon Monoxide Binding Rates

HEMOXYCarrier® presents interesting NO and CO transport properties, having a lower binding rate than human Hb and polymerized bovine Hb (Oxyglobin, Biopure, Boston, MA) as shown in Fig. (**8**). However, NO binding rate is not fully indicative of its NO scavenging capacity because it does not reflect steady state NO bioavailability. Notably phospholipid encapsulated Hb, which is not vasoactive has a > 10 fold NO binding constant. The finding that CO binding is somewhat greater than for other HBOCs suggests that this material may decrease CO bioavailability.

In summary, HEMOXYCarrier® derived from a marine organism presents biophysical transport properties appropriate for an oxygen carrier for transfusion medicine. HEMOXYCarrier® is derived from molecules designed by evolution to maintain life in hypoxic conditions for prolonged periods, hence is suitable for transporting and delivering oxygen. The extremely low P50 of this molecular Hb targets its oxygen delivery to highly hypoxic tissue and it is unknown how this may affect the function of metabolically active tissues. HEMOXYCarrier® is stable without needing chemical modification over a wide range of ionic compositions and osmolarities and has superoxide dismutase like antioxidant properties [12, 14]. The current findings taken as a whole do not provide a compelling explanation for HEMOXYCarrier®'s lack of local vasoactivity in the hamster dorsal window chamber model. However, these preliminary data on rat and hamster demonstrates that the product has different properties in comparison to previous HBOC formulations and thus warrants more in depth investigations and its continued pursuit as an oxygen transporter for transfusion medicine or other applications where oxygen delivery is needed.

CONFLICT OF INTEREST

F.Z. and M.R. are founders and hold stock in Hemarina which produces the substance being investigated. Materials for the studies were provided by Hemarina. Biotrial was contracted by Hemarina to perform the rat studies presented in this publication. E.D. and C.D.L.R. are employees of Biotrial. Hamster studies were performed at University of California, San Diego. All other authors declare that they do not have any conflicts of interest with this work.

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REFERENCES

[1] Winslow RM. Current status of oxygen carriers ('blood substitutes'): 2006. Vox Sanguinis 2006; 91: 102-10.

[2] Alayash AI. Oxygen therapeutics: Can we tame haemoglobin? Nature Reviews Drug Discovery 2004; 3: 152-9.

[3] Jahr JS, Akha AS, Holtby RJ. Crosslinked, Polymerized and PEG-conjugated hemoglobin-based oxygen carriers: Clinical safety and efficacy of recent and current products. Curr Drug Discov Technol 2012; 9(3): 158-65.

[4] Winslow RM. Alternative oxygen therapeutics: products, status of clinical trials, and future prospects. Curr Hemat Reports 2003; 2: 503-10.

[5] Sakai H. Present situation of the development of cellular-type hemoglobin-based oxygen carrier (hemoglobin-vesicles). Curr Drug Discov Technnol 2012; 9(3): 188-93.

[6] Salazar Vázquez BY, Hightower CM, Martini J, *et al.* Vasoactive hemoglobin solution improves survival in hemodilution followed by hemorrhagic shock. Crit Care Med 2011; 6: 1461-66.

[7] Sakai H, Hara H, Yuasa M, *et al.* Molecular dimensions of Hb-based O_2 carriers determine constriction of resistance arteries and hypertension. Am J Physiol 2000; 279: 908-15.

[8] Matheson B, Kwansa HE, Bucci E, Rebel A, Koehler RC. Vascular response to infusions of a nonextravasating hemoglobin polymer. J Appl Physio 2002; 93: 1479-86.

[9] Cabrales P, Zhou Y, Harris DR, Palmer AF. Tissue oxygenation after exchange transfusion with ultrahigh-molecular-weight tense- and relaxed-state polymerized bovine hemoglobins. Am J Physiol Heart Circ Physiol 2010; 298: 1062-71.

[10] Cabrales P, Tsai AG. Plasma viscosity regulates systemic and microvascular perfusion during acute extreme anemic conditions. Am J Physiol Heart Circ Physiol 2006; 291: 2445-52.

