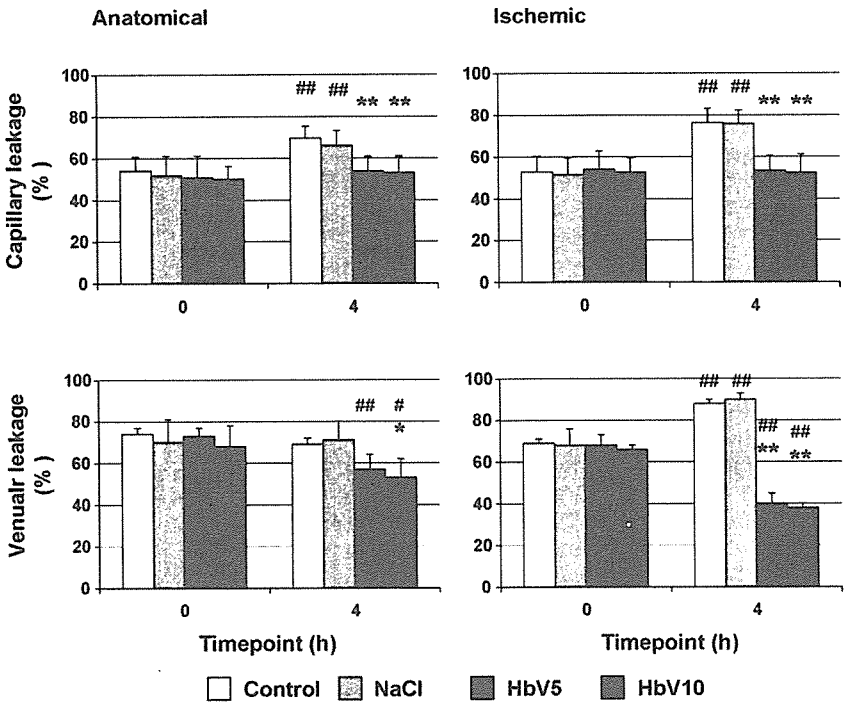

Figure 3. Macromolecular leakage in the anatomically perfused and ischemic tissues at baseline and after top-load infusion of 0.9% NaCl, and hemoglobin vesicles suspended in NaCl at Hb concentrations of 5 g/dL (*HbV5*) and 10 g/dL (*HbV10*). Data represent mean values and SD. #$p < .05$, ##$p < .01$ vs. baseline; *$p < .05$, **$p < .01$ vs. control.

line and control, not significant between HbV groups).

In the control group, capillary macromolecular leakage was increased by 29% in the anatomically perfused tissue and by 44% in the ischemic tissue over time (both $p < .01$), whereas it remained virtually unchanged after the infusion of the HbV solutions ($p < .01$ vs. control, Fig. 3). Compared with the control animals, the HbV solution decreased venular leakage by ~20% in the anatomically perfused tissue (not significant for HbV5, $p < .05$ for HbV10) and by ~55% in the ischemic tissue ($p < .01$).

All inflammatory markers revealed an almost two-fold accumulation in the ischemic tissue compared with the anatomically perfused part ($p < .01$), which was completely abolished by the HbV solutions ($p < .01$ vs. control, Fig. 4). In the anatomically perfused tissue, the proinflammatory cytokine levels were virtually not affected by any solution, whereas the leukocyte counts were reduced to <25% after the infusion of HbVs ($p < .01$).

DISCUSSION

The principal findings of this study were that the hypoxia-related activation of TNF-α and IL-6 and accumulation of leukocytes in the ischemic tissue could be completely abolished by the top-load infusion of HbVs at both 5 g/dL and 10 g/dL Hb concentrations.

This effect was paralleled by restoring the highly diminished partial oxygen tension in this tissue to virtually normoxic values. Therefore, it appears most conceivable that the attenuation of inflammation was accomplished by the capacity of HbVs to improve oxygen delivery to critically ischemic tissues (9–12). By definition, oxygen delivery to an organ or tissue compound is determined by the volumetric blood flow and the oxygen content in the feeding artery, which in turn is the product of the amount of oxygen carriers and their oxygen saturation. Although it added oxygen carriers to the circulation, the top-load infusion of the HbV solutions resulted in a net hemodilution, which may partly be explained by their low Hb concentration and which was partly compensated by an improved alveolar gas exchange, as suggested by the behavior of the systemic arterial Po_2, Pco_2, and pH values. According to the oxygen dissociation curve (18), arterial oxygen saturation was 80% at baseline. Four hours after HbV infusion, it was 87% for the native Hb and 95% for the artificial Hb, which was not sufficient to obtain any relevant systemic arterial oxygen content increase.

Nevertheless, the HbVs may have raised the oxygen content in the collateral arterioles nourishing the ischemic tissue. Due to their high oxygen affinity, the HbVs may have diminished the oxygen release in the upstream vasculature, which has been estimated to reach as much as 40–50% of the systemic arterial oxygen content (14). Both experimental (19–21) and theoretical (22) studies have shown that oxygen delivery may be shifted to the downstream direction if oxygen carriers with high oxygen affinity are infused. Presumably, the contribution of HbVs to the overall oxygen release was very low in the present study due to their small proportion (~6% of total Hb for HbV5, ~12% for HbV10), and, as indicated by the tissue oxygen tension data and the oxygen dissociation curve (18), because they still kept approximately 75% of their oxygen after having circulated through the ischemic vascular territory. Therefore, the HbVs may act primarily by redistributing the release of RBC-bound oxygen in favor of the ischemic tissue.

All variables expressing capillary blood flow in the ischemic tissue were substantially improved after the infusion of the HbV solutions but not saline. In addition to causing an increase in capillary RBC perfusion, HbVs, because of their small size, may perfuse capillaries that are no longer accessible by RBCs. Indeed, HbVs were observed in capillaries showing a cessation of RBC flux (18). One reason for the enhanced capillary perfusion may be the increased plasma viscosity. A dependency of capillary perfusion on plasma viscosity has been described for conditions of severe hemodilution (23). The effect has been ascribed to shear-stress-induced, nitric oxide-mediated arteriolar vasodilation, which is required to maintain capillary pressurization (24). However, no such vasodilation could be observed in the present study, thus calling for an alternative mechanism to explain the capillary hemodynamic findings.

