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# A Fourteen-Day Observation and Pharmacokinetic Evaluation after a Massive Intravenous Infusion of Hemoglobin-Vesicles (Artificial Oxygen Carriers) in Cynomolgus Monkeys

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## Abstract

Hemoglobin-vesicles (HbV) are a cellular type hemoglobin-based oxygen carrier in which a concentrated hemoglobin solution is encapsulated within a phospholipid vesicle (liposome). Although it was previously revealed that HbV possesses a higher biocompatibility, low toxicity and no accumulation in the body in an animal model, these assessments were limited to the use of rodents, including mice, rats and rabbits as models. The aim of this study was to observe the effects of the administration of HbV in a nonhuman primate. For this purpose, cynomolgus monkeys were used as the model and the systematic response, serum biochemical analysis and pharmacokinetic properties were monitored for 14 days after a massive intravenous injection of HbV at a putative dose (1400 mg Hb/kg, 17.5 mL/kg). All of the monkeys tolerated the massive amount of injected HbV and survived, and no abnormal behavior was observed. The systematic response and serum biochemical analysis were overall normal, except for a transient elevation in alanine aminotransferase levels. In addition, the levels of phospholipids, total cholesterol and total bilirubin, metabolites of hemoglobin and lipid components of HbV, were increased after HbV administration. In the pharmacokinetic study, HbV was retained for a sufficient period to permit it to function as an alternative to red blood cells and showed good metabolic properties without accumulation in the bloodstream. In conclusion, this is the first report of biological reactions and a pharmacokinetic evaluation for a 14 day period after a massive intravenous injection of HbV in a primate. The results obtained in this study provide useful information, not only for the development of further optimized HbV but also for designing relevant and rational protocols for clinical trials.

**Keywords:** Hemoglobin; Liposome; Artificial blood; Cynomolgus monkey

**Abbreviations:** RBC: Red Blood Cells; HBOCs: Hemoglobin-Based Oxygen Carriers; HbV: Hemoglobin-Vesicles; Hb: Hemoglobin; PEG: Polyethylene glycol; DPPC: 1,2-dipalmitoyl-*sn*-glycero-3-Phosphatidylcholine; DHSG: 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate; $PaO_2$: arterial blood oxygen tension; $PaCO_2$: arterial blood carbon dioxide tension; AST: Aspartate Aminotransferase; ALT: Alanine Aminotransferase; γ-GTP: γ-glutamyltransferase; ALP: Alkaline Phosphatase; BUN: Blood Urea Nitrogen; TG: Triglyceride; HDL-Cholesterol: High-Density Lipoprotein; MCV: Mean Corpuscular Volume; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MPS: Mononuclear Phagocyte System

## Introduction

There is now little doubt that the transfusion of red blood cells (RBC) is an indispensable procedure in the treatment of patients with massive hemorrhages. However, conventional RBC transfusions still have the potential for blood-type mismatching, infections by unrecognized pathogens, hepatitis, human immunodeficiency virus or West Nile virus etc. In addition, it is difficult to maintain a steady supply of RBC at a time of a disaster during military conflicts and problems associated with its short 2-3 week preservation period. To overcome these problems, various artificial oxygen carriers, such as perfluorocarbon-based oxygen carriers, synthetic $Fe^{2+}$ porphyrin-based materials, acellular type hemoglobin-based oxygen carriers (HBOCs) and cellular type HBOCs, have been under development worldwide [1-4]. Pre-clinical and clinical trials dealing with the above systems indicate that perfluorocarbon-based oxygen carriers can induce chronic pneumonitis due to their insufficient excretion from the body and their accumulation in the lung [5], and some acellular type HBOCs lead to the development of myocardial lesions and an increase in mortality rates in humans [6]. Therefore, perfluorocarbon-based oxygen carriers and acellular type HBOCs can be excluded as possible candidates for artificial oxygen carriers even though they proceeded to the stage of clinical trials.

