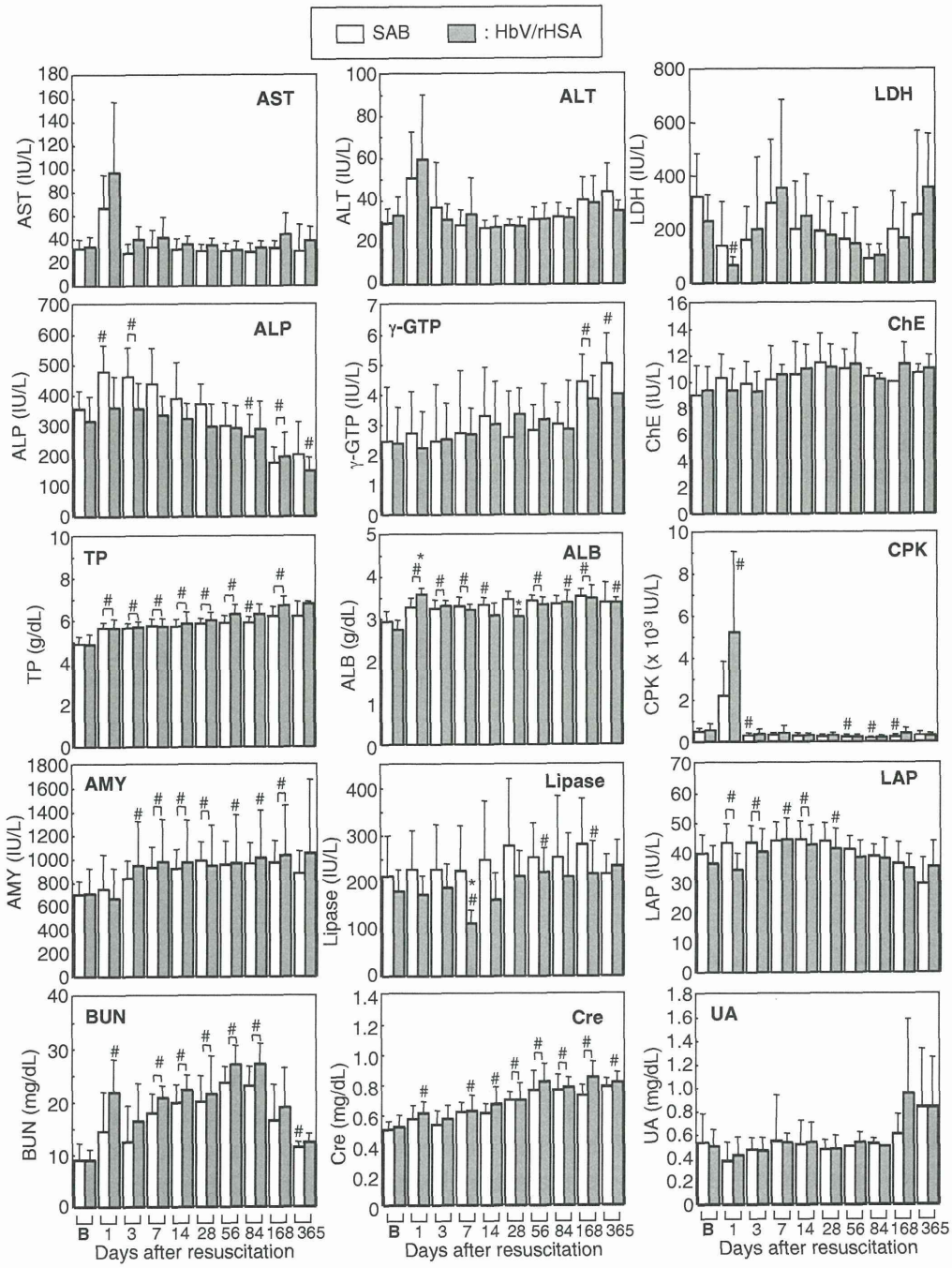

Fig. 28.6 Plasma biochemical tests reflecting the organ functions such as liver, pancreas, and kidneys during one year after 40 percent hemorrhagic shock and resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # Significantly different from baseline ($p < 0.01$); * significantly different versus the autologous shed blood group ($p < 0.01$). *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *LDH* lactate dehydrogenase, *ALP* alkaline phosphatase, *GGTP* γ-glutamyltransferase, *ChE* cholinesterase, *TP* total protein, *ALB* albumin, *CPK* creatine phosphokinase, *AMY* amylase, *LAP* leucin amino peptidase, *BUN* blood urea nitrogen, *Cre* creatinine, *UA* uric acid

were considered significant amounts of HbV phagocytized by macrophages in the spleen and Kupffer cells in the liver were observed. In chronic phase study, the HbV/rHSA group on Day 28 showed brown pigment deposition in the spleen and the Kuppfer cells of the liver (Fig. 28.8). These findings were not observed at other time points of the HbV/rHSA groups and SAB groups. No significant changes were seen in the pancreas, lung, heart, and kidney (Fig. 28.9).