The HbV-related improvement in capillary perfusion was accompanied by a reduction of capillary diameters. Although due to technical reasons, the capillary diameters may have been underestimated because of the presence of HbVs to a certain extent, this response of capillary diameters is somewhat surprising,

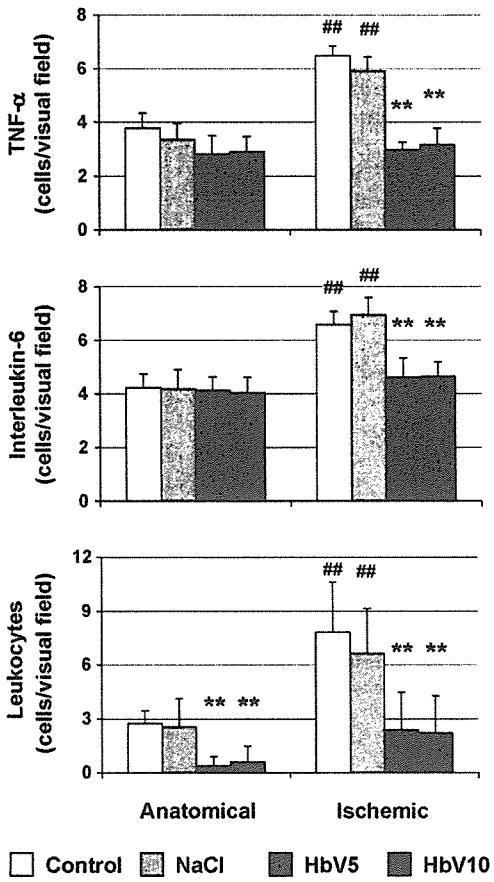

Figure 4. Density of cells stained for tumor necrosis factor (*TNF*)-α and interleukin-6 and of leukocytes in the anatomically perfused and ischemic tissues 5 hrs after surgery and 4 hrs after top-load infusion of 0.9% NaCl, and hemoglobin vesicles suspended in NaCl at Hb concentrations of 5 g/dL (*HbV5*) and 10 g/dL (*HbV10*). Data represent mean values and SD. #$p < .05$, ##$p < .01$ vs. anatomical; *$p < .05$, **$p < .01$ vs. control.

because the reduction of macromolecular leakage and inflammatory variables in the HbV-treated animals suggests less endothelial swelling and less edema formation, which would both widen the capillary lumen. Therefore, the capillary narrowing in these animals can only be explained by intraluminal capillary depressurization due to a decrease in postcapillary resistance.

Postcapillary resistance may be greatly enhanced due to the adhesion of leukocytes in this vascular segment (25), representing an early step of the inflammation cascade. During hypoxia, leukocyte adhesion has been reported to be mediated by HIF-1α activation (4). The activation of the leukocyte-endothelium interaction is paralleled by an increased permeability of the vascular wall, resulting in macromolecular leakage (17, 25). In the ischemic tissue, macromolecular leakage was significantly increased on both the capillary and the postcapillary level over time. This was attenuated by the injection of HbV, which provides further support to the assumption that HbV improved capillary perfusion by reducing postcapillary resistance, possibly by avoiding hypoxia-induced leukocyte adhesion.

However, even though not as marked as in the ischemic tissue, capillary leakage also increased in the normoxic, anatomically perfused part of the flap over time, which may be due to the traumatization of the tissue as a consequence of surgical manipulation (26). In this tissue, the reestablishment of normoxia may not account for the improvement in macromolecular leakage following HbV injection. However, the concept of reducing postcapillary resistance by attenuating leukocyte adhesion may also apply for this tissue, because similarly, yet less pronounced than in the ischemic tissue, the HbV infusions led to a reduction in capillary diameters and improved capillary perfusion and attenuation of leukocyte accumulation. In both tissues, capillary leakage may have been diminished secondarily due to the higher capillary flow velocity. Possibly, the HbVs may have diminished the capability of leukocytes to adhere to the endothelial lining due to the enhanced plasma viscosity, thus increasing wall shear stress (27).

This assumption is supported by our previous study, in which both macromolecular leakage and capillary hemodynamics in the ischemic flap tissue were improved with the administration of vesicles void of oxygen-carrying capacity (12). The proposed viscosity-related effect on postcapillary resistance may be of particular importance in the case of ischemia-reperfusion injury after reoxygenation of the critically ischemic tissue, which may have taken place in the animals receiving HbV, as evidenced by the improved partial tissue oxygen tension.

CONCLUSIONS

Hypoxia-induced inflammation in the critically ischemic hamster flap tissue was abolished by the injection of HbVs, which was accompanied by an improvement in capillary perfusion, presumably through a reduction in postcapillary vascular resistance. The effect may have been achieved by reestablishing virtually normoxic conditions by increasing oxygen delivery and plasma viscosity. Since capillary perfusion is a major contributor of oxygen delivery itself, HbVs may have interrupted a vicious circle consisting of hypoxia, inflammation, and secondary microcirculatory deterioration, a phenomenon that has been deemed responsible for the poor outcome after critical illness. Although our data may not be extrapolated to other tissues or organs in general, they are in line with findings reported in a previous shock study, in which resuscitation with HbV solution was able to substantially suppress the shock-related plasma TNF-α increase, thus emphasizing the potential of HbVs in preventing the systemic inflammatory response syndrome (28).

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One-Year Observation of Wistar Rats after Intravenous Infusion of Hemoglobin-Vesicles (Artificial Oxygen Carriers)

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Abstract: Hemoglobin-vesicles (HbV) or liposome-encapsulated Hb are artificial oxygen carriers. Our previous studies of the bolus infusion of HbV into Wistar rats showed that HbV was captured by the reticuloendothelial system from the blood stream and degraded completely with no deteriorative effect for 2 weeks. However, one authority on artificial organs research suggested conducting a one-year observation because he experienced, with one lipid-emulsified perfluorocarbon (PFC), that rats died within one year from a pulmonary abnormality after receiving the PFC emulsion due to the unstable dispersion state (personal communication). We thought this would never happen for HbV because the dispersion state of HbV is stable with PEG-modification. To confirm this, we made one-year observations after HbV infusion as suggested. Five male

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Wistar rats intravenously received 20 ml/kg HbV suspended in saline ([Hb] = 10 g/dL). They were housed in separated cages and provided with food and water *ad libitum*. All rats survived one year, and were apparently healthy. Their body weights (821 ± 75 g) reflected obesity from their confinement in small cages. No histopathological abnormality was found in the lung. Plasma biochemical analyses showed overall normal organ functions. In our previous report, plasma lipid levels increased transiently at 1 or 2 days; then they reverted to the control level at 7 days. One year later, the rats showed much higher plasma lipid levels, a symptom of hyperlipidemia that is attributable to obesity and aging. It seemed the transient increases at the early days had no impact compared with the levels of hyperlipemia of the old rats.