Hemoglobin-vesicles (HbV) are a type of cellular type HBOCs in which a concentrated human hemoglobin (Hb) solution is encapsulated in a liposome, the surface of which is covered with polyethylene glycol (PEG) [7]. The cellular structure of HbV most closely mimics the characteristics of a natural RBC such as the cell membrane function, which physically prevents Hb from coming into direct contact with the components of the blood and vasculature during its circulation. In addition, HbV has been shown to possess several superior characteristics

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to RBC transfusions including the absence of viral contamination [8], a long-term storage period of over 2 years at room temperature [9] and no need of cross-matching etc. Furthermore, the transport of oxygen by HbV is equivalent to RBC, after adjusting for the amount of allosteric effector [10], and HbV and RBC have comparable pharmacological effects in hemorrhagic shock animal models [11-13]. Based on these facts, HbV appears to have potential for use as an alternative to RBCs, and has considerable promise for use in clinical settings.

Since the dosage of HbV for use as a substitute for RBC is significantly greater than that for other commercial drugs, comparatively massive amounts of HbV and its associated components, including Hb and lipids, are introduced into the body. Because of the fact that massive amounts of HbV are used, Hb and lipids derived by HbV during metabolism and disposition could result in the accumulation of such components in blood or organs, and might cause a variety of adverse effects, such as hypertension, renal disease, arterial sclerosis and hyperlipidemia [14,15]. Therefore, it becomes necessary to develop an in-depth understanding of the biological reactions associated with a massive HbV administration at the preclinical trial stage. To date, several preclinical trials have evaluated the histology, biochemical analysis and pharmacokinetic properties after the administration of a putative dose of HbV. The results show that HbV possesses a low toxicity and is promptly metabolized (no accumulation in the body) even after a massive infusion [16-21]. Although pre-clinical studies (toxicity, pharmacology and pharmacokinetic etc.) have been carried out in multiple species including rodents, domestic animals and nonhuman primates, all of the aforementioned *in vivo* studies of HbV were limited to the use of rodents, such as mice, rats and rabbits. In considering nonhuman primates as animal models, rhesus monkeys and cynomolgus monkeys are particularly good models for observing the biological reactions of drugs at the preclinical trial stage. In addition, the results of such studies can be helpful in designing the most relevant and rational protocols for clinical studies. Therefore, before HbV proceeds to the stage of clinical trials, biological reactions after the administration of a massive amount of HbV need to be done using nonhuman primates.

Based on this background, we conducted the further evaluations of systemic response, serum biochemical analysis and pharmacokinetic properties for 14 days after massive intravenous injection of HbV at a putative dose in cynomolgus monkeys.

## Materials and Methods

All studies were conducted at the Shin Nippon Biomedical Laboratories (Kagoshima, Japan). The study protocol was approved by the Shin Nippon Biomedical Laboratories Animal Care and Use Committee (IACUC707-004). All experiments were performed according to the guidelines, principles, and procedures for the care and use of laboratory animals of Shin Nippon Biomedical Laboratories.

### Preparation of HbV particles and HbV test solution

HbV particles were prepared under sterile conditions, as previously reported [22]. Briefly, an Hb solution was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dl) contained 14.7 mM of Pyridoxal 5'-phosphate as an allosteric effector to regulate oxygen affinity ($P_{50}$). The lipid bilayer was a mixture of 1, 2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine (DPPC), cholesterol, and 1, 5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate (DHSG) at a molar ratio of 5/5/1, and 1, 2-distearoyl-*sn*-glycero-3-phosphatidyl-ethanolamine-*N*-PEG (0.3 mol%). The HbV particles were suspended in a physiological salt solution, filter-sterilized (Dismic, Toyo-Roshi, Tokyo, Japan; pore size, 450 nm), and nitrogen gas was bubbled through the solution for storage. The properties of the HbV solution used in this study (Lot #: 1010) are shown in Table 1. Before the experiments, the HbV solution was mixed with a 25% recombinant human serum albumin solution (Nipro Corp., Osaka, Japan) to adjust the albumin concentration of the vesicle-suspension medium to 5 g/dL. Under these conditions, the colloid osmotic pressure of the suspension is maintained constant at approximately 20 mm Hg [22]. This solution was used for all experiments as the HbV test solution.