[11] Zal F, Green BN, Lallier FH, Vinogradov SN, Toulmond A. Quaternary structure of the extracellular haemoglobin of the lugworm *Arenicola marina*: a multi-angle-laser-light-scattering and electrospray-ionisation-mass-spectrometry analysis. Eur J Biochem 1997; 243: 85-92.

[12] Rousselot M, Delpy E, Drieu La Rochelle C, *et al. Arenicola marina* extracellular hemoglobin: a new promising blood substitute. Biotechnol J 2006; 1: 333-45.

[13] Royer WE Jr., Omartian MN, Knapp JE. Low resolution crystal structure of Arenicola erythrocruorin: influence of coiled coils on the architecture of a megadalton respiratory protein. J Mol Biol 2007; 365: 226-36.

[14] Thuillier R, Dutheil D, Trieu MT, *et al.* Supplementation with a new therapeutic oxygen carrier reduces chronic fibrosis and organ dysfunction in kidney static preservation. Am J Transpl 2011; 11(9): 1845-60.

[15] Gladwin MT, Crawford JH, Patel RP. The biochemistry of nitric oxide, nitrite and hemoglobin in blood flow regulation. Free Radic Biol Med 2004; 36(6): 707-17.

[16] Sriram K, Salazar Vázquez BY, Yalcin O, Johnson PC, Intaglietta M, Tartakovsky DM. The effect of small changes in hematocrit on nitric oxide transport in arterioles. Antioxid Redox Signal 2011; 14: 175-85.

[17] Suematsu M, Goda N, Sano T, *et al.* Carbon monoxide: an endogenous modulator of sinusoidal tone in the perfused rat liver. J Clin Invest 1995; 96: 2431-37.

[18] European Commission- Enterprise and Industry: EudraLex. The rules governing medicinal products in the European Union. Vol 4. Good manufacturing practice. Medicinal products for human and veterinary use. Part II: Basic requirements for active substances used as starting materials. Brussels, Belgium, 2010. http://ec.-europa.eu/health/files/eudralex/vol 4/2007_09_gmp_part2_en.pdf

[19] Tsai AG, Cabrales P, Manjula BN, Acharya SA, Winslow RM, Intaglietta M. Dissociation of local nitric oxide concentration and vasoconstriction in the presence of cell-free hemoglobin oxygen carrier. Blood 2006; 108: 3603-10.

[20] Endrich B, Asaishi K, Gotz A, Messmer K. Technical report- a new chamber technique for microvascular studies in unanesthetized hamsters. Res Exp Med 1980; 177: 125-34.

[21] Friesenecker B, Tsai AG, Instaglietta M. Capillary perfusion during ischemia-reperfusion in subcutaneous connective tissue and skin muscle. Am J Physiol 1994; 267: 2204-12.

[22] Van Hoosier GL, McPherson CW. Laboratory hamsters. Academic Press: Orlando 1987.

[23] Sakai H, Sato A, Sobolewski P, Takeoka S, Frangos JA, Kobayashi K, Intaglietta M, Tsuchida E. NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers. Biochim Biophys Acta 2008; 1784: 1441-47.

[24] Hourdez S, Weber RE. Molecular and functional adaptations in deep-sea hemoglobins. J Inorg Biochem 2005; 99: 130-41.

[25] Flohe L, Otting F. Superoxide dismutase assays. Methods Enzymol 1984; 105: 93-104.

[26] Hagege A, Baldinger T, Martin-Jouet M, *et al.* Assessment of the contribution of inductively coupled plasma mass spectrometry to metalloprotein analysis. A novel approach for studies of multiproteic complexes. Rapid Commun Mass Spectrom 2004; 18: 735-38.