Keywords: Blood substitute; Liposome; Metabolism; Reticuloendothelial system; Safety study

INTRODUCTION

Phospholipid vesicles or liposomes encapsulating hemoglobin (Hb-vesicles, HbV) can serve as an O_2 carrier with comparable ability to that of red blood cells (RBC) [1–5]. Advantages of the Hb-based O_2 carriers (HBOCs) are the absence of blood-type antigens and transmission of known and unknown blood-borne diseases, the possibility of improving rheological properties of blood flow according to patients' needs, and stable long-term storage [6]. These characteristics will enable the use of HBOCs both in elective and emergency situations. In this sense, the infusion of HBOCs becomes superior to the conventional blood transfusion, which still entails the potential for mismatching, viral infection by HIV and hepatitis virus, and problems inherent in the short 2–3 week preservation period. According to clinical conditions in which HbVs are expected to be applied, an organism is faced with the metabolism of large amounts of both Hb and lipids because the HbV dose rate is considerably large. The HbV particles, as well as phospholipid vesicles, which are infused in the blood stream are ultimately captured by phagocytes in the reticuloendothelial system (RES, or mononuclear phagocytic system, MPS) [7–9].

Through histopathological studies of rats receiving 20 ml/kg of HbV infusion, our previous reports clarified that the HbV particles were captured and metabolized within 7 days in RES, mainly in the spleen and liver [7,8]. 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. Phagocytic activity decreased and then increased transiently, but tended to revert to the original level.

Plasma biochemical analyses for one week showed normal values overall, except that amylase and lipase activities showed reversible changes. However, no morphological changes were apparent in the pancreas. Plasma bilirubin and iron did not increase in spite of the fact that a large amount of Hb was metabolized in the macrophages. Lipid components increased transiently showing the maximum at 1 or 2 days, and returned to the control level at 7 days. They should be derived from membrane components of HbV that are liberated from macrophages that entrap HbV. Considering that result along with the previous report of prompt metabolism of HbV in the RES by histopathological examination, we conclude that HbV infusion transiently modified the values of the analytes at the bolus infusion rate of 20 ml/kg without any irreversible damage to the corresponding organs [7,8].

However, one authority in the research field of artificial organs strongly suggested that we conduct a one-year-observation because he experienced, unexpectedly, that rats died of pneumonitis within one year after receiving one lipid-emulsified perfluorocarbon (PFC) solution (personal communication) due to the insufficient dispersion stability [10]. In the case of HbV, the dispersion stability is ensured by the surface modification with polyethylene glycol (PEG) [6,10], and we thought this would never happen for our HbV. However, according to his suggestion, we conducted a simple test of topload bolus infusion of HbV at the dose rate of 20 ml/kg into Wistar rats. We confirmed the rats' survival, body weight increase, and conducted hematological, histological and plasma biochemical analyses, thereby verifying the safety of HbV.

MATERIALS AND METHODS

Preparation of Polyethylene Glycol (PEG)-Modified Hb-Vesicles (HbV)

The PEG-modified HbV was prepared in a sterile condition as reported previously in the literature [6,11,12]. 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 hemoglobin (38 g/dl) contained 14.7 mM of pyridoxal 5′-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) as an allosteric effector at a molar ratio of Hb/PLP = 2.5. The lipid bilayer was composed of Presome PPG-I [a mixture of 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-dipalmitoyl-L-glutamate-*N*-succinic

acid and 1,2-distearoyl-*sn*-glycero-3-phosphatidylethanolamine-*N*-PEG at a molar ratio of 5/5/1/0.033 (Nippon Fine Chemical Co. Ltd., Osaka, Japan)]. The HbV was suspended in saline at the Hb concentration of 10 g/dl, and sterilized using filters (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}), 28 Torr.

HbV Infusion and Procedure for Serum Clinical Laboratory Tests

All animal studies were approved by the Animal Subject Committee of School of Medicine, Keio University, 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 five male Wistar rats (264 ± 8 g; Saitama Experimental Animals Supply Co. Ltd., Kawagoe, Japan). They were anesthetized using diethylether inhalation, and the HbV suspension was infused into the tail vein at a dose rate of 20 ml/kg. All rats were housed individually in cages and provided with food and water *ad libitum* in a temperature-controlled room on a 12 h dark/light cycle.

Each rat's body weight was measured after 1, 3, 6, 9, and 12 months. At 12 months, the rats were anesthetized with 1.5% sevoflurane inhalation (Maruishi Pharmaceutical Co. Ltd., Osaka, Japan) using a vaporizer (TK-4 Biomachinery; Kimura Medical Instrument Co. Ltd., Tokyo). The animals were laparotomized and about 6 ml of blood was withdrawn from the caudal vena into a heparinized syringe for plasma biochemical tests. Then they were sacrificed by acute bleeding from the abdominal aorta. The organs were resected and were fixed in a 10% formalin neutral buffer solution (Wako Pure Chemical Industries Ltd., Tokyo) for staining with hematoxylin/eosin and Berlin blue. The withdrawn blood was centrifuged to obtain plasma, and the serum specimens were stored at − 80°C until routine clinical laboratory tests at BML, Inc. (Kawagoe, Japan). The selected analytes were total protein, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), gamma-glutamyltransferase (gamma-GTP), creatine phosphokinase (CPK), amylase, lipase, uric acid (UA), urea nitrogen (BUN), creatinine (CRE), total cholesterol, triglyceride (TG), free fatty acid (FFA), phospholipids, total bilirubin, and Fe^{3+}. The assay methods are described in our previous report [13].

For the comparative histopathological study, five male Wistar rats (31 weeks, body weight, 472 ± 8 g) were used.

Data Analysis

All control results and one-week observations after HbV infusion are taken from our previous report [8]. 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. Changes were considered statistically significant if $p < 0.01$.

RESULTS

All five rats survived one year; they were apparently healthy. The body weight before infusion was 264 ± 8 g, which increased monotonously to 821 ± 75 g (Fig. 1). However, all rats became obese because of their confinement in small cages individually for one year.

Plasma biochemical analysis clarified that no abnormal values were detected that reflect liver functions such as AST, ALT, and LDH, and those of kidney such as UA and CRE (Fig. 2). In a previous report, lipase activity increased transiently at 1 and 2 days, but it was quite normal at one year.

Plasma lipid components, cholesterol, phospholipids, and triglyceride were significantly higher than the control values and those at 1–3 days after the bolus infusion of HbV in the previous report [8], as shown in Fig. 3. According to the data of the breeders, hyperlipidemia was confirmed for one-year-old rats housed in pairs in cages, not individually. Total bilirubin and iron concentrations were not higher at one year.

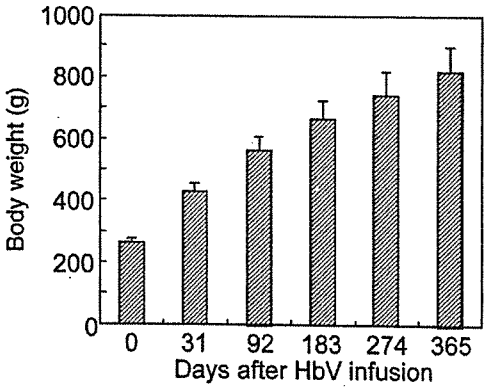

Figure 1. Changes in body weight of the Wistar rats during one year following intravenous bolus infusion of HbV at a dose rate of 20 ml/kg. All the rats were housed individually in cages and provided with food and water *ad libitum*.