28.4 Discussion

Our primary finding in this study is that HbV suspended in an albumin solution showed a similar resuscitative ability to that of SAB. Cardiovascular function such as MAP, PAP, CVP, PCWP recovered after resuscitation, and there was not significant differences between all groups (HbV/rHSA group, rHSA group, and SAB group). We have reported the efficacy as a resuscitative fluid in hemorrhagic shock in canine model (Yamamoto et al. 2012). In the previous report, up-regulation of PAP in HbV group after resuscitation was significantly higher than the other groups (SAB, rHSA, and Lactate Ringer solution groups). We thought HbV has the constrictive potential to the pulmonary circulation. In this study, PAP recovered after resuscitation in all groups and there were no significant differences between groups. From these findings, we considered that spleen plays a primary role to mitigate the influence of HbV. Since spleen is a large RES organ, it could play a role as a filter of the particle that might influence on endothelium. Furthermore, it was the first time to clarify the long term safety of HbV using canine model as evidenced by the result that HbV did not disturb the cardiopulmonary circulation, and all the dogs survived for 1 year without any remarkable side effect for each organs.

In the acute phase study, the rHSA group tended to delay the recovery of decreased MAP, however, HbV/rHSA group showed the prompt response that was similar to SAB group. The change of DO_2 showed the similar characteristics to the change of MAP, and HbV contributed 26–29 % of the total DO_2 values. These findings showed that the resuscitative ability of HbV was better than that of rHSA and was equivalent to SAB, because of the enough ability of oxygen carrying capacity. Regarding the rHSA group, MAP tended to show lower values than the other groups, and SVR showed significantly lower value than the HbV/rHSA group. The remarkable increase of cardiac output (CO) was caused by the reduced blood viscosity and the increased HR to compensate for the inadequate oxygen carrying capacity. Due to the sufficient compensatory mechanism to this level of hemorrhagic shock and resuscitation, the blood gas parameters showed non-significant changes between rHSA and the other groups. By contrast, lactic acid showed lower value in the rHSA group than in other groups. Because all the dogs in the rHSA groups survived for 4 h, the rHSA fluid alone possesses a resuscitative ability to restore blood volume in spite of the lack of oxygen carrying capacity. Unlike a human spleen, a canine spleen is an important blood reservoir capable of

