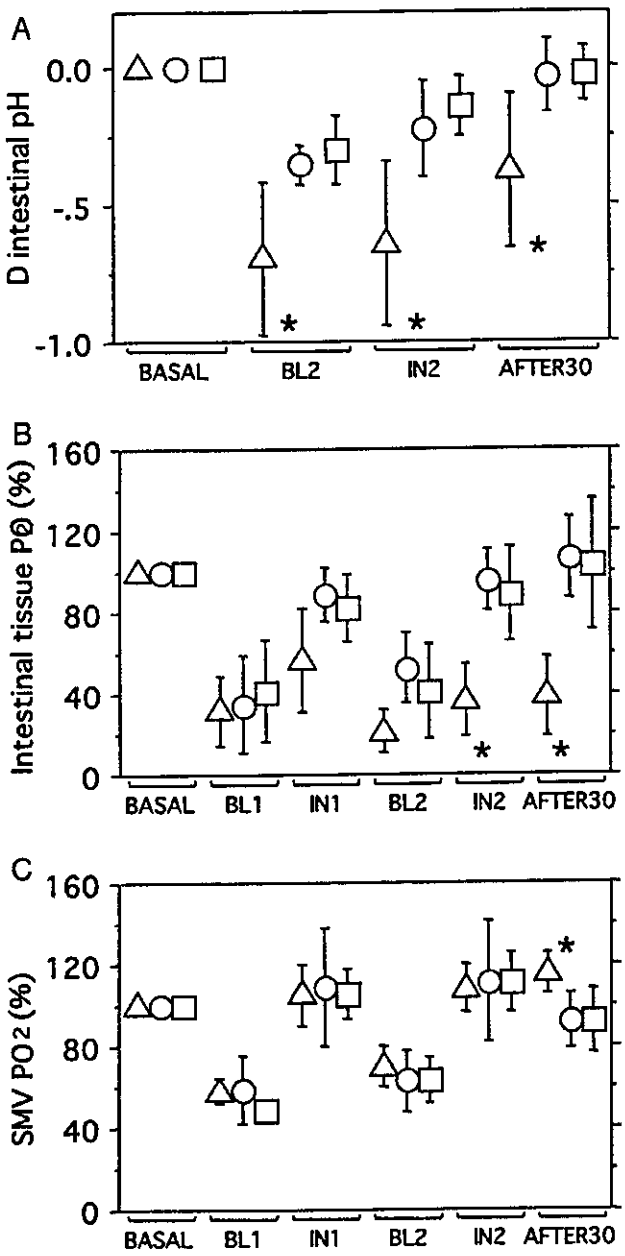

Figure 5. Parameters representing intestinal perfusion are shown as percentage changes or difference from basal values in rabbits. Time points: before bleeding (BASAL), after first bleeding (BL1), after first infusion (IN1), after second bleeding (BL2), after second infusion (IN2), and at 30 min after the second infusion (AFTER30). Triangle, alb group; circle, HbValb group; square, RBCalb group. *$p < 0.05$ *vs.* HbValb and RBCalb groups.

HbValb group, close to the RBCalb group. These data collectively indicate the proficient oxygen transporting capability of HbV and its potential efficacy in shock resuscitation. One of the powerful advantages of HbV is that its properties, such as oxygen binding and release, viscosity, and colloid osmotic pressure, can be manipulated by changing the amount of allosteric effector in HbV and the plasma expander in which to suspend HbV. We believe that currently ongoing optimization of these properties will further improve the efficacy of HbV in shock resuscitation.

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Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model

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Abstract

Phospholipid vesicles encapsulating Hb (Hb-vesicles: HbV) have been developed for use as artificial O_2 carriers (250 nm ϕ). As one of the safety evaluations, we analyzed the influence of HbV on the organ functions by laboratory tests of plasma on a total of 29 analytes. The HbV suspension ([Hb] = 10 g/dl) was intravenously infused into male Wistar rats (20 ml/kg; whole blood = 56 ml/kg). The blood was withdrawn at 8 h, and 1, 2, 3, and 7 days after infusion, and the plasma was ultracentrifuged to remove HbV in order to avoid its interference effect on the analytes. Enzyme concentrations, AST, ALT, ALP, and LAP showed significant, but minor changes, and did not show a sign of a deteriorative damage to the liver that was one of the main organs for the HbV entrapment and the succeeding metabolism. The amylase and lipase activities showed reversible changes, however, there was no morphological changes in pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Cholesterols, phospholipids, and β-lipoprotein transiently increased showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from the membrane components of HbV that are liberated from macrophages entrapping HbV. Together with the previous report of the prompt metabolism of HbV in the reticuloendothelial system by histopathological examination, it can be concluded that HbV infusion transiently modified the values of the analytes without any irreversible damage to the corresponding organs at the bolus infusion rate of 20 ml/kg.