### Animal and HbV test solution injection

Four cynomolgus male monkeys (5.57-5.93 kg) were treated with HbV test solution. From at least 10 days before the HbV administration, all animals were acclimatized to experimental conditions, such as fixture to a positioner, administration, blood sampling, and were maintained in a temperature-controlled room with a 12-hrs dark/light cycle with *ad libitum* access to water. The food intake was restricted to approximately 108 g per day with treats twice per week.

On day 0, the monkeys without anesthesia were fixed to the positioner. Subsequently, intravenous cannula were introduced into the cephalic vein of the forearm for infusion, and each animal received a single intravenous infusion of the HbV test solution, administered as a transfusion of 17.5 mL/kg (1400 mg Hb/kg), representing approximately 20% of total blood volume, at a rate of 1 mL/min as well as previous studies [4,19,21].

### Measurement of blood pressure

Under unanesthetized conditions, monkeys were fitted with a blood pressure cuff on the upper arm, and both systolic and diastolic blood pressure were measured using a non-invasive blood pressure monitor (BP-8800NC, Omron Colin, Tokyo, Japan) at 7 days before administration (baseline). And 1,6 hour, 1, 3, 7, 14 days after HbV test solution administration.

### Blood sampling and measurement of blood gas, hematology and serum chemistry

At 7 days before administration (baseline), 1, 3, 7 and 14 day after the HbV test solution administration, arterial blood samples were collected from the femoral artery. Immediately after withdrawal, the arterial blood oxygen tension ($PaO_2$), arterial blood carbon dioxide tension ($PaCO_2$), the pH of all arterial blood samples were measured using a blood gas analyzer (i-STAT 300F; Abbott Point of Care Inc., Princeton, NJ). The venous blood samples were collected from the femoral vein at 7 days before administration (baseline), 7 and 14 day after the HbV test solution administration for the evaluation of serum chemistry. The venous blood was centrifuged (1710 g, 10 min) to obtain serum. The serum samples were then ultracentrifuged to remove HbV (50000 g, 30 min), because HbV interferes with some of the laboratory tests [23]. All serum samples were analyzed by a JCA-BM6070 or JCA-BM6050 (JEOL Ltd., Tokyo, Japan) instrument. The analyses performed were total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ -glutamyltransferase (γ -GTP), alkaline

| Solution | Hemoglobin (g/dL) | Lipids (g/dL) | $P_{50}$ (mm Hg) | Diameter (nm) | Methemoglobin (%) |
|---|---|---|---|---|---|
| HbV | 10.1 | 9.8 | 20.2 | 252.8 | 7.4 |

$P_{50}$, arterial blood oxygen tension at which hemoglobin is half-saturated with oxygen

**Table 1:** Solution properties of Hemoglobin-vesicles (HbV).

phosphatase (ALP), blood urea nitrogen (BUN), creatinine, uremic acid, lipase, amylase, triglyceride (TG), phospholipids, total cholesterol, high-density lipoprotein (HDL)-cholesterol, total bilirubin, sodium, potassium, calcium, chloride, glucose and inorganic phosphate.

### Pharmacokinetic study

Under unanesthetized conditions, blood samples were collected from the femoral vein at multiple time points after administration of the HbV test solution (7 days before administration (baseline), immediately after finishing the administration (~1 min), 10 min, 30 min, 1, 3, 6, 12 hrs, 1, 2, 3, 7 and 14 days after administration) and the plasma was separated by centrifugation (1710 g, 15 min). The concentration of HbV in the plasma was evaluated by measuring the concentration of Hb in plasma, because HbV remains in the plasma phase after centrifugation. The Hb concentration of HbV in plasma was determined by a cyanomethemoglobin method (hemoglobin B test kit Wako: Wako Chemicals, Saitama, Japan).