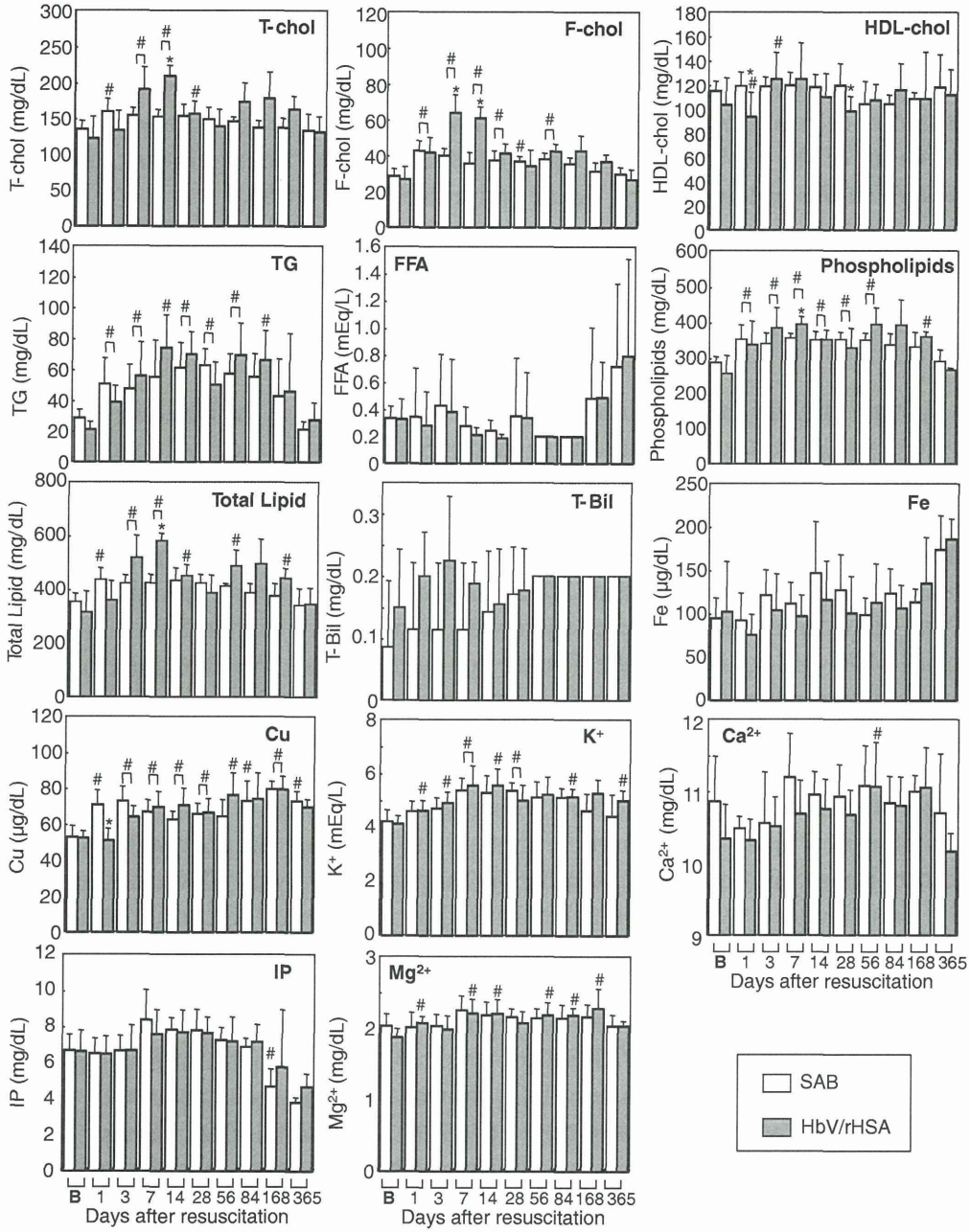

Fig. 28.7 Plasma biochemical tests relating the metabolism of the components of HbV (lipids and Hb), microelements, and electrolytes during one year after 40 percent hemorrhagic shock and resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # Significantly different from baseline ($p < 0.01$); * significantly different versus the autologous shed blood group ($p < 0.01$). *T-chol* total cholesterol, *F-chol* free cholesterol, *HDL-chol* high density lipoprotein cholesterol, *TG* triglyceride, *FFA* free fatty acid, *T-Bil* total bilirubin, *IP* inorganic phosphate