Keywords: Biomimetic material; Blood; Drug delivery; In vivo test; Liposome; Nanoparticle

1. Introduction

Liposomes or phospholipid vesicles have been extensively studied for the application of drug delivery system, and some are now approved for a clinical use as antifungal or anticancer therapies [1]. Another promising application is to use vesicles for encapsulating a concentrated human Hb. The resulting Hb-vesicle (HbV) can serve as an O_2 carrier with ability comparable to red blood cells (RBC) [2–4]. The advantages of the Hb-based O_2 carriers (HBOCs) are the absence of blood-type antigens and transmission of known and unknown blood-borne disease, the possibility to improve the rheological properties of blood flow according to the needs of patients, and stability for long-term storage. These characteristics will make it possible to use the HBOCs both in elective and emergency situations [5,6]. In this sense, the infusion of HBOCs becomes superior to the conventional blood transfusion that still has the potential of mismatching, infection such as HIV and hepatitis virus, and the problems of only 2–3 week preservation period. The acellular Hb modifications including polymerized Hb and polymer-conjugated Hb are now undergoing the final stages of clinical trials [7,8]. However, the cellular structure of HbV (particle diameter, ca. 250 nm) most closely mimics the characteristics of natural RBC such as the cell membrane function of physically preventing direct contact of Hb

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with the components of blood and vasculature during circulation [9]. In comparison with some acellular Hb modifications, the Hb encapsulation in vesicles suppresses hypertension induced by vasoconstriction, a theory that is suggested to be due to the high affinity of Hb with nitric oxide and carbon monoxide as vasorelaxation factors [10,11]. Moreover, the surface modification of HbV with polyethylene glycol (PEG) chains not only prolongs the circulation half-life [12] but also prevents the intervesicular aggregation and guarantees the homogeneous dispersion in the plasma phase that provides a prompt blood flow in the microcirculation and the resulting sufficient tissue oxygenation [13,14].

According to the clinical conditions HbVs are supposed to be applied for, the organism is faced with the metabolism of a large amount of both Hb and lipids, because the dose rate of HbV is significantly large. The HbV particles, as well as phospholipid vesicles, infused in the blood stream are finally captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [4,15]. In a previous report, we clarified by the histopathological studies of rats receiving 20 ml/kg of HbV infusion that the HbV particles were captured and metabolized within 7 days in RES mainly in the spleen and liver [16]. Transmission electron microscopy provided a clear image of the HbV particles in the phagosomes 1 day after infusion, but they disappeared within 7 days. Staining with the anti-human Hb antibody, Berlin blue, and hematoxylin/eosin showed prompt metabolism of Hb molecules with no morphological changes in the liver and spleen. The phagocytic activity decreased and then transiently increased, but tended to return to the original level. From these studies, we did not see any irreversible damage to the organs.

Serum laboratory tests are the most common diagnostic tools to monitor organ functions clinically. However, both the PEG-modified HbV particles and the chemically modified Hb solutions remained in the plasma even after usual centrifugation to remove RBC, showing significant interference effects due to the light absorption by Hb and light scattering by the particles. These interference effects hindered the accurate evaluation of plasma laboratory tests and have been regarded as a serious issue for the development of HBOCs [17,18]. However, quite recently we have clarified by an in vitro experiment that the simple removal of PEG-modified HbV as a precipitate by ultracentrifugation (50,000 *g*, 20 min) or by conventional centrifugation in the presence of a high-molecular-weight dextran diminished most of the interference effects [19]. Using this simple procedure, we aimed to evaluate the safety of HbV by the laboratory tests of plasma after bolus intravenous infusion of HbV at a rate of 20 ml/kg, the same experimental model as in the previous study [16].

2. Materials and methods

2.1. Preparation of PEG-modified HbV

The PEG-modified HbV was prepared in a sterile condition as previously reported in the literature [10, 20–22]. Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Society of Red Cross, Japan (Tokyo, Japan). The encapsulated purified Hb (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP, Sigma) as an allosteric effector at a molar ratio of PLP/Hb = 2.5. The lipid bilayer was composed of a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, and 1,5-bis-*O*-hexadecyl-*N*-succinyl-L-glutamate at a molar ratio of 5/5/1 (Nippon Fine Chem. Co., Osaka, Japan), and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-poly(ethylene glycol) (NOF Co., Tokyo, Japan, 0.3 mol% of the total lipid). The HbCO solution and the lipid powder were mixed and stirred for 12 h at 4°C. The resulting multilamellar vesicles were extruded through membrane filters with a final filter pore size of 0.22 μm. Thus prepared PEG-modified HbV was suspended in saline at the Hb concentration of 10 g/dl, and filtrated (pore size: 0.45 μm). The physicochemical parameters of the HbV are as follows: particle diameter, 251 ± 80 nm; [Hb], 10 g/dl; [metHb], <3%; [HbCO], <2%; phospholipids, 4.0 g/dl; cholesterol, 1.7 g/dl; and oxygen affinity (P_{50}), 30 Torr. The endotoxin content was precisely measured by modified *Limulus* Amebocyte lysate gel-clotting analysis that has been developed by our group recently, and confirmed that the endotoxin content was less than 0.1 endotoxin unit/ml [23].