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

Hiromi Sakai,[1,2] Yuji Suzuki,[3] Keitaro Sou,[4] Mayumi Kano[3]

[1]Organization for University Research Initiatives, Waseda University, Tokyo, Japan
[2]Waseda Bioscience Research Institute in Singapore, Biopolis, Singapore
[3]Nihon Bioresearch Inc., Hashima, Japan
[4]Center for Advanced Biomedical Sciences, Waseda University, Tokyo, Japan

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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_2 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 relates 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.
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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.[1] 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 dyspensa, tachypenia, tachycardia, hypotension and hypertension, chest pain, and back pain.[2–5] It is believed that these hypertensive reactions are a consequence of complement activation: so-called complement activation-related pseudoallergy.[4] 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.[4,5]

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.[6] 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.
Correspondence to: H. Sakai; e-mail: hirosakai@aoni.waseda.jp
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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,[7,8] as a priming fluid for extracorporeal membrane oxygenator,[9] and as a targeted oxygen carrier for ischemic tissues[10–12] and tumors.[13] Actually, Hb encapsulation shields the toxic effects of stroma-free Hbs, especially preventing unwanted reactions of Hb with endogenously produced NO,[14,15] thereby preventing NO-related vasoconstriction and hypertension.[16]

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.[17] 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.[18] The present formulation of HbV contains a different type of negatively charged lipid, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG).[6] It has been confirmed that DHSG significantly reduces the complement activation using a rat model,[19,20] and human plasma.[21] Nevertheless, it remains unknown how pigs respond to the injection of HbV. Pigs are known to respond sensitively to the injection of liposomes.[4] 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[22]; but in some pathological conditions PIMs appear.[23,24]

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.[6] 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_{5000} (DSPE-PEG; NOF Corp., Tokyo, Japan) at the molar composition of 5/4/0.9/0.03. 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_2O and O_2 (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) × 80 (in dyne·s·cm^{-5}), and systemic vascular resistance (SVR) was calculated as {(MBP − RAP)/CO} × 80 (in dyne·s·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,[5] 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 that 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 ascertained 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 × g for 15 min to obtain blood plasma. It was then stored at −80°C and was transferred to SRL (Tokyo) for quantitative measurements of thromboxane B_2 (TXB2) 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^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 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 ± 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 ± 6 mmHg at the baseline to 129 ± 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 ± 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 ± 6 mmHg and 110 ± 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.