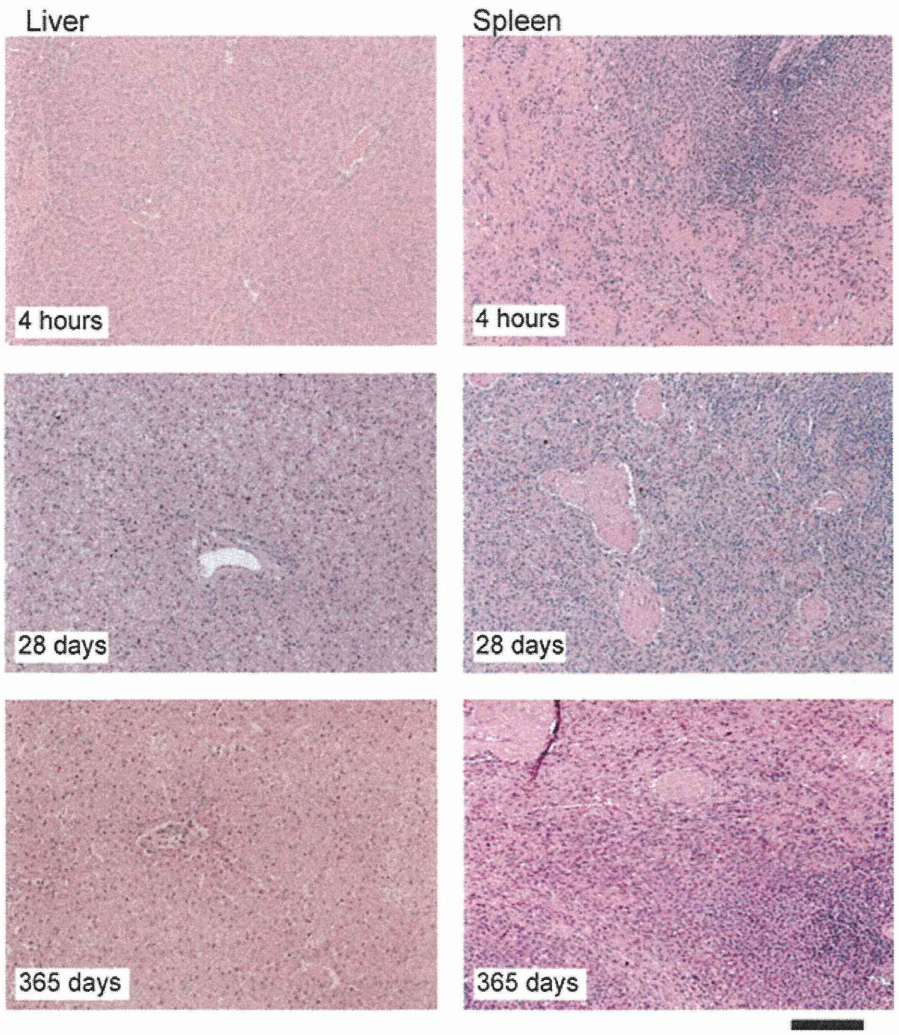

Fig. 28.8 Histology of spleen and liver of HbV/rHSA group at 4 h, and 28 and 365 days after resuscitation. The presence of spleen macrophages and liver Kupffer cells phagocytizing HbV particles was shown at 4 h. The liver and spleen at 28 days contained slight brown pigment deposition. No significant change is noted at 365 days. Scale bar, 100 μm. Hematoxylin and eosin stains

maintaining Hct at a stable level by "autotransfusion" in response to a blood loss such as hemorrhagic shock (Hoit et al. 1991). This might have caused the unexpectedly moderate resuscitative ability of the rHSA solution and without causing severe shock state. In previous report, while animals undertook splenectomy and 50 % hemorrhagic shock, in rHSA group, animals could maintain proper lactate level and showed good recovery of pH during resuscitation and observation phase (Yamamoto et al. 2012). In this respect, resuscitation potential of albumin solution was high, given the patient had enough cardiac reserve to restore organ perfusion, preventing hypoxia. In HbV/rHSA group, cardiac output, heart rate, and lactate level showed almost identical change compared with SAB group.

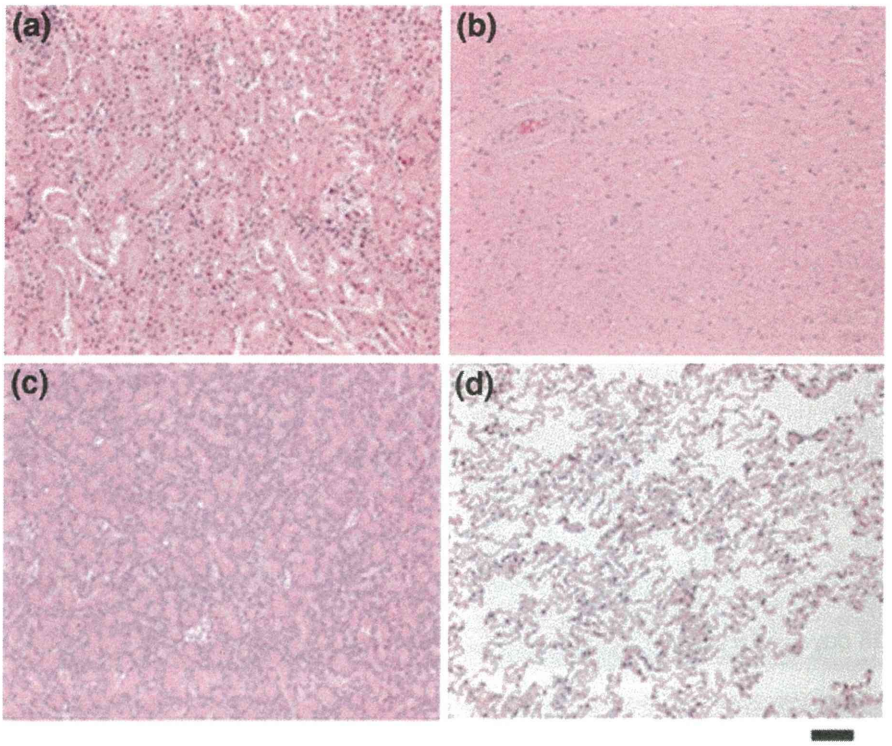

Fig. 28.9 Histology of kidneys (**a**), heart (**b**), pancreas (**c**), and lungs (**d**) of HbV/rHSA group at 365 days after resuscitation. No significant change is noted in these organs. Scale bar, 100 μm. Hematoxylin and eosin stains

It has to be emphasized that acute and chronic safety of HbV was shown in this study using the canine model in which a large amount of HbV was transfused.