2.2. HbV infusion and procedure for the plasma laboratory tests

All animal studies were approved by the Animal Subject Committee of Keio University School of Medicine and performed according to NIH guidelines for the care and use of laboratory animals (NIH publication #85-23 Rev. 1985). The experiments were carried out using 40 male Wistar rats (200–210 g, Saitama Experimental Animals, Kawagoe, Japan). They were anesthetized with diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg ($n = 5$ for every time point). Ten rats were used to obtain the control values. All the rats were housed in cages and provided with food and water *ad libitum* in a temperature controlled room on a 12 h dark/light cycle.

After 8 h, and 1, 2, 3, and 7 days, the rats were anesthetized with 1.5% sevoflurene inhalation (Maruishi Pharm. Co., Osaka, Japan) using a vaporizer (Model

TK-4 Biomachinery, Kimura Med., Tokyo). Polyethylene tubes (PE-50, Natsume Co., Tokyo) were implanted in the carotid artery for withdrawing blood into heparinized syringes for the Hct, HbV concentration, and plasma laboratory tests. The animals were finally laparotomized and sacrificed with acute bleeding from the abdominal aorta and the liver and spleen were obtained for weight measurements. The control rats received the same procedure for the measurements.

A part of the withdrawn blood (6 ml) was centrifuged to obtain plasma which was turbid and red/brown colored due to the presence of PEG-modified HbV particles especially in the samples taken at 8 h, 1 and 2 days after infusion. The plasma was ultracentrifuged (50,000 g, 20 min) to remove the HbV particles. The obtained transparent plasma specimens were stored at −80°C until the laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ -glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), cholinesterase (ChE), leucin amino peptidase (LAP), creatine phosphokinase (CPK), amylase, lipase, total cholesterol (Total-Chol.), cholesteryl ester (Chol.Ester), free cholesterol (Free-Chol.), HDL-cholesterol (HDL-Chol.), β-lipoprotein, triglyceride (TG), free fatty acid (FFA), phospholipids, total lipids, uric acid (UA), blood urea nitrogen (BUN), creatinine (CRE), K^+, Ca^{2+}, inorganic phosphate (IP), and Fe^{3+}. In our previous study, it was confirmed that the concentrations of the plasma components in terms of the above analytes did not change after the ultracentrifugation at 50,000 g for 20 min [19]. Since rat albumin is slightly insensitive to the bromcresol green method, the values were corrected according to Takano et al. [24].

2.3. Histopathological examination of pancreas

After sacrificing the animals by acute bleeding from the abdominal aorta, the pancreas was resected for a histopathological study. The organs were fixed in a 10% formalin neutral buffer solution (Wako Chem. Co., Tokyo) immediately after the resection, and the paraffin sections were stained with hematoxylin/eosin.

2.4. Data analysis

Differences between the control and a treatment group were analyzed using a one-way ANOVA followed by Fisher's protected least-significant difference (PLSD) test. The changes were considered statistically significant if $p<0.05$.

3. Results

All the rats receiving the bolus infusion of HbV at a dose rate of 20 ml/kg tolerated the infusion and survived until intentional sacrifice. There was no noticeable change in appearance such as piloerection.

3.1. Hct and circulation persistence of HbV

The control Hct was 42±1%, and it decreased slightly to 40±1% at 1 day after HbV infusion. The estimated Hb concentration of HbV in plasma just after infusion was about 6 g/dl, and it gradually decreased to 4.4±0.3 g/dl at 8 h, 1.9±0.2 g/dl at 1 day, 1.3±0.1 g/dl at 2 days, and 0.8±0.01 g/dl at 3 days (Fig. 1). At 7 days, HbV was not detected at all in the plasma phase.

3.2. Spleen and liver weights

The changes in the spleen and liver weights were expressed as percents of the body weight (Fig. 1). The liver weight ratio (control, 4.81±0.17%) showed a significant increase 1 day after the infusion (5.29±0.27%, $p<0.01$), and then it returned to the original level at 2 days. Spleen weight ratio significantly increased from 0.32±0.05% to 0.66±0.06% 3 days after the infusion ($p<0.01$), however, it was reduced to 0.41±0.02% at 7 days.

3.3. Plasma laboratory tests

The plasma fraction after centrifugation of the blood sample for 3 days after the HbV infusion was turbid due to the presence of PEG-modified HbV. However, ultracentrifugation of the plasma produced transparent and light-yellow plasma phase and PEG-modified HbV was precipitated at the bottom in a tube. There was no sign of the presence of Hb in the supernatant, indicating that there was no hemolysis of both RBC and HbV.