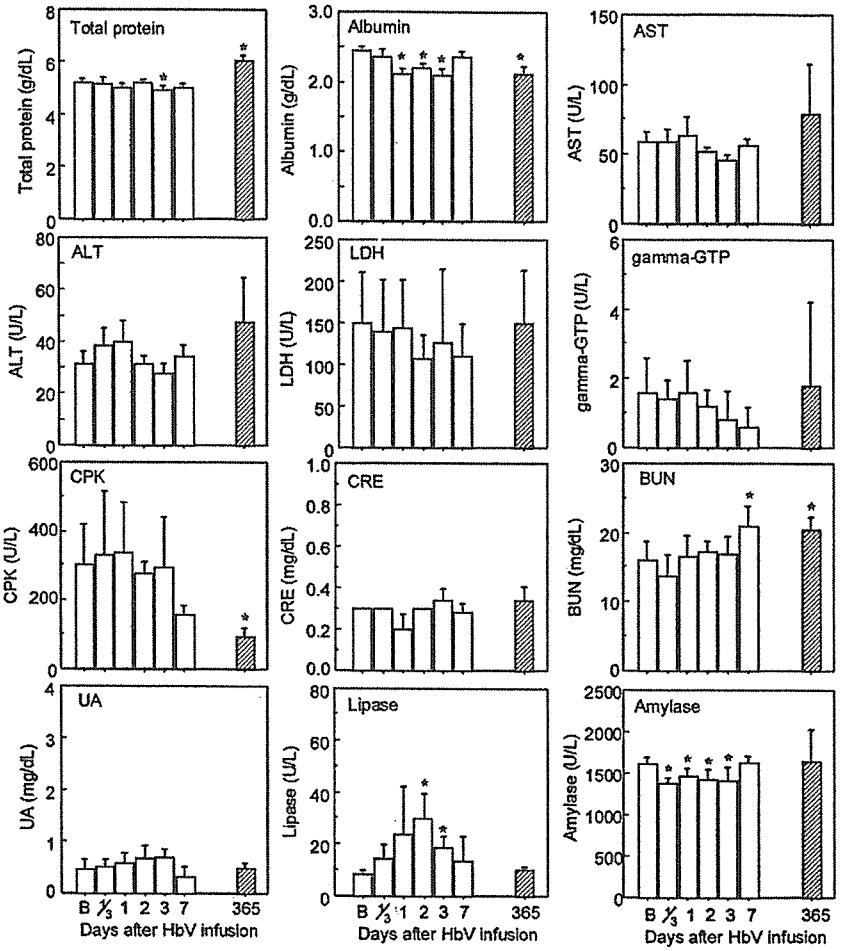

Figure 2. Changes in plasma biochemical parameters after intravenous bolus infusion of HbV into Wistar rats at a dose rate of 20 ml/kg. Data for 7 days (white bars) are cited from our previous report [8]. B, baseline.

No apparent anatomical abnormalities were detected in the lung (Fig. 4), liver, pancreas, heart, testis, stomach, intestine, kidney, muscle, bone marrow, or skin. One exception was that one rat showed an adenoma in the pituitary gland. Berlin-blue staining revealed considerable amounts of hemosiderin deposition in the spleen, pancreas, kidney, and adrenal glands. Rats that received no HbV also showed considerable amounts of hemosiderin in the same organs.

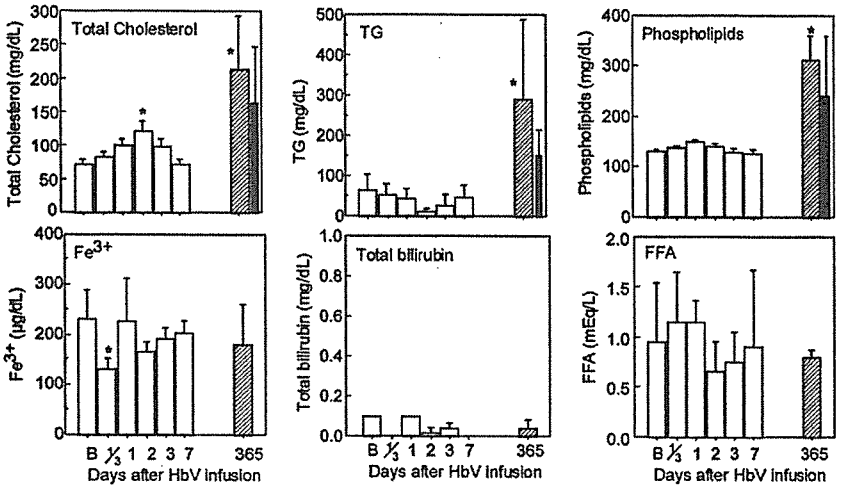

Figure 3. Changes in plasma levels of lipid components, iron, and bilirubin after intravenous bolus infusion of HbV into Wistar rats at a dose rate of 20 ml/kg. Data for 7 days (white bars) are cited from our previous report [8]. B, baseline. The values of black bars are from one-year-old rats housed in pairs in cages, not individually (cited from the breeder's data) [17].

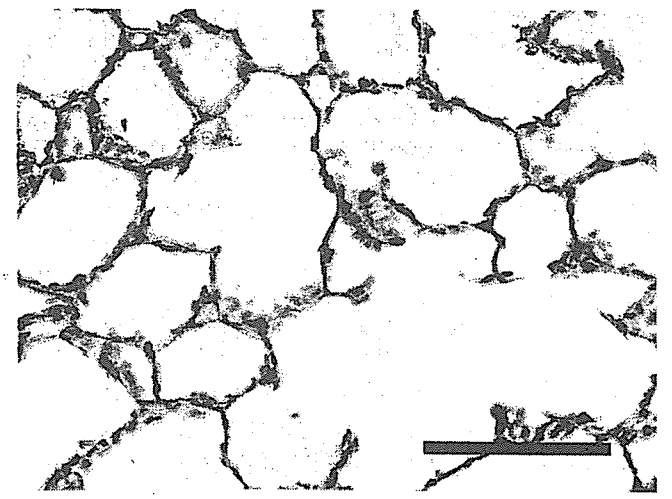

Figure 4. Histology of lung one year after bolus infusion of HbV stained with hematoxylin/eosin. Scale bar, 100 μm.