### Data analysis

Friedman's test was used for comparison of baseline and subsequent values. Data are means ± SD for the indicated number of animals. A probability value of $p<0.05$ was considered to indicate statistical significance.

## Results and Discussion

### Body weight and behavior

All monkeys survived up to 14 days after HbV administration and none were on the verge of death. Body weight before administration was 5773 ± 161 g, which increased slightly to 5893 ± 206 g at 14 days after HbV administration. These data indicate that the effect of HbV on physiological functions and the suppression of growth are negligible. Moreover, no abnormal behavior was observed, i.e., no changes in appearance of any reduction in appetite after HbV administration. It can therefore be concluded that HbV is not toxic to the cerebral nervous system.

### Arterial pressure

Table 2 shows average values for systolic blood pressure and diastolic blood pressure for the 14 day follow-up period for the animals. Similar to reported data for the previous study [24], no significant changes were observed in either systolic or diastolic blood pressure immediately after HbV administration and during the experiment. It has been reported that acellular type HBOCs such as polymerized Hb or intramolecular cross-linked Hb can induce hypertension due to scavenging of the endogenous vasorelaxation factors nitric oxide and carbon monoxide [25-27]. On the other hand, the similarity of the HbV structure to RBC prevents the Hb from contact with endothelial cells, which retard the reaction with endogenous nitric oxide and carbon monoxide [28,29]. Therefore, changes in systolic and diastolic blood pressure after HbV administration would not be expected.

### Blood gas parameters and electrolytes

Table 3 summarizes the blood gas parameters for the 14 day follow-up period. No abnormal values or significant differences between before and after HbV administration were observed. In addition, no changes in electrolytes were observed during the experiment except for inorganic phosphate (Table 4). Although inorganic phosphate levels were significantly different at 14 day after HbV administration as compared to before HbV administration, these changes were within the normal range. Previous reports also showed, in the case of the healthy rat, that HbV administration did not affect blood gas parameters and electrolytes even though they infused HbV (950 mg Hb/kg/day) for 14 consecutive days, which was equal to 2.5 times the actual blood volume [18]. These collective findings indicate that HbV administration has no effect on blood gas parameters and electrolytes in all mammalian species.

### Serum laboratory test

In a previous study, it was reported that an HbV suspension showed considerable interference effects in some analysis including colorimetric and turbidimetric analyses and chemical reactions that occur in some assays [21]. However, they can be easily removed from the plasma by ultracentrifugation, which substantially diminished these interfering components in clinical chemistry [23]. Therefore, no interference by HbV was detected in 22 serum laboratory tests, including electrolytes, as shown in Table 4, since it was removed from the serum by ultracentrifugation.

The parameters reflecting liver function (total protein, albumin AST, ALT, γ-GTP and ALP) are shown in Figure 1. Among these parameters, no changes in total proteins, AST, γ-GTP and ALP were found at 7 and 14 days after HbV administration. While ALT was slightly increased at 7 day after HbV administration, their values returned to original levels within 14 day after HbV injection. Sakai et al. (2004) previously reported, in a healthy rat model, that ALT increased slightly after an HbV infusion at a dose of 2000 mg Hb/kg [16]. The liver is one of the main organs of the metabolism of HbV, because HbV particles are ultimately captured by the mononuclear phagocyte system (MPS), such as Kupffer cells [17-19]. Therefore, an extra load on the liver during the metabolism of the massive amounts of HbV might result in elevated ALT. In addition, albumin was also slightly changed at 14 days after HbV injection. However, it is possible that the effect of HbV administration on liver function was of no consequence, because these changes of ALT and albumin were transient and/or normal range and no changes in other parameters reflecting liver function were found (Figure 1).