It has been reported that resuscitation from hemorrhagic shock with acellular type HBOCs such as polymerized or intramolecular cross-linked Hb causes the elevation of MAP beyond the baseline values. The hypertension may be presumably due to the high affinity for nitric oxide of acellular type HBOCs and their smaller size that enables nitric oxide trapping in the proximity of the endothelium (Sakai et al. 2000a; Yu et al. 2008; Nakai et al. 1998; Driessen et al. 2001; Kasper et al. 1998; Natanson et al. 2008). In this study, the abnormal increase of MAP after infusion of resuscitative fluid was not seen. MPAP showed increase immediately after the infusion, and gradual decrease after 30 min from resuscitation. PVR showed the stable values after the infusion. As HbV/rHSA group did not show the specific elevation of MAP, MPAP, SVR and PVR, we considered that the cellular HbV presumably trap nitric oxide slowly as erythrocyte does. (Sakai et al. 2011; Arazov et al. 2011). On the other hand, it has been reported that the infusion of the other cellular type HBOCs cause the elevation of PVR in a beagle dog model and the elevation of SVR in a goat model (Pape et al. 2008; Kansaku et al. 2008). These circulatory abnormalities are not related the high affinity for nitric oxide because Hb is encapsulated, but may be presumably due to the activation of complement and platelet caused by the lipid component of the membrane

encapsulating the Hb (Abe et al. 2006; Sou and Tsuchida 2008). In the previous report, 50 % hemorrhagic shock model in beagle dog that underwent splenectomy showed transient increase of PAP after resuscitation while in the present study we could not find this change. The difference between previous study and acute phase of the present study is whether splenectomy was conducted. Spleen might have the ability to compromise the transient increase of PAP during resuscitation. Further study is required.

Pathological examination of the liver and spleen of 4 h after resuscitation showed accumulation of HbV (Sakai et al. 2001). Because the circulation half-life of HbV is about 35 h (Sou et al. 2005), the spleen had already started to show accumulation of HbV 4 h after resuscitation. The lung and kidney did not show any abnormalities such as embolism in the capillaries derived from the aggregation of vesicles (Rudolph et al. 1995). In the chronic phase study, Hct showed complete recovery to the baseline 7 days after resuscitation, although difference between SAB group remained significant until two months after experiment. While HbV have disappeared from circulating blood by 7 days after infusion because the circulation half-life of HbV is about 35 h, phagocytized HbV might made effect on the delay of erythropoiesis recovery. In contrast, WBC and Platelet didn't show any significant differences between SAB and HbV/rHSA groups. This fact showed that HbV didn't make an influence on the kinetics of WBC and platelet.

Hemorrhagic shock and resuscitation induce ischemia, hypoxia, and reperfusion injury, all of which influence organ functions. Many precedent papers have described the elevation of plasma enzyme levels, such as AST, ALT, and LDH after resuscitation with HBOCs or transfusion (Sakai et al. 2004c; Marks et al. 1987; Lehnert et al. 2003; Bosman et al. 1992; Mota-Filipe et al. 1999; McDonald et al. 2002; Young et al. 2007). In the present study, the elevations of these plasma enzyme levels were also seen for the SAB group, and these are the common reaction for this kind of shock study.

Lipase activity, but not amylase, significantly decreased in the HbV group, whereas no histopathological abnormality was seen in the pancreas. In our previous tests of daily repeated infusion for 14 days or bolus injection using rats, a transient increase in lipase activity was observed. This was thought to be due to the up regulation of lipase in response to the infusion of a large amount of lipids from the liposomes (Stuecklin-Utsch et al. 2002). However, in this study, the result was in conflict with the past results. The reason is not clear, but the difference of species might be one possible reason.

Liposome-encapsulated Hb without PEG-modification aggregated in the plasma and showed a slight accumulation in the kidneys (Rudolph et al. 1995). However, our PEG-modified HbV does not induce intervesicular aggregation, and does not have any deteriorating influence on the kidneys. In this study, no abnormal value was noted for BUN, Cre, and UA and there was no histopathological abnormality in the kidneys in the HbV/rHSA group.