DISCUSSION

Our group has studied safety issues of HbV extensively [1,3,7–9,14,15]; no deteriorative or irreversible side effects have been observed after HbV infusion. Therefore, it was not surprising for us that all the rats survived one year after receiving intravenous bolus infusion of HbV (20 ml/kg) with no pathological abnormality in the lung, even though this was the first study to confirm one-year survival after HbV infusion. We conducted this experiment because it was communicated by Dr. A. Nosé that one lipid-emulsified perfluorocarbon (PFC) solution induced a chemical pneumonitis, causing death within one year (personal communication) [10]. In his recent review paper, he explains the mechanism that the unstable oxygen-carrying particles, such as PFC emulsion, would be covered with plasma proteins, and they would be trapped in the pulmonary capillary, resulting in the chemical pneumonitis [16]. On the other hand, in our experiments of HbV we did not observe any pathological abnormalities in the lung in one year. This should be due to the dispersion stability and blood compatibility of HbV achieved by the surface modification of HbV with PEG chains.

Serum biochemical analyses showed overall normal values. Albumin, AST, ALT, LDH, gamma-GTP, CRE, BUN, UA, lipase, and amylase were similar to the baseline values. The significant decreases in CPK and the significant increases in total proteins should be attributed to aging according to background data of the animal breeders [17,18]. In the previous report, plasma levels of lipid components increased transiently: they were liberated from macrophages that entrap HbV. They subsequently showed the maximum value at 1 or 2 days, and reverted to the control level at 7 days [8]. On the other hand, the present study showed significantly higher lipid levels in the rats at one year. All the rats gained weight significantly and apparently became obese from their confinement in small cages individually for one year with *ad libitum* access to food and water. The body weights of rats (821 ± 75 g) that received HbV were significantly higher than those of normal 52–week-old Wistar rats of the breeders (703 ± 61 g, caged in pairs [17]; 490 ± 50 g [18]). It seemed that the plasma lipid increase at 1 and 2 days after the HbV infusion had no impact compared with the levels of hyperlipemia because of obesity of the old rats.

Anatomical examination clarified that there were no apparent abnormalities in the vital organs including the lung. However, one rat showed an adenoma in the pituitary gland. This can result from aging, according to the literature [19–21] and information obtained from the animal breeders [17], describing that aged rats often show pituitary adenoma. Berlin-blue staining showed considerable amounts of hemosiderin

deposition in the spleen, liver, pancreas, kidney, and adrenal glands. It has been reported that hemosiderin deposition in the rat increases with age, especially in the liver and spleen [22–25], and pancreas in corpulent rats [26]. Actually, rats that did not receive HbV (31 weeks old) also showed considerable amounts of hemosiderin in those organs. Therefore, we speculate that hemosiderin deposition in this case is attributable to aging.

In conclusion, all rats survived for one year after bolus infusion of HbV at the dose rate of 20 ml/kg with no deteriorative side effects. All significant changes that were observed are attributable to the age and obesity of the rats. Further study is necessary to observe survival after a clinically relevant dose of HbV infusion such as that administered for resuscitation from hemorrhagic shock or a high level of blood exchange.

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Influence of O_2-carrying plasma hemoprotein "albumin-heme" on complement system and platelet activation *in vitro* and physiological responses to exchange transfusion

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Abstract: Recombinant human serum albumin (HSA) including the synthetic iron(II)-porphyrin (FeP), albumin-heme (HSA-FeP), is a unique O_2-carrying plasma hemoprotein as a red blood cell substitute. We have investigated the possible influence of HSA-FeP on the complement system and platelet activation *in vitro*. The amounts of the serum complement titer CH_{50} and terminal complement complex SC5b-9 of human blood serum, incubated with HSA-FeP (10, 20, and 40 vol %), were almost the same as those of the corresponding samples with HSA. The effect of HSA-FeP on the platelet reactivity has been demonstrated by conformational changes in the membrane glycoprotein IIb/IIIa and surface expression of an α-granule membrane protein P-selectin. Platelet activation in response to the ADP-stimulation was not influenced by the presence of HSA-FeP. It can be concluded that the albumin-heme solution does not facilitate the immunological reaction and platelet activation. Moreover, a 20% exchange transfusion with HSA-FeP into anesthetized rats has been performed to evaluate the circulation and blood parameters for 6 h. Time course changes in all parameters showed features identical to the control group (without infusion) and HSA group. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res 80A: 000–000, 2007

Key words: albumin-heme; complement system; exchange transfusion; platelet activation; RBC substitute

INTRODUCTION

There has been significant progress in red blood cell (RBC) substitutes in the past decade, and several hemoglobin (Hb)-based products are currently in clinical II/III trials.[1–4] We have also developed a unique albumin-based O_2-carrier "albumin-heme," which is composed of recombinant human serum albumin (HSA) including synthetic heme (2-[8-{*N*-(2-methylimidazolyl)}-octanoyloxymethyl]-5,10,15,20-tetrakis(α,α,α,α-*o*-pivalamido)phenylporphinatoiron(II)) (FeP, Scheme 1) (HSA-FeP).[5–7] The HSA-FeP solution has a high compatibility with the blood cell components, and its O_2-transporting capability was evaluated by animal experiments.[8,9] Nevertheless, animal studies cannot predict the potential effect on human responses. Especially, immunological reactions and platelet activity need not be the same as those in animals. In this study, we report the possible influence of HSA-FeP on the human complement system and platelet activation *in vitro*.

It is known that the complement cascade is activated in trauma patients with or without hemorrhagic shock.[10] The large volume administration of HSA-FeP as a blood alternative to the human body may affect the total serum complement activity. We have measured the amounts of complement titer CH_{50} and the terminal complement complex SC5b-9 of the human blood serum after incubation with HSA-FeP.

On the other hand, the platelet activation process involves an ordered sequence of events. In particular, the

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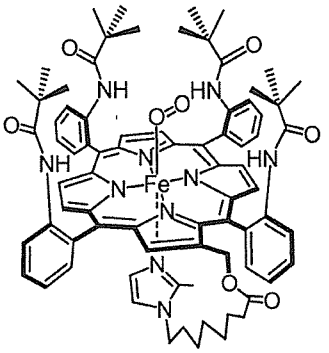

Scheme 1. Chemical structure of synthetic iron(II)-porphyrin (FeP) incorporated into HSA.

conformational changes in the membrane glycoprotein IIb/IIIa seem to be the most sensitive marker in the first event.[11–13] The activated GPIIb/IIIa creates a functional receptor for fibrinogen, which provides the link between adjacent platelets to form aggregates. The activation of this receptor can be detected by the specific monoclonal antibody PAC-1 that competes with fibrinogen.[11] The second event is the secretion of activation mediators, for example, ADP, serotonin, and thromboxane, resulting in further recruitment of platelets at the injured site.[14] The secretion process is accompanied by the rapid translocation of the α-granule membrane protein P-selectin (CD62P) to the outer membrane.[15,16] If the HSA-FeP solution activates the platelet, it may lead to amplifying the blood aggregation and inflammatory response. The PAC-1 binding and P-selectin surface expression have been assayed at the various levels of the ADP-stimulation.

Furthermore, we carried out the 20% exchange transfusion with the HSA-FeP solution into anesthetized rats and monitored the time courses of the circulation parameters (MAP, HR) and blood parameters (pH, PaO_2, PvO_2, $PaCO_2$) for 6 h.