| | Pre | after HbV administration | | | | | |
|---|---|---|---|---|---|---|---|
| | | (hour) | | (day) | | | |
| | | 1 | 6 | 1 | 3 | 7 | 14 |
| Systolic blood pressure (mmHg) | | | | | | | |
| Ave.<br>(range) | 118.5 ± 8.3<br>(110-130) | 124.5 ± 4.7<br>(120-129) | 123.3 ± 15.4<br>(103-139) | 109.3 ± 11.1<br>(99-125) | 110.5 ± 14.3<br>(94-129) | 104.3 ± 10.3<br>(93-114) | 109 ± 10.1<br>(101-122) |
| Diastolic blood pressure (mmHg) | | | | | | | |
| Ave.<br>(range) | 64.3±4.9<br>(58-70) | 66.8±5.7<br>(60-72) | 66.3±6.6<br>(57-71) | 56.3±9.1<br>(48-69) | 63.3±10.0<br>(53-77) | 56.5±5.1<br>(53-64) | 55.3±6.1<br>(50-64) |

Data are mean ± S.D. (n=4)

**Table 2:** Changes in mean systolic and diastolic blood pressure after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

| | pre | Day after HbV administration | | | |
|---|---|---|---|---|---|
| | | 1 | 3 | 7 | 14 |
| $PO_2$ (mmHg) | | | | | |
| Ave. (range) | 96.3±4.9 (90-101) | 93.3±2.2 (90-95) | 93.8±6.2 (87-100) | 98.5±6.0 (92-106) | 90.8±17.7 (66-107) |
| $PCO_2$ (mmHg) | | | | | |
| Ave. (range) | 29.7±2.4 (27.1-32.9) | 31.9±2.3 (29.4-34.9) | 31.0±2.1 (28.0-32.8) | 30.5±2.9 (26.3-32.9) | 28.5±1.8 (26.3-30.6) |
| pH | | | | | |
| Ave. (range) | 7.36±0.09 (7.26-7.46) | 7.44±0.07 (7.36-7.52) | 7.47±0.02 (7.44-7.49) | 7.44±0.02 (7.41-7.45) | 7.38±0.08 (7.27-7.47) |

Data are mean ± S.D. (n=4)

**Table 3:** Changes in arterial blood oxygen tension ($PaO_2$), arterial blood carbon dioxide tension ($PaCO_2$) and pH after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

| | pre | Day after HbV administration | |
|---|---|---|---|
| | | 7 | 14 |
| Sodium (mEq/dL) | | | |
| Ave. (range) | 153.9±1.5 (152-155) | 146.9±2.1 (145-152) | 148.7±2.4 (144-150) |
| Pottasium (mEq/dL) | | | |
| Ave. (range) | 4.3±0.5 (3.6-4.7) | 3.9±0.2 (4.1-5.1) | 4.4±0.4 (3.7-4.4) |
| Calcium (mEq/dL) | | | |
| Ave. (range) | 10.3±1.5 (8.3-12.0) | 10.8±1.7 (8.3-11.3) | 9.9±1.4 (9.4-12.9) |
| Chloride (mEq/dL) | | | |
| Ave. (range) | 112.2±2.0 (111-115) | 108.8±1.8 (107-115) | 112.4±3.6 (106-111) |
| Glucose (mg/dL) | | | |
| Ave. (range) | 91.8±24.4 (72-122) | 91.5±10.2 (70-127) | 91.0±24.9 (80-102) |
| Inorganic phosphate (mg/dL) | | | |
| Ave. (range) | 5.2±0.9 (4.0-5.9) | 5.7±0.7 (5.0-6.6) | 6.0±0.8** (4.7-6.4) |

** p<0.01 vs. pre-treatment. Data are mean ± S.D. (n=4)

**Table 4:** Summary of electrolytes after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in male cynomolgus monkeys.