As for the analytes that reflect the liver function, the total protein (control, 5.2±0.1 g/dl) and albumin (2.46±0.06 g/dl) slightly decreased to, e.g., 4.9±0.2 and 2.11±0.10 g/dl, respectively, with statistically significant differences ($p<0.01$) for 3 days after the HbV infusion (Fig. 2). They tended to return to its original level at 7 days ($p<0.05$). AST (control, 60±7 U/l) decreased to 46±3 U/l ($p<0.05$) and returned to the original level at 7 days. ALT (control, 32±5 U/l) only slightly increased to 40±8 U/l 1 day after the HbV infusion ($p<0.01$), but it returned to its original level 2 days after the infusion. LDH (control, 150±60 U/l) did not change significantly. ALP (control, 1265±231 U/l) decreased at 2 days (812±149 U/l) and 3 days (872±98 U/l) ($p<0.01$), but it returned to the control level at 7 days. γ-GPT (control, 1.6 U/l) and LAP (31±1 U/l) showed significant but minimal reductions

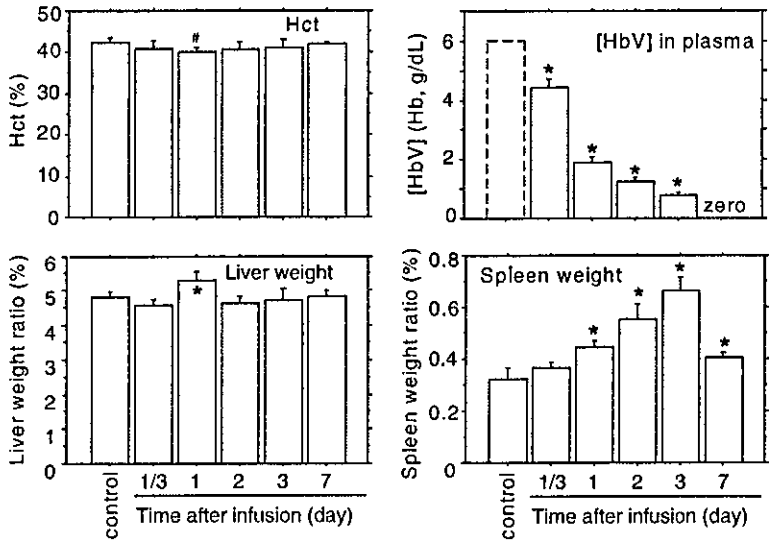

Fig. 1. Changes in hematocrit, concentration of HbV in plasma, and spleen and liver weights after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. The control value of [HbV] is the estimated concentration of HbV immediately after the infusion and expressed as with a dashed line. The spleen and liver weights are expressed as the ratio to the body weight (%).

($p<0.05$). ChE (control, 76 ± 18 U/l) did not show a noticeable change. Plasma total bilirubin (≤0.1 mg/dl) and Fe^{3+} showed some reductions but were maintained at a low level for 7 days in spite of the metabolism of a large amount of Hb.

CRE (control, 0.3 mg/dl) was maintained at a low level for 7 days. BUN (control, 16 ± 3 mg/dl) showed a slight increase at 7 days (21 ± 3 mg/dl) (Fig. 3). UA (control, 0.47 ± 0.19 mg/dl) increased to 0.70 ± 0.16 mg/dl at 3 days, however, it returned to a non significant level at 7 days. Amylase (control, 1613 ± 74 U/l) significantly decreased for 3 days after the infusion ($p<0.01$), but returned to its original level at 7 days. Lipase (control, 9 ± 1 U/l) showed significant increases ($p<0.01$) after the HbV infusion, and it tended to decrease after 3 days, and was reduced to a non-significant level at 7 days. CPK (control, 304 ± 116 U/l) decreased at 7 days ($p<0.05$), but did not show a noticeable increase during the experiment. As for the electrolyte concentrations, K^{+}, Ca^{2+}, and IP did not show any significant changes.

The most consistent changes were seen in the lipid components (Fig. 4). Total-Chol. (control, 73 ± 7 mg/dl), Free-Chol. (18 ± 2 mg/dl), Chol.Ester (59 ± 8 mg/dl), and HDL-Chol. (32 ± 4 mg/dl) showed significant increases and maximum values at 2 days ($p<0.01$). Free-Chol. increased to 39 ± 4 mg/dl, about twice the control value. However, it tended to decrease at 3 days, and returned to its control level at 7 days. β-Lipoprotein (control, 110 ± 42 mg/dl) slightly increased at 1 day (160 ± 33 mg/dl), but returned to its original level at 3 days. TG (control, 64.4 mg/dl) significantly decreased to 12.4 mg/dl at 2 days ($p<0.01$), but tended to increase to its original level at 7 days. Phospholipid (control, 132 ± 8 mg/dl) significantly increased to 150 ± 9 mg/dl at 1 day ($p<0.01$), and then returned to the original level at 3 days.

3.4. Histopathological examination of pancreas

The histology of pancreatic tissue 2 days after the infusion of HbV is shown in Fig. 5. There was no significant morphological change in spite of the increment of the pancreatic lipase activity.