The plasma lipid components; T-Chol, F-Chol, and Total lipid significantly increased after the infusion in HbV/rHSA group. They should be derived from HbV because it contains a large amount of cholesterol, and they would be liberated

after the HbV particles are captured and degraded in the reticuloendothelial system (RES) (Sakai et al. 2001, 2004a). Extensive studies of circulation kinetics and organ distribution of isotope-labeled HbV clarified that HbV accumulates preferentially in the RES (Awasthi et al. 2004; Sou et al. 2005). It is reported that once liposome is captured in the Kupffer cells, the diacylphosphatidylcholine is metabolized and is reused as a cell membrane component or excreted in the bile (Dijkstra et al. 1985). Cholesterol is finally catabolized as bile acids in the parenchymal hepatocytes. There should be no direct contact of HbV and the hepatocyte because HbV (diameter, 250 nm) cannot diffuse across the fenestrated endothelium into the space of Disse (Goda et al. 1998). Cholesterol of the vesicles should reappear in the blood mainly as lipoprotein cholesterol after entrapment in the Kupffer cells and should then be excreted in the bile after entrapment of the lipoprotein cholesterol by the hepatocytes (Kuipers et al. 1986). Actually, it was confirmed that ^{3}H labeled cholesterol was excreted in feces by the experiment of rat (Taguchi et al. 2009). In terms of PEG-lipid, we reported previously that PEG chain disappeared within 14 days in the liver and spleen by the experiment of rat (Sakai et al. 2009). In the present study, the plasma lipid components increased until 7 days, and recovered at 14 days, and so, it is supposed that the lipid components of HbV would be completely metabolized within 14 days.

During the metabolism of Hb, we would expect a release of bilirubin and iron. But they did not increase in the plasma. The released heme from Hb in HbV could be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages (Sakai et al. 2004a; Finch and Huebers 1982). Bilirubin would normally be excreted in the bile as a normal pathway, and no obstruction or stasis of the bile should occur in the biliary tree. Normally, iron from a heme is stored in the ferritin molecule (Grady et al. 1989). Both ferritin and hemosiderin release iron. They are anticipated to induce hydroxyl radical production followed by lipid peroxidation (O'Connell et al. 1989). The iron release rate from hemosiderin, however, is substantially less than that from ferritin (Bennett and Kay 1981). Consequently, the excess amount of iron would then normally be stored in an insoluble and less toxic form as hemosiderin. We found iron deposit in the spleen and liver in long term study. The finding was the same with hemosiderosis often observed in patients who have received repeated blood transfusions.

The liver and the spleen are important organs for degradation of HbV in RES. Pathological examination of the liver showed evidence of Kupffer cells phagocytizing HbV; it disappeared within 7 days in the liver. In the spleen, substantial accumulation of HbV was confirmed in macrophages in the red pulp zone in the same manner as that in previous studies of bolus injection, daily repeated injections, and exchange transfusion (Sakai et al. 2001, 2004b, c). In the present study, hemosiderin deposition was detected in the liver of the HbV/rHSA group at 28 days. These results indicate that heme was metabolized in Kupffer cells of the liver and does not indicate a disability of liver function, as supported by the normal plasma enzyme levels.

We investigated lung, heart, liver, spleen, pancreas, kidney, adrenal glands, testis, trachea, esophagus, small intestine, and colon. Throughout the pathologic survey, we found no fibrotic change in organs. Perfluolochamical artificial oxygen carrier remained in the organs for over two years and induced fibrotic change in the liver (Kitazawa et al. 1982). Perfluorochamicals were said biological inert but it didn't have metabolic pathways. Therefore the material accumulate in the RES system and lymphnode. HbV were made of substantially biodegradable materials. Newly developed materials were minus charged lipid (DHSG) and PEG. Judging from the chemical structure, DHSG could be hydrolysed by non specific dehydrogenase, and the same pathways might be applied to PEG. We could not find any accumulation of these materials pathologically but further study was required.

In conclusion, resuscitation with HbV suspended in rHSA showed rapid recovery of hemodynamic parameters. There was no obvious side effect in hematological tests, plasma biochemical parameters, and histopathological examination within 1 year in comparison to the SAB group. Although transient but substantial accumulation of HbV in phagocytic cells raises concerns of the impact on the defensive function of the body, the present results using beagle dogs reassure that HbV suspended in rHSA shows a similar resuscitative ability and safety to that of SAB.

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