It is well-known that the Hb derived from hemolysis causes renal toxicity by the dissociation of tetramic Hb subunits into two dimers, extravasaion, and precipitation in tubules [15]. In the present study, BUN, creatinine and uremic acid levels, which reflect renal function, were slightly changed during the 14 days after the HbV injection, but these changes were still within the normal ranges (Figure 2). This could be due to the characteristics of HbV in which its structure is maintained intact in the circulation, while Hb derived from HbV was completely degraded by MPS. In fact, several results observed in this study (as well as in previous reports) support this conclusion: (i) there was no evidence of the presence of Hb in the supernatant after ultracentrifugation and hemoglobinuria in this study (data not shown), (ii) HbV is circulated in the form of stable HbV in the blood circulation until metabolized by MPS [19].

Amylase and lipase activity was measured to analyze pancreatic function (Figure 2). Amylase levels were essentially unchanged. On the other hand, lipase levels were temporally increased seven days after HbV, but returned to the basal level within 14 days. The same tendency was observed in a previous report using healthy rats [16]. This elevated lipase activity could be due to the damage to the pancreas by HbV. However, when the pancreas is damaged (e.g. acute necrotizing pancreatitis), lipase activity typically becomes dramatically elevated from 10 to 475-540 IU/L [30]. Thus, the small elevation in lipase levels observed here can likely be attributed to the induction of pancreatic enzymes by the presence of a large amount of lipids associated with HbV. This speculation was also supported by our present results that only lipase activity increased followed by elevation of TG and phospholipid shown in Figure 4, but amylase did not.

The concentration of the TG and HDL-cholesterol were increased slightly, and the levels of phospholipids and total cholesterol, metabolites of the lipid components of HbV, were significantly increased after HbV administration (Figure 3). In addition, total bilirubin, which is related to the metabolic routes of Hb, were temporarily increased at 7 days after HbV administration, but returned to basal levels within 14 days after the HbV administration (Figure 3). They are likely derived from the HbV particles because they contain a large amount of cholesterol (1200 mg/dL), DPPC (1840 mg/dL) and Hb (10000 mg/dL). During the metabolism of Hb derived from RBC, bilirubin is released and excreted in the bile. In this study, total bilirubin increased at 7 day after HbV administration, indicating that Hb derived from HbV is metabolized and excreted via the normal pathway. On the other hand, cholesterol of the vesicles should reappear in the blood mainly in the form of a lipoprotein-cholesterol complex after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by hepatocytes [31]. Furthermore, it was reported that phospholipids in liposomes are metabolized in MPS and reused as cell membranes or are excreted into the bile [32,33]. In a study using healthy mice and rats, we demonstrated that the outer lipid components, especially cholesterol, were mainly eliminated in the feces *via* biliary excretion after metabolization by MPS [19]. Therefore, it would be desirable to understand whether the lipid components in HbV behaved the same as endogeneous lipid after the metabolization of HbV in MPS.