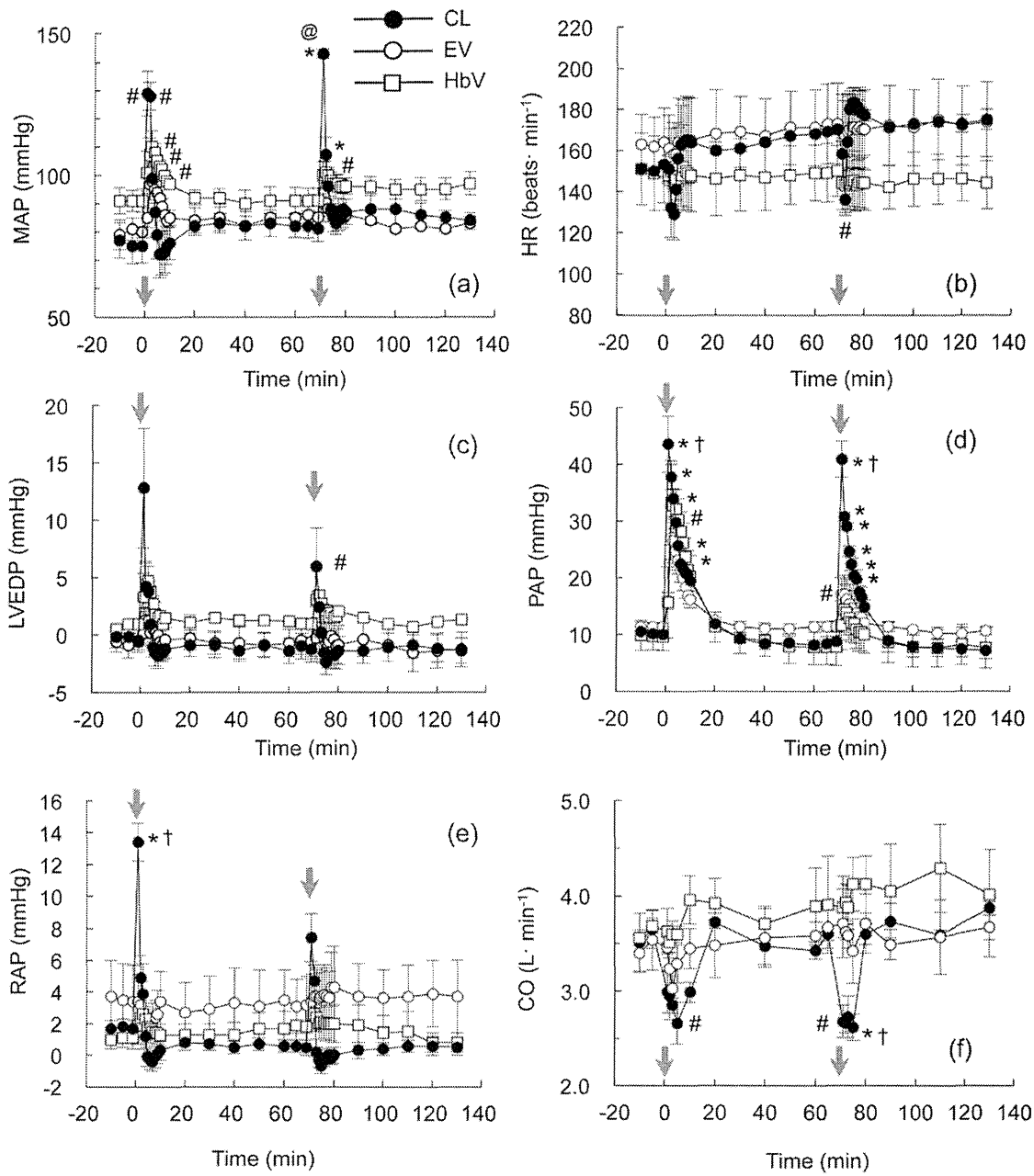

FIGURE 1. Systemic cardiovascular responses to the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg): closed circles, CL; open circles, EV, open squares, HbV; (a) mean arterial pressure, MAP; (b) heart rate, HR; (c) left ventricular end-diastolic pressure, LVEDP; (d) 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 ± 5.2 mmHg at 1 min after the first injection, and it returned to the original level in 4 min. It repeatedly increased to 5.9 ± 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 ± 1 mmHg at the baseline to 44 ± 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 ± 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 ± 4 and 17 ± 3 mmHg at 3 min after the first and the second injections. PAP of the HbV group, respectively, increased to 33 ± 4 and 14 ± 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 ± 0.5 mmHg at the baseline to 13.4 ± 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 ± 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 3.5–3.7 $L \cdot min^{-1}$ on average. It started to decrease 1 min after the first injection and showed the minimum value of 2.66 ± 0.22 $L \cdot min^{-1}$ 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 ± 0.14 $L \cdot min^{-1}$ 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 $dyne \cdot s \cdot cm^{-5}$ on the average at the baseline to 1197 ± 208 $dyne \cdot s \cdot cm^{-5}$ 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 ± 109 $dyne \cdot s \cdot cm^{-5}$ 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 ± 74 and 726 ± 53 $dyne \cdot s \cdot cm^{-5}$ 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 $dyne \cdot s \cdot cm^{-5}$ on average, at the baseline to 3378 ± 262 $dyne \cdot s \cdot cm^{-5}$ 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 ± 309 $dyne \cdot s \cdot cm^{-5}$ 1 min after the second injection ($p < 0.001$); it returned to the original level in 10 min. Both EV and HbV groups showed smaller increases in SVR after the first injection, with no increase after the second injection.