4. Discussion

The clinical indications for the use of the HbV suspension as an artificial O_2 carrying fluid are estimated to be mainly preoperative or perioperative hemodilution, or resuscitation from hemorrhagic shock in emergency situations [25], both of which result in exchanging more than 20% of the original blood with the HbV suspension. Thus, the dose amount is extremely greater than that of stealth liposomes for drug delivery systems. HbV particles in the blood stream are finally captured by RES in the same manner as the conventional phospholipid vesicles [15]. In a previous study, we confirmed by the histopathological examination in a rat model that HbV particles were captured in the phagosomes of liver Kupffer cells and spleen macrophages without tissue damage, and they had completely disappeared within 7 days [16]. The transient splenomegaly and hepatomegaly in Fig. 1 seemed associated with the entrapment of HbV. The total weight change of

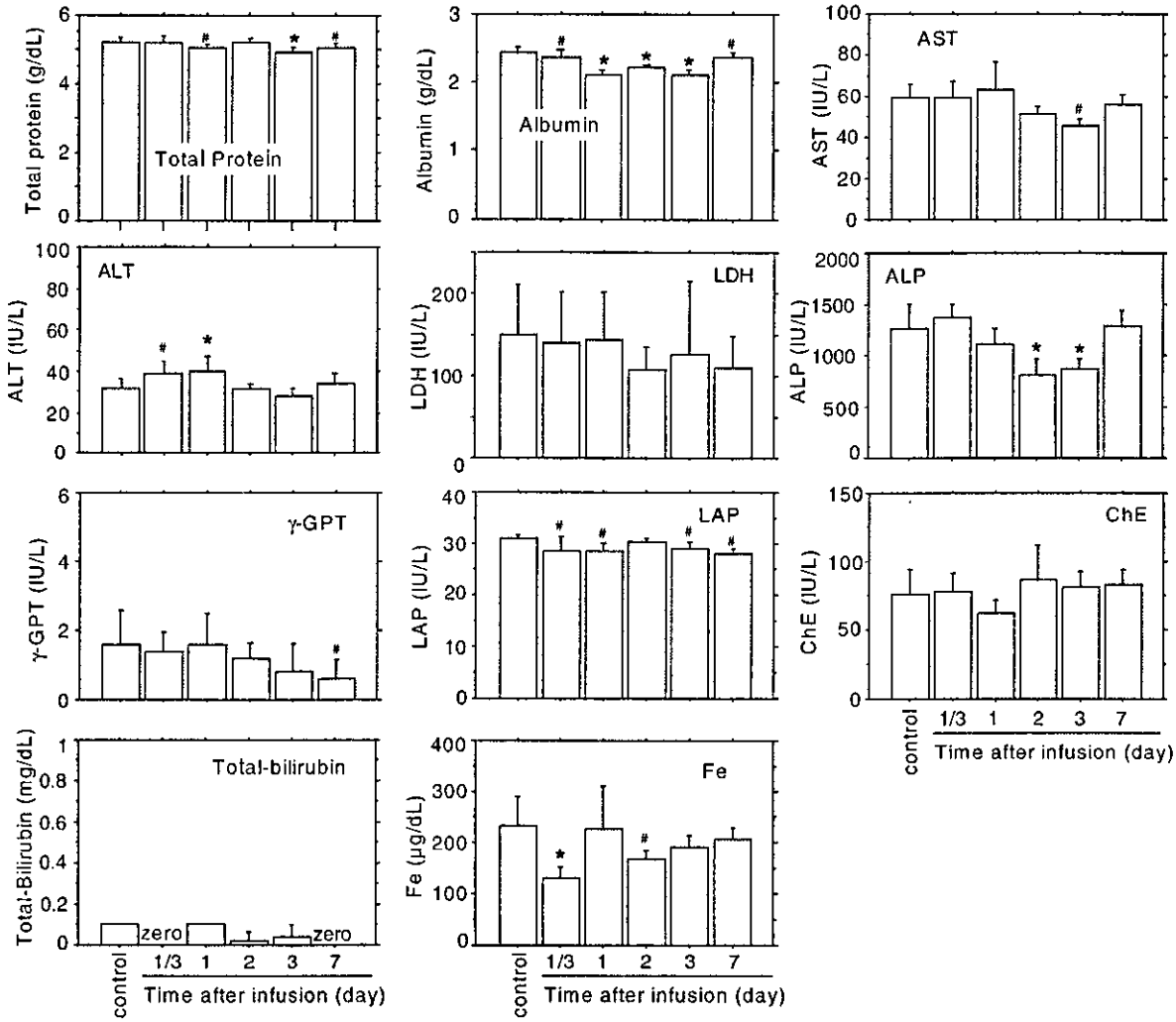

Fig. 2. Plasma laboratory tests representing the liver function and metabolism of Hb after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), γ-glutamyltransferase (γ-GTP) alkaline phosphatase (ALP), leucin amino peptidase (LAP), cholinesterase (ChE).