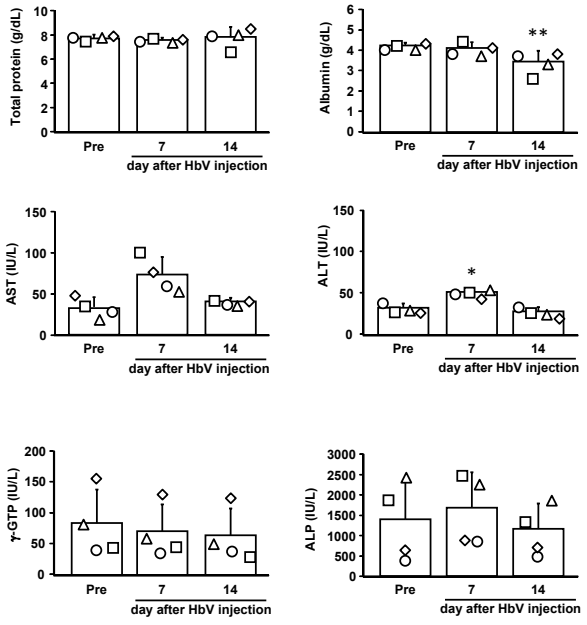


**Figure 1: Serum laboratory tests representing liver function after the administration of hemoglobin-vesicles at a dose of 1400 mg Hb/kg in cynomolgus monkeys.**
Data are mean ± SD. (n=4) * p<0.05, ** p<0.01 vs. pre-treatment.
The individual values are representing as following symbols (○; No.1, □; No.2, Δ; No.3, ◊; No.4).
AST; aspartate aminotransferase, ALT; alanine aminotransferase, γ--GTP; γ-glutamyltransferase, ALP; alkaline phosphatase.

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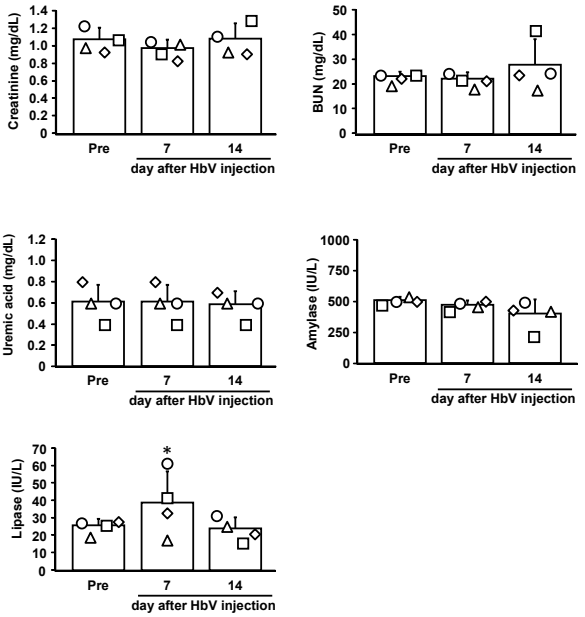


**Figure 2: Serum laboratory tests representing renal and pancreatic function after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.**
Data are mean ± SD. (n=4) * p<0.05 vs. pre-treatment.
The individual values are representing as following symbols (○; No.1, □; No.2, △; No.3, ◊; No.4).
BUN; blood urea nitrogen.

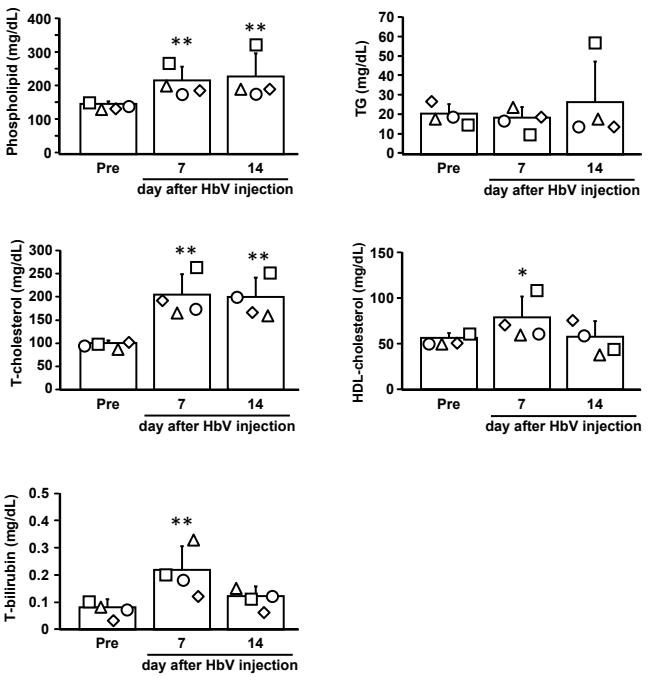


**Figure 3: Serum laboratory tests representing the metabolism of lipid and hemoglobin after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.**
Data are mean ± SD. (n=4) * p<0.05, ** p<0.01 vs. pre-treatment.
The individual values are representing as following symbols (○; No.1, □; No.2, △; No.3, ◊; No.4).
TG; triglyceride, T-cholesterol; total cholesterol, HDL-cholesterol; high-density lipoprotein cholesterol, T-bilirubin; total bilirubin.