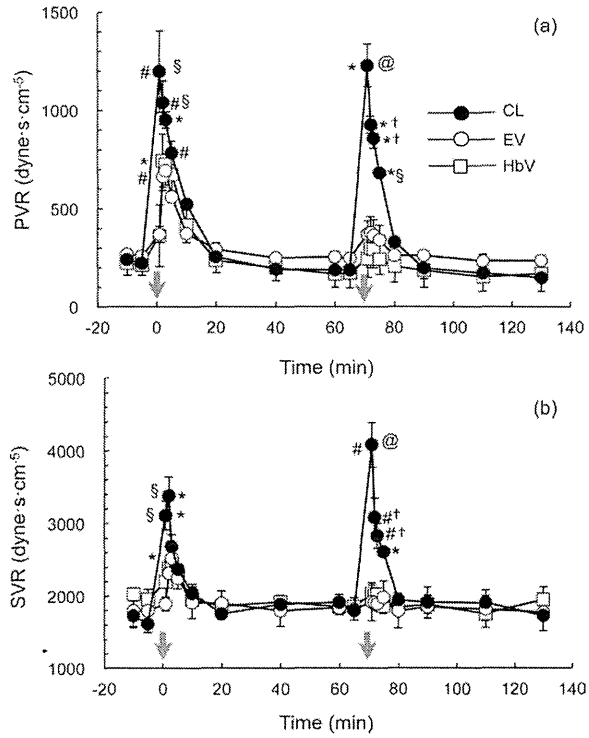

FIGURE 2. Changes in (a) pulmonary vascular resistance, PVR, and (b) systemic vascular resistance, SVR, at the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV; open squares, HbV. The CL group showed stronger and repeated changes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

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_2

The number of PLTs of the CL group decreased significantly from 44.7 ± 9.3 × 10^4 μL^{-1} at a baseline to 13.5 ± 4.9 × 10^4 μL^{-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 ± 3.0 × 10^4 μL^{-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 ± 18 × 10^2 μL^{-1} at the baseline to 62 ± 13 × 10^2 μL^{-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 ± 10 × 10^2 μL^{-1} 1 min after the second injection ($p < 0.05$). It returned to

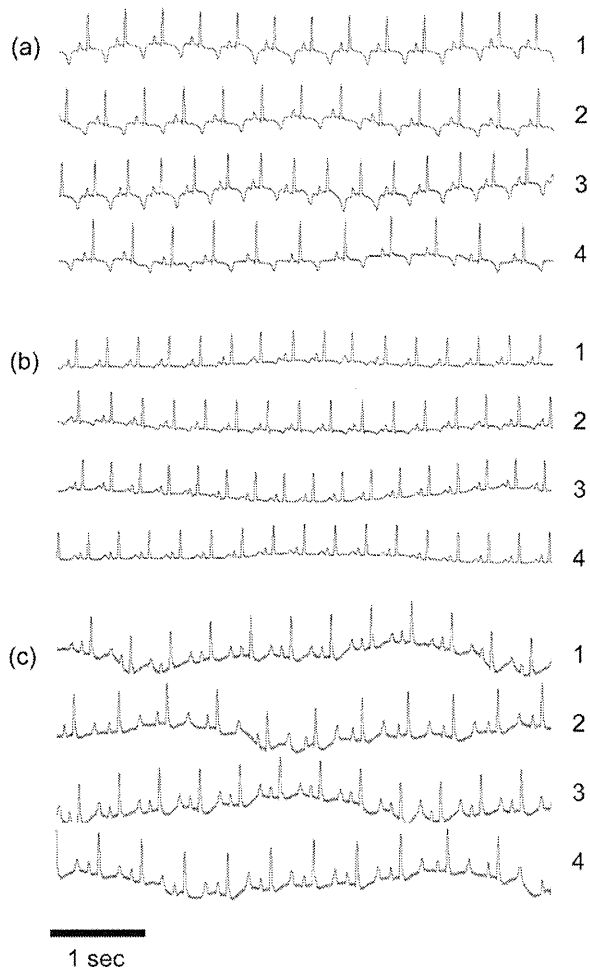

FIGURE 3. Electrocardiographs obtained before and immediately after injection of the first and second injections of liposomes: (a) CL group, (b) EV group, and (c) HbV group. 1, before the first injection; 2, after the first injection; 3, before the second injection; 4, after the second injection.