these organs is 0.8% of the body weight (1600 mg for 200 g body weight), which should correspond to not only the accumulated HbV (635 mg for 20 ml/kg) but also to the increased amount of phagocytic or parenchymal cells and/or RBC. The organ weight ratios tended to return to their original levels as HbV disappeared from the blood stream, and there was no deteriorative sign of morphological change in the main organs such as the liver, spleen, lung, kidney, and heart. To confirm the safety more in detail, we analyzed for the first time, the plasma laboratory tests on 29 analytes without any interference effect of the PEG-modified HbV simply by removing it from plasma by ultracentrifugation [19].

Our results indicated no irreversible sign of organ damage after the bolus infusion of HbV at a dose rate of 20 ml/kg (cf. whole blood = 56 ml/kg). Especially, liver is one of the main organs of the trapping and metabolism of HbV. However, we did not see an increase in the physiological meaning of the parameters representing the liver function. As for the parameters representing the renal function, there were slight changes in CRE, BUN, and UA without any physiological meanings. CPK did not significantly change, indicating that the intactness of the cardiac function and skeletal muscular function should be preserved.

Amylase and lipase that represent pancreatic function showed slight changes. The amylase activity slightly decreased while the lipase activity significantly increased from 9 ± 1 IU/l at control to 30 ± 9 IU/l at 2 days. The lipase activity was measured by an enzymatic method that was specific for pancreatic lipase. Therefore, the increment should not be attributed to the hepatic or

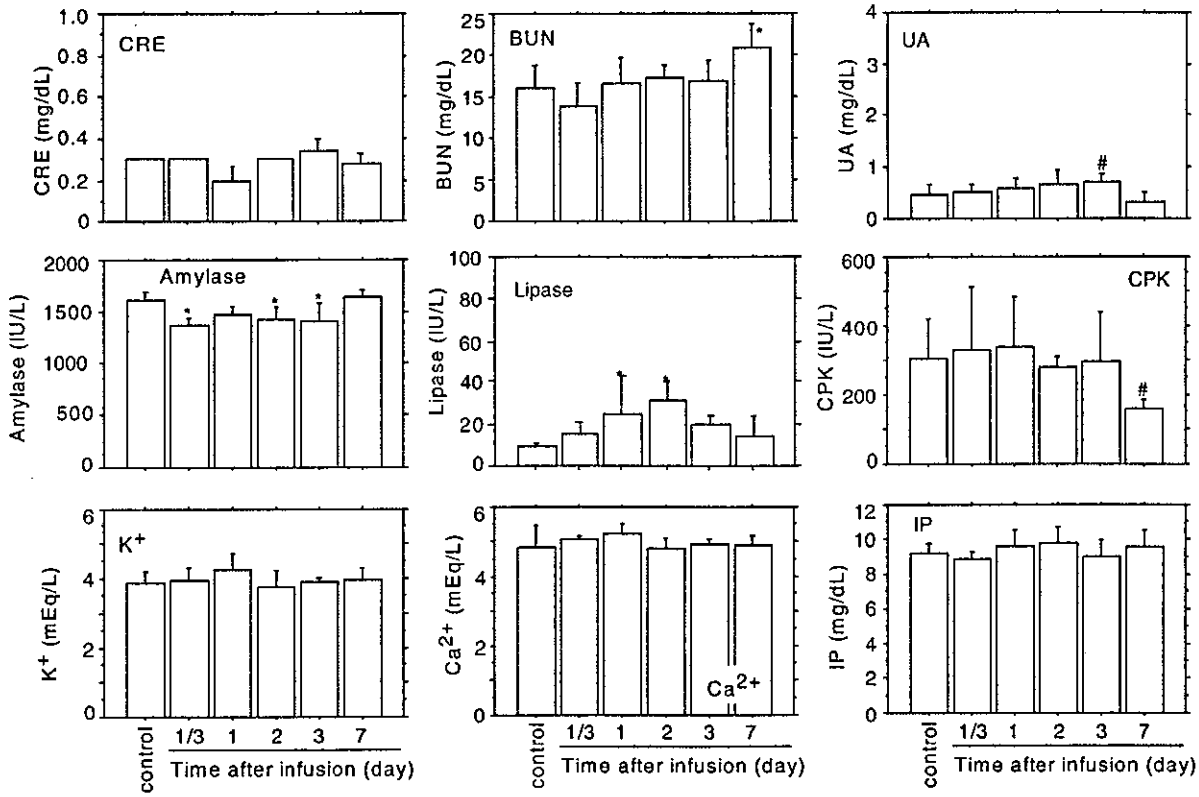

Fig. 3. Plasma laboratory tests representing renal, pancreatic and myocardial function, and electrolytes after infusion of HbV (20 ml/kg). The values are mean±SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: creatinine (CRE), blood urea nitrogen (BUN), uric acid (UA), creatine phosphokinase (CPK), inorganic phosphate (IP).