MATERIALS AND METHODS

Materials

The recombinant human serum albumin (HSA, 25 wt %) was obtained from the NIPRO (Osaka). The HSA-FeP solution ([HSA]: 5.0 wt %, pH 7.4, [FeP]: 3.0 m*M*, COP: 21 mmHg, osmolarity: 300 mOsm, viscosity: 1.1 cP, endotoxin: <0.1 EU/mL, O_2-binding affinity ($P_{1/2}O_2$): 33 Torr) was prepared using our previously reported procedure.[8]

CH_{50} and SC5b-9

The human blood serum or plasma was well mixed with the HSA-FeP solution (the final concentration is 10, 20, and 40 vol %) and incubated for 1 h at 37°C. The CH_{50} value was determined by a 50% hemolysis assay based on Mayer's method with a commercial kit (New One point CH50 (KW), Japan BCG Supply, Tokyo). The SC5b-9 in the sample was determined using enzyme-linked immunosorbent assay kits (QUIDEL, Mountain View, CA).

PAC-1 and CD62P

The expression of PAC-1 and CD62P on platelets was measured as previously described.[17] Briefly, sodium citrate human whole blood was mixed with the HSA-FeP or HSA solution (the final concentration is 10, 20 and 40 vol %) and incubated for 10 min at 37°C. After adjusting the platelet concentration to

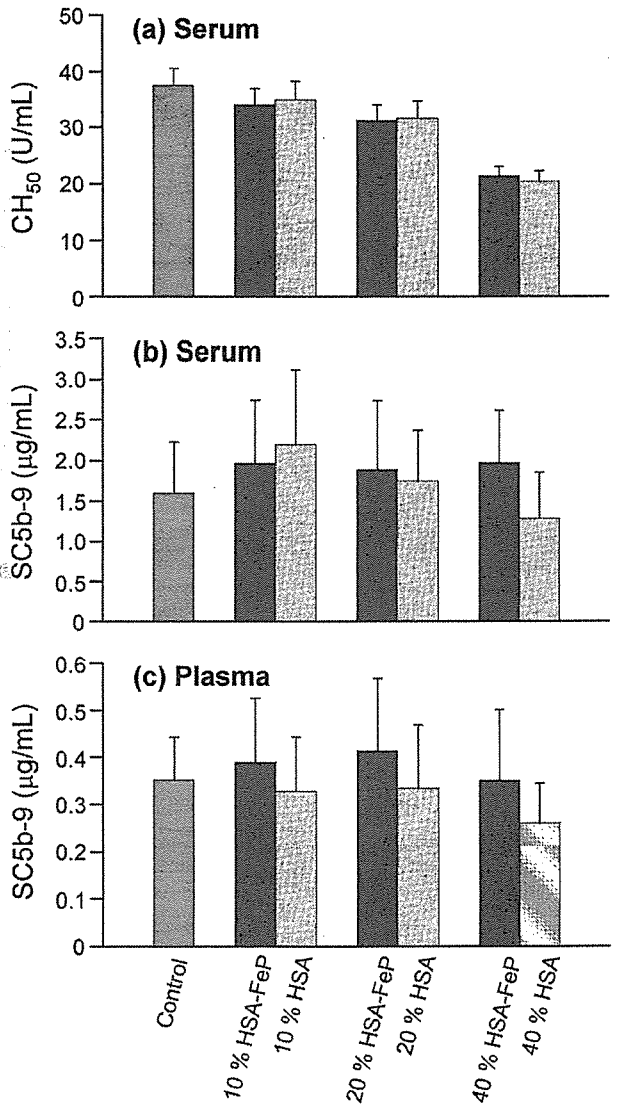

Figure 1. Influence of HSA-FeP on human serum complement after incubation for 1 h at 37°C; (a) serum complement titer (CH_{50}) in human blood serum, (b) terminal complement complex (SC5b-9) in human blood serum, and (c) SC5b-9 in human plasma. Each value represents the mean ± SD ($n = 5$).

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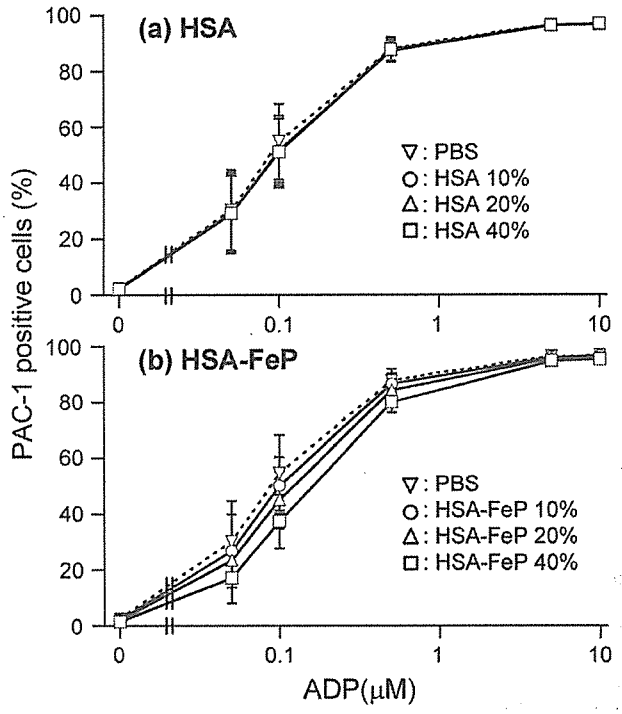

Figure 2. PAC-1 positive platelets in human whole blood mixed with (a) HSA-FeP and (b) HSA for 10 min at 37°C in response to various levels of ADP-stimulation. Each value represents the mean ± SD ($n = 5$).

$4 \times 10^5/20$ μL by Hepes Tyrode's buffer (pH 7.3), a cocktail of FITC-conjugated PAC-1, PE-conjugated anti-CD62P, and PerCP-conjugated anti-CD42a was added in equal amounts. A certain amount of ADP was added (the final ADP concentration is 0.05, 0.1, 0.5, 5, and 10 μ*M*). The FITC-conjugated anti-mouse IgM, PE-conjugated anti-mouse IgG, and PerCP-conjugated anti-mouse IgG were used as the negative controls. All antibodies were purchased from BD Bioscience-Pharmingen (San Jose, CA). The mixture was reacted in the dark for 20 min at room temperature and fixed with 1% paraformaldehyde. The samples were analyzed by flow cytometry (LSR, BD, San Jose, CA). Fluorescence data from 10,000 platelet events were collected in the logarithmic mode. The platelet population was identified by the number of CD42a positive events. The increased activation of GPIIb/IIIa and expression of CD62P were demonstrated by the percent of PAC-1 and CD62P positive cells in the platelets, respectively.