126 ± 23 × 10^2 μL^{-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 ± 12.2 $pg{\cdot}mL^{-1}$ at the baseline to 20,000 ± 4500 $pg{\cdot}mL^{-1}$ at 1 min after injection of CL ($p < 0.001$) (Fig. 5). It decreased considerably to 617 ± 337 $pg{\cdot}mL^{-1}$ at 60 min after injection. After the second injection of CL, the TXB2 level repeatedly increased significantly to 9830 ± 1530 $pg{\cdot}mL^{-1}$. However, both the EV and HbV groups showed significantly smaller increases. The EV group showed small increases of TXB2 to 260 ± 109 $pg{\cdot}mL^{-1}$ at 1 min after the first injection, and 83 ± 7 $pg{\cdot}mL^{-1}$ after the second injection. The HbV groups showed small increases to 567 ± 96 $pg{\cdot}mL^{-1}$ at 1 min after the first injection, and 129 ± 25 $pg{\cdot}mL^{-1}$ at 1 min after the second injection.

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.

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.

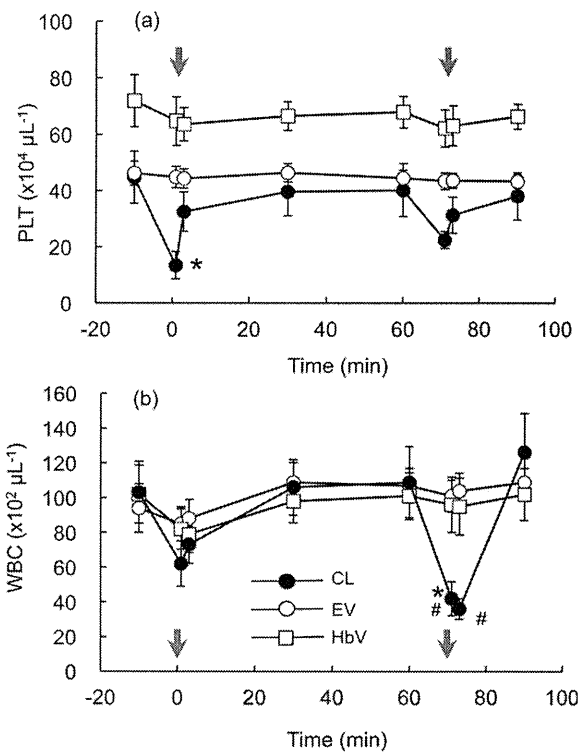

FIGURE 4. Changes in (a) platelet count, PLT and (b) white blood cell count, WBC, in response to the repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV, open squares, HbV. For PLT, one pig in the HbV group was omitted because of an 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.