lipoprotein lipase. However, this level of increment was significantly smaller than the reported value for the Wistar rats of pancreatitis. Hofbauer et al. [26] reported that acute necrotising pancreatitis increased lipase activity from 10 to 475–5430 IU/l. It was reported that the injection of liposome amphotericin B raised the serum lipase activity, and one possible reason was speculated to be the enzyme induction in the pancreas by the presence of a large amount of lipids from the liposomes [27], because pancreatic lipase hydrolyze not only TG but also phosphatidylcholine [28]. This speculation was also supported by our results that the profiles of the transient increases in the lipid components coincided with that of lipase, but not with amylase. The cause of this modification is not clear at the present time. Histopathological analysis showed no significant pathological change in the pancreas. However, the pancreatic function should carefully be monitored in the ongoing safety studies.

Significant and consistent increases were seen in the lipid components with maximum at 1 or 2 days. They should be derived from the HbV particles because they contain a large amount of cholesterol (ca. 1200 mg/dl) and DPPC (1840 mg/dl) in the infused suspension ([Hb] = 10 g/dl). The gradual increases in cholesterols by 2 days after infusion and no Hb release from HbV in the plasma indicate that they should be liberated from RES after HbV are captured by RES and destroyed in the phagosomes. This is also supported by the fact that the maximum concentrations were seen at 2 days when the HbV in the plasma had mostly disappeared from the blood. It has been reported that the infused lipid components of the phospholipid vesicles are trapped in the Kupffer cells, and diacylphosphatidylcholine is metabolized and reused as a component of the cell membrane, or excreted in the bile and in the exhaled air [29–31]. Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocytes because HbV is so large that it cannot diffuse across the fenestrated endothelium into the space of Disse [11]. Cholesterol from HbV should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells [32], and then excreted in the bile after entrapment of the corresponding lipoprotein by the hepatocytes [33]. We speculate that the main components of the lipid bilayer membrane of HbV, the phospholipids and cholesterol, would gradually be redistributed

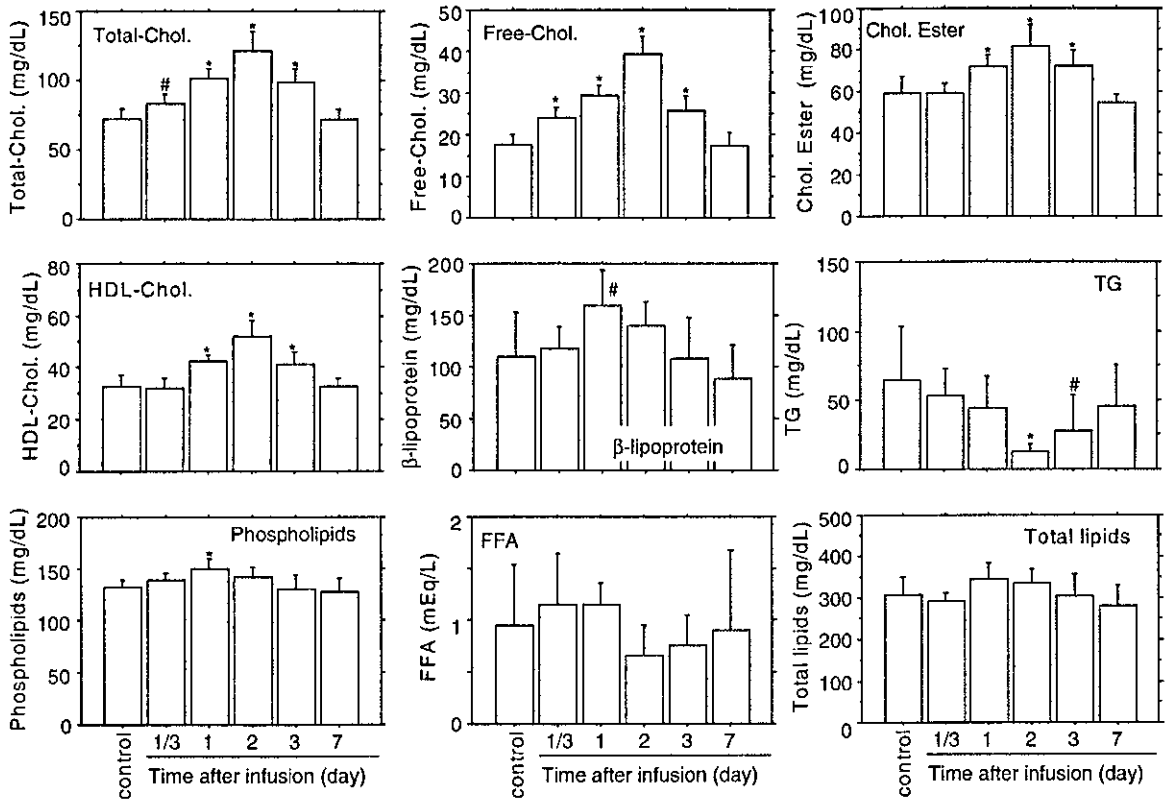

Fig. 4. Plasma laboratory tests representing lipid metabolism after infusion of HbV (20 ml/kg). The values are mean ± SD. $^{*}p<0.01$; $^{\#}p<0.05$ vs. control values. Abbreviations: total cholesterol (Total-Chol.), free cholesterol (Free-Chol.), cholesteryl ester (Chol.Ester), HDL-cholesterol (HDL-Chol.), triglyceride (TG), free fatty acid (FFA).