All subjects enrolled in this research had responded to an Informed Consent which has been approved by The Committee on Human Research of Hokkaido Red Cross, and that this protocol was found acceptable by them.

Exchange transfusion

The animal experiments using Wistar rats (304 ± 7.2 g) were carried out according to our previously reported protocol.[9] After stabilization of the animal, the 20% exchange transfusion was performed by 1 mL blood withdrawal via the common carotid artery and 1 mL HSA-FeP infusion from the femoral vein (each 1 mL/min); a total of four repeating cycles ($n = 6$, HSA-FeP group). A blood sample was taken from the artery (0.3 mL) and vein (0.2 mL) at the following five times; (1) before, (2) immediately after, (3) 1 h after, (4) 3 h after, and (5) 6 h after the exchange transfusion. MAP, HR, O_2-pressure (PaO_2), CO_2-pressure ($PaCO_2$) and pH for the arterial blood, and the O_2-pressure (PvO_2) of the venous blood were measured. As a reference group, the 5 g/dL HSA solution was similarly administered to the rats ($n = 6$, HSA group).[9] Furthermore, six rats without infusion (operation only) were also used as a control group.[9]

All animal handlings were in accordance with the NIH guidelines for the care and use of laboratory animals. The protocol details were approved by the Animal Care and Use Committee of Keio University.

Data analysis

All data were represented by mean ± standard deviation (SD). Statistical analyses were performed by repeated analysis measures of variance (ANOVA) using a StatView (SAS Institute). Values of $p < 0.05$ were considered significant.

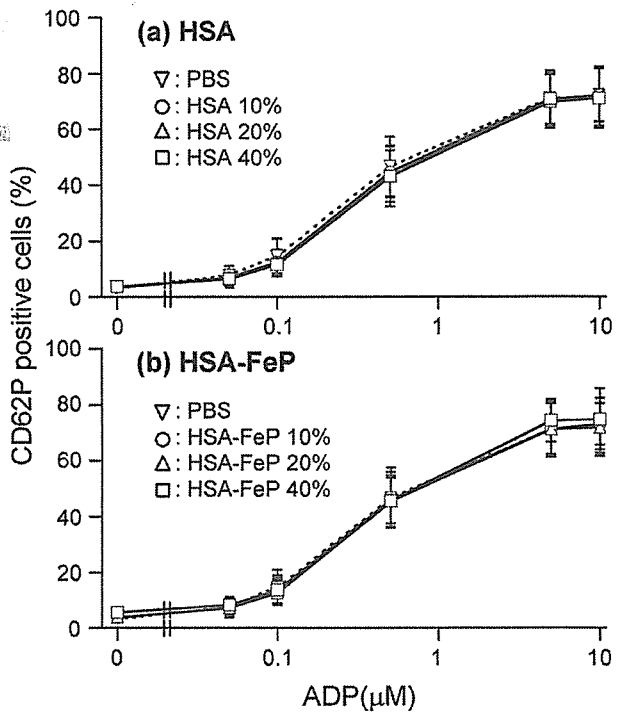

Figure 3. P-selectin expression on platelets in human whole blood mixed with (a) HSA-FeP and (b) HSA for 10 min at 37°C in response to various levels of ADP-stimulation. Each value represents the mean ± SD ($n = 5$).

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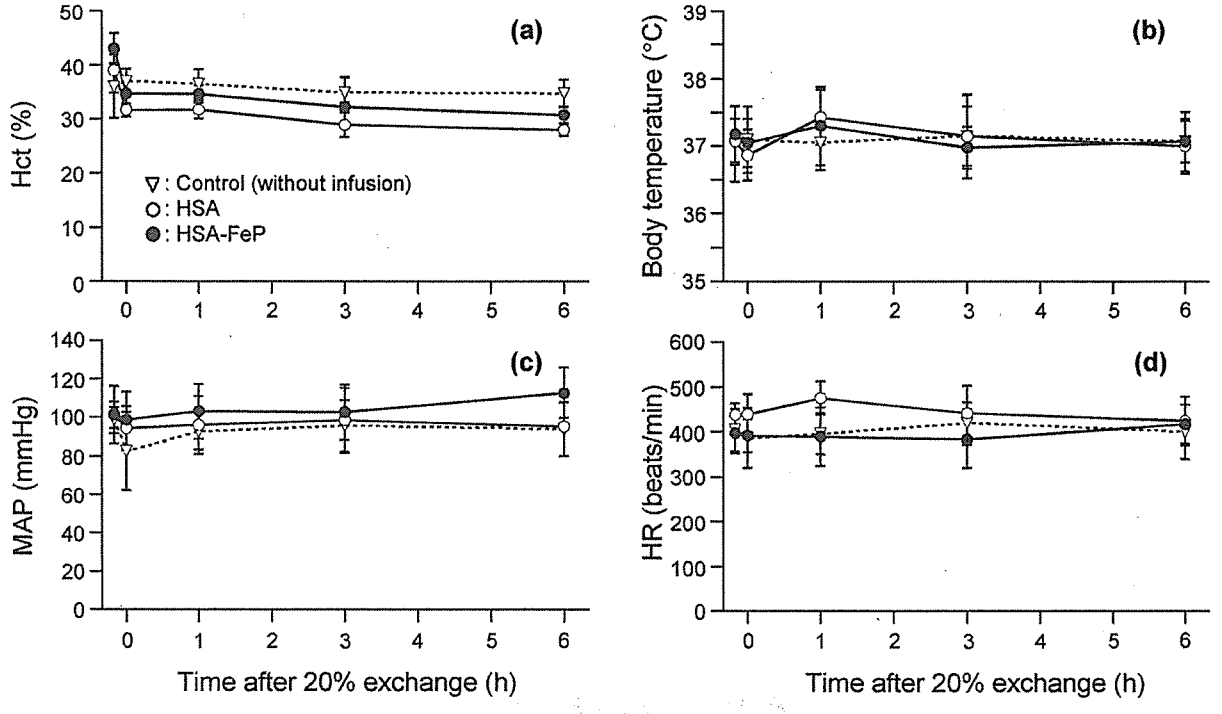

Figure 4. Time courses of (a) hematocrit (Hct), (b) body temperature, (c) mean arterial pressure (MAP), and (d) heart rate (HR) in anesthetized rats after 20% exchange transfusion with HSA-FeP or HSA solution. Each value represents the mean ± SD ($n = 6$).

RESULTS AND DISCUSSION

Complement system

Changes in the complement consumption are generally demonstrated by the complement titer CH_{50}. Ohtani et al. reported that the recombinant HSA, which is used for host albumin for HSA-FeP, shows the same immunochemical properties as the plasma HSA.[18] Thus the recombinant HSA should be a good reference in this experiment. The CH_{50} of the human blood serum incubated with 10, 20, and 40 vol % of the HSA-FeP solution were reasonably reduced in proportion to the each dilution ratio: 91, 83, and 57% of the control level [Fig. 1(a)]. The differences are almost the same as those observed in the HSA group, suggesting that the decrease in the CH_{50} with HSA-FeP did not involve any specific interaction.