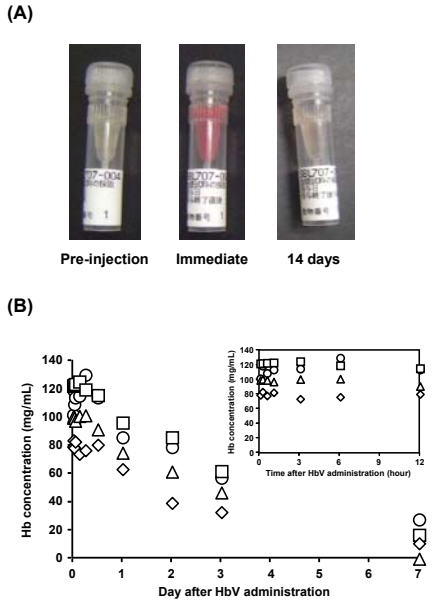


**Figure 4: (A) The appearance of plasma before hemoglobin-vesicle administration (left), immediately after finishing administration (middle) and 14 days after hemoglobin-vesicle administration (right). (B) Time course for plasma concentration of hemoglobin represents hemoglobin-vesicle concentration in plasma after hemoglobin-vesicle administration at a dose of 1400 mg Hb/kg in cynomolgus monkeys.**
The individual values are representing as following symbols (○; No.1, □; No.2, △; No.3, ◊; No.4).

## Pharmacokinetics

Since HbV is known to be dispersed in the plasma, we measured the Hb concentration in plasma using a cyanomethemoglobin method to examine the retention of HbV in the circulation in cynomolgus monkeys. Figure 4B shows the time course for the concentration of Hb in plasma after the administration of HbV at a dose of 1400 mg Hb/kg in cynomolgus monkeys. Although the plasma concentration of Hb varies widely among individuals, HbV is sufficiently retained in the blood in the monkey (Half-life; 47-72 hour). On the other hand, no Hb was detected in plasma when analyzed by the cyanomethemoglobin method (data not shown) and its appearance (Figure 4A) at 14 days after HbV administration was the same as that before HbV administration. Therefore, HbV appears to be completely cleared from plasma within 14 days after HbV administration. Interestingly, in the present study using nonhuman primates, a saturation phenomenon for HbV elimination was observed for the first 12 hour after HbV administration (Figure 4B), and the plasma HbV concentration started to decrease following a 1-compartment model at 12 hour after HbV administration. HbV in the bloodstream is finally captured and metabolized by phagocytes in the MPS, especially Kupffer cells in the liver [17,19]. Therefore, the saturation phenomenon observed in this study appears to be due to the saturation of Kupffer cells. These data indicate that HbV possesses both good retention in the blood and metabolic properties even in a nonhuman primate.

## Conclusions

In addition to functioning as a substitute for RBCs, HbV would be expected to have a variety of other applications, based on its oxygen transport characteristics, such as in cardiopulmonary bypass priming solutions [34], wound healing in critically ischemic skin [35], acute

ischemic strokes [36] and as a radiation therapy agent [37]. Therefore, HbV has considerable promise for use in the clinic in the future. This study is the first report about the observation of biological reaction and pharmacokinetic evaluation after massive intravenous injection of HbV at a putative dose in a nonhuman primate. As a result of this study, no severe adverse effects were observed in terms of systemic response and in serum biochemical analyses. In addition, pharmacokinetic evaluation showed that HbV is retained in the blood long enough to function as an RBC substitute in nonhuman primates as well as rodents [19,38-40]. The results obtained in present study not only clarify the effect of HbV on physiological responses in primates but also provide helpful information related to designing the most relevant and rational protocol for a clinical study.

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