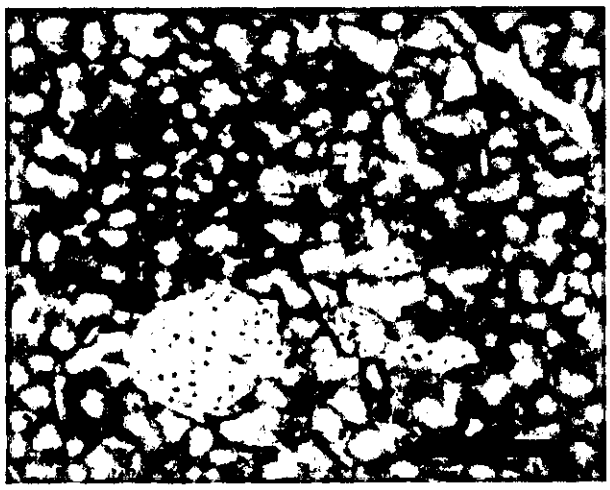

Fig. 5. Histology of pancreas 2 days after the infusion of HbV (20 ml/kg). Bar indicates 100 μm (HE stain).

or metabolized in the same manner. However, a precise pharmacokinetic study is necessary using radiolabeled materials to demonstrate the metabolic and excretory routes of the lipids. Transient, but significant increases in the lipid components raise the necessity of a further study to clarify the influence of a large dose of HbV especially on a lipemic model.

During the metabolism of Hb, there should be a release of bilirubin and iron. However, they did not increase for 7 days. In a previous study, the anti-human Hb antibody staining was effective for detecting the special and temporal distribution of human Hb of HbV both in the spleen and liver [16], and we made it clear that human Hb disappeared within 7 days. The released heme from Hb in HbV may probably be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells in the liver and in the spleen [11,34]. Bilirubin should be excreted in the bile as a normal pathway, and there should be no obstruction or stasis of bile in the biliary tree. Berlin blue staining revealed the presence of hemosiderin 3 and 7 days after HbV infusion, and it disappeared after 14 days [16]. A similar observation was reported for a polymerized Hb that was captured by the Kupffer cells while showing subsequent hemosiderin formation [35]. Normally, iron from a heme is stored in the ferritin molecule [36]. Ferritin in the lysosomal membrane may form paracrystalline structures and

eventually aggregate in mass with an iron content as high as 50%. These are hemosiderins composed of degraded protein and coalesced iron. Both ferritin and hemosiderin release iron molecules, and they are anticipated to induce hydroxyl radical production and succeeding lipid peroxidation [37,38]. However, iron release from hemosiderin is substantially less than that from ferritin, thus iron molecules in hemosiderin are relatively inert [39]. Plasma iron, mostly bound to transferrin, remained constant after HbV infusion. The iron concentration should be coordinately regulated through the "iron regulatory proteins" that sense the levels of iron for hematopoiesis and metabolic needs [40], and the excess amount of iron should be stored in an insoluble and less toxic form as hemosiderin. Together with the time course of the histopathological changes, the results of the plasma laboratory tests indicate that the metabolism of heme and the recycling or excretion of iron molecule is within the physiological capacity and suggested to be on the physiological pathway that has been well characterized for the metabolism of senescent RBC [41].

5. Conclusion

In this study, the plasma laboratory tests after the infusion of HbV (20 ml/kg) did not demonstrate an irreversible sign for a deteriorative damage to the organs. Plasma bilirubin and iron, which were considered to be released during the metabolism of the Hb molecule, did not increase during the observation period. This may be due to the moderate rate of Hb metabolism in RES after the entrapment of HbV with a moderate length of circulation time. The lipid components significantly increased at 2 or 3 days after infusion. These may be derived from the membrane component of HbV entrapped in RES. The complete normalization of the lipid components indicates that they are metabolized in a normal metabolic and/or recycling pathway. The precise biodistribution and fate of the components should be confirmed by a radioisotope technique. Our results have demonstrated the safety of HbV using only healthy rats, while rats in hemorrhagic shock, septic shock, or lipemia have to be tested in the ongoing safety studies. It should also be emphasized that the data cannot be extrapolated to large animals or humans, which may react differently to such a large dose of HbV.

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