On the other hand, the mean amounts of SC5b-9 in the human blood serum or plasma after the incubation with HSA-FeP were slightly higher than the control levels. However, all such differences were not significant within the experimental errors. Similar observations were found in the HSA group independent of the mixing ratio (10, 20, and 40 vol %). This implies that HSA-FeP does not enhance the production of SC5b-9.

Platelet activation

PAC-1 recognizes an epitope on the GPIIb/IIIa complex of activated platelets near the fibrinogen receptor.[19] We measured the percent of PAC-1 positive cells in the blood sample incubated with the HSA or HSA-FeP solution. When the ADP is absent, the fraction of the active cells was negligibly small, that is, 1.37–2.39%, independent of the mixing ratio of HSA or HSA-FeP (10, 20, and 40 vol %) (Fig. 2). The addition of a certain amount of ADP increased the PAC-1 positive cells, for instance, 96.9 % at 10 μM in the PBS group. It is rather remarkable that the coexistence of HSA and HSA-FeP (10–40 vol %) did not disturb this concentration dependence of the ADP-stimulation (Fig. 2).

The P-selectin (CD62P) on the activated platelet interacts with vascular endothelial cells to induce hemostasis. The percent of the P-selectin positive cells in the sample with HSA or HSA-FeP solution was 3.30–5.57% independent of the mixing ratio (10, 20, and 40 vol %) (Fig. 3). The addition of ADP enhances the numbers of active cells, and the concentration dependence curves observed in the HSA and HSA-FeP groups ([ADP] = 0.05–10 μM) were all identical to that of the PBS group. These results revealed that the platelet activation in response to the ADP-stimulation was not influenced by HSA-FeP. We concluded that albu-

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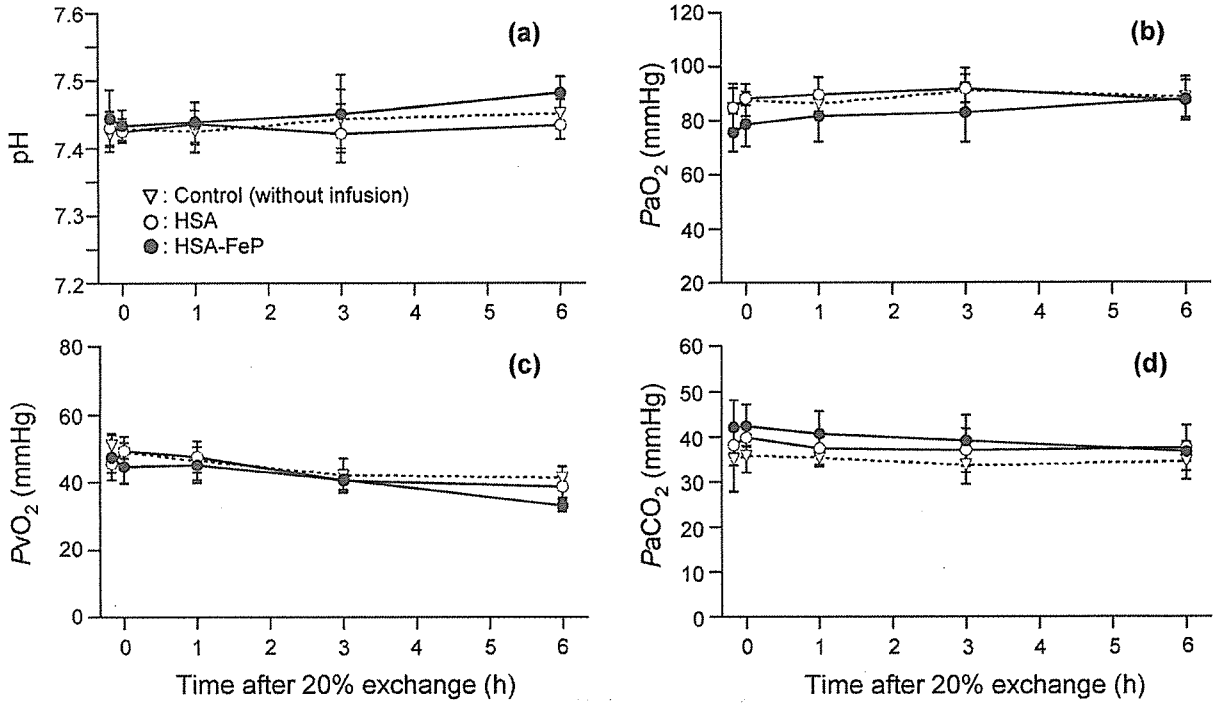

Figure 5. Time courses of (a) blood pH, (b) arterial blood O_2-pressure (PaO_2), (c) venous blood O_2-pressure (PvO_2), and (d) arterial blood CO_2-pressure ($PaCO_2$) in anesthetized rats after 20% exchange transfusion with HSA-FeP or HSA solution. Each value represents the mean ± SD ($n = 6$).

min-heme does not facilitate the immunological reaction and platelet activation in human blood at least based on the present test situation and degree.

Circulation parameters

After the 20% exchange transfusion with the HSA or HSA-FeP solution, Hct decreased ~80% of the basal values [Fig. 4(a)]. The body temperature of each group was maintained constant within 36.9–37.4°C during the experiments [Fig. 4(b)]. The time courses of MAP and HR of the HSA-FeP group were almost the same as those of the control or HSA group for 6 h [Fig. 4(c,d)]. Our previous studies showed that the infusion of HSA-FeP did not induce vasoconstriction and hypertension because of the low permeability of the albumin scaffold through the vascular endotheterlium.[20] It has been again demonstrated that absolutely no vasoactive response occurs after the 20% volume infusion of HSA-FeP.

Blood gas parameters

Changes in the blood gas parameters during the 20% exchange transfusion are shown in Figure 5(a–d). Differences in pH among the three groups were in the narrow range of 7.42–7.48 [Fig. 5(a)]. The PaO_2, PvO_2, and $PaCO_2$ values of the control, HSA, and HSA-FeP groups were also constant in the range of 78.9–91.8, 33.2–49.5, and 33.9–42.6 mmHg, respectively, by the end of the measurements [Fig. 5(b–d)]. These results revealed that HSA-FeP satisfies the initial preclinical safety as an RBC substitute.

In summary, one of the most prominent characteristics of the HSA-based O_2-carrier is its high blood compatibility, and no effect on the human immunological reaction and platelet activation. The appearance of the animals demonstrated no change for 6 h after the 20% exchange transfusion with HSA-FeP. The physiological responses in the HSA-FeP group were identical to those of the control and HSA groups. These results allow us to now undertake further advanced preclinical testing of this entirely synthetic O_2-carrier.

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