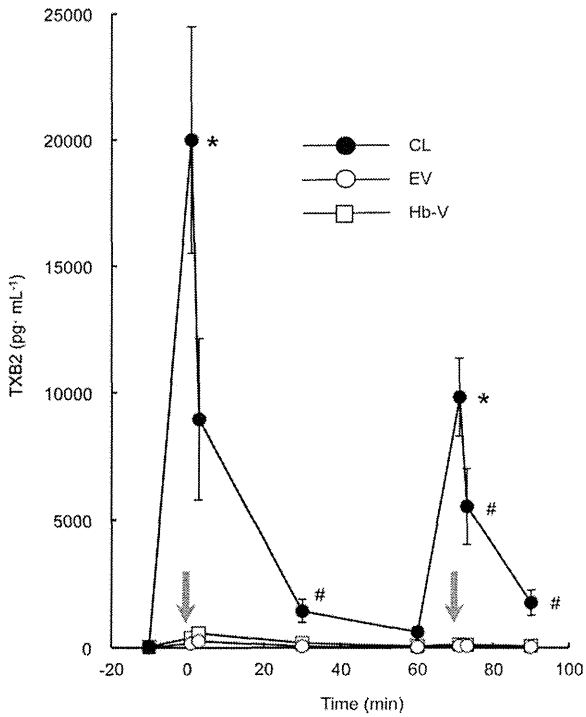

FIGURE 5. Changes in the plasma level of thromboxane B_2 (TXB2) in response to repeated injections of liposome suspensions (first, 0.05 mL/kg; second, 0.5 mL/kg); closed circles, CL; open circles, EV, open squares, HbV. Arrows indicate the time points of the first and the second injections (0, 70 min). * $p < 0.001$ 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.[6] 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.[17,25] Commonly used negatively charged lipid includes fatty acids and PG. However, rat experiments clarified that PG induces complement activation.[5,20,21,26–28] Pape et al.[29] 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[30] 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.[31] Actually, in the present experiment, we observed leukocytopenia and thrombocytopenia in the CL group only. Arachidonic acid cascade is activated and thromboxane A_2 (TXA2) is produced to induce strong systemic vasoconstriction. This scheme of responses is evident from the significant increases of plasma TXB2, a metabolite of TXA2, 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

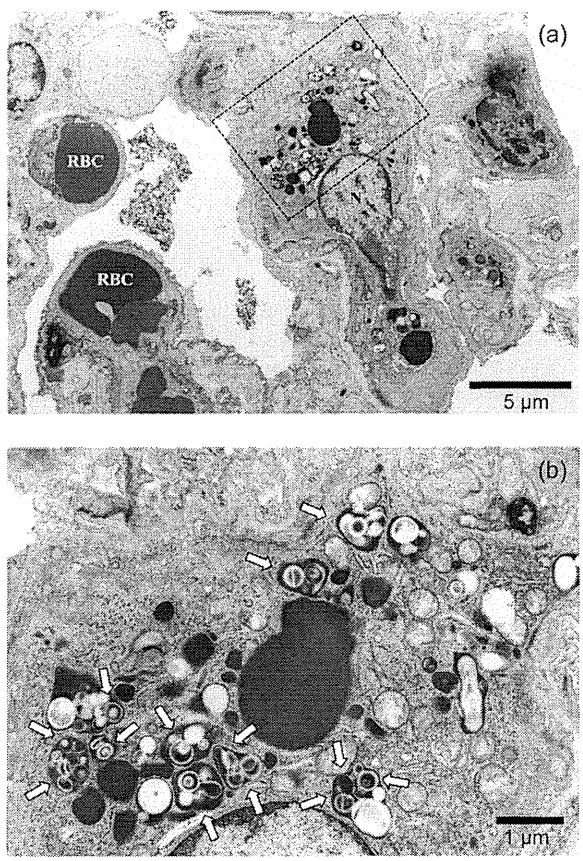

FIGURE 6. Transmittance electron micrographs of a lung tissue in the HbV group, which was sacrificed immediately after the experiment of two injections of HbV. (a) Whole view of a pulmonary intravascular macrophage, PIM, and the surrounding tissues. "N" denotes the nucleus of the PIM. RBCs are the red 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.[5] 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,[21] in rodents,[20] and pigs, as shown in this study, even though a previous report shows that dense PEGylation, like 5 mol % PEG_{2000}-DSPE, prevented complement activation.[32] 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.[22] 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.[23,33] 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.[34] 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.[35] Usually detection of liposomes is difficult and requires a maker 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.[35,36] Reportedly, injection of liposomes into sheep also induces pulmonary hypertension because of phagocytosis by PIMs and release of TXB2.[37–39] Even though Szebeni et al.[4,5] 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.[40] 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.[5] 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.[41,42] A report describes that liposomes without a negatively charged lipid reduce pulmonary hypertension,[39] 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.[23,33] A morphometric study of human lung tissues showed no macrophages or macrophage-like cells in the pulmonary capillaries.[43] 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.[23,24] It might be necessary to consider patients' hepatic condition before injection of liposomes.[44]

CONCLUSIONS

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