Figure Legends

Figure 1. Changes in mean pulmonary arterial pressure (MPAP), mean arterial pressure (MAP), pulmonary capillary wedge pressure (PCWP), central venous pressure (CVP), heart rate (HR), cardiac output (CO), systemic vascular resistance (SVR), and pulmonary vascular resistance (PVR) during hemorrhagic shock and resuscitation with infusion of rHSA alone, shed SAB and HbV/rHSA. The values are mean ±SD. #,§: significantly different between HbV/rHSA group ($p<0.05$)

Figure 2. Changes in pH, PaO_2, $PaCO_2$, base excess (BE), lactate, and PvO_2 during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB and HbV/rHSA. The values are mean ± SD. There was no significant difference between rHSA or SAB and HbV/rHSA group.

Figure 3. Change in hemoglobin (Hb) concentration, hematocrit (Hct), and lactic acid (LA) level during hemorrhagic shock and resuscitation with infusion of rHSA alone, SAB, and HbV/rHSA. The values are mean ± SD. Composition of Hb concentration in the whole blood in HbV/rHSA group (B).

Figure 4. (**Top**) Changes in oxygen delivery (DO_2), and oxygen consumption (VO_2) during hemorrhagic shock and resuscitation with infusion of rHSA alone, sSAB and HbV/rHSA. **(Bottom)** The rates of oxygen delivery derived from dissolved oxygen in plasma (DO_2(Plasma)), hemoglobin of RBCs (DO_2(RBC)), and HbV (DO_2(HbV)) in total oxygen delivery (DO_2) of the HbV/rHSA group and oxygen consumption derived from dissolved oxygen in plasma (VO_2(Plasma)), hemoglobin of RBCs (VO_2(RBC)), and HbV (VO_2(HbV)) in total oxygen consumption (VO_2) of the HbV/rHSA group. The values are mean ± SD. # significantly different vs. the HbV/rHSA group ($p < .05$).

Figure 5. One-year observation of changes in body weight, hematocrit (Hct), white blood cells count (WBC), and platelet count (PLT) after resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # significantly different vs. the autologous shed blood group ($p < .01$).

Figure 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 ($p < .01$); * significantly different vs. the autologous shed blood group ($p < .01$). AST = aspartate aminotransferase; ALT = alanine aminotransferase; LDH = lactate dehydrogenase; ALP = alkaline phosphatase; γ GTP = 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.

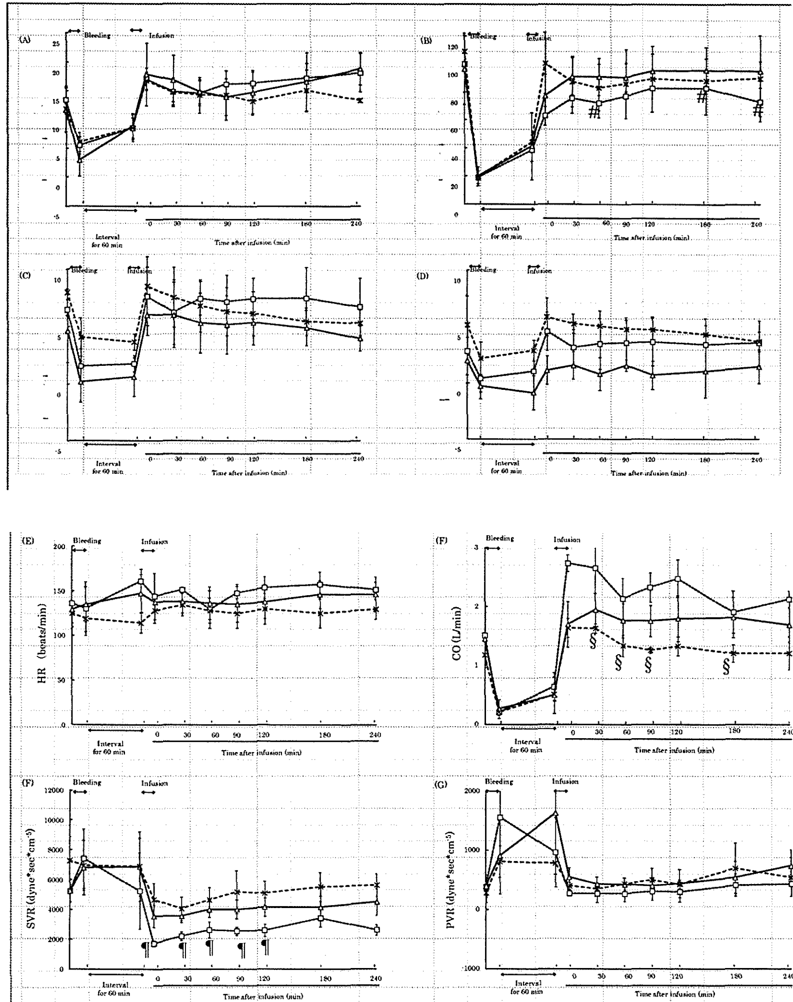
Figure 7. Plasma biochemical tests relating the metabolism of the components of HbV (lipids and Hb), microelements, and electrolytes during one year after 40 percent hemorrhagic shock and resuscitation with infusion of SAB and HbV/rHSA. The values are mean ± SD. # Significantly different from baseline ($p < .01$); *

significantly different vs. the autologous shed blood group ($p < .01$). T-chol = total cholesterol; F-chol = free cholesterol; HDL-chol = high density lipoprotein cholesterol; TG = triglyceride; FFA = free fatty acid; T-Bil = toal bilirubin; IP = inorganic phosphate.

Figure 8. Histology of spleen and liver of HbV/rHSA group at 4 hours, and 28 and 365 days after resuscitation. The presence of spleen macrophages and liver Kupffer cells phagocytizing HbV particles was shown at 4 hours. The liver and spleen at 28days contained slight brown pigment deposition. No significant change is noted at 365days. Scale bar, 100 μm. Hematoxylin and eosin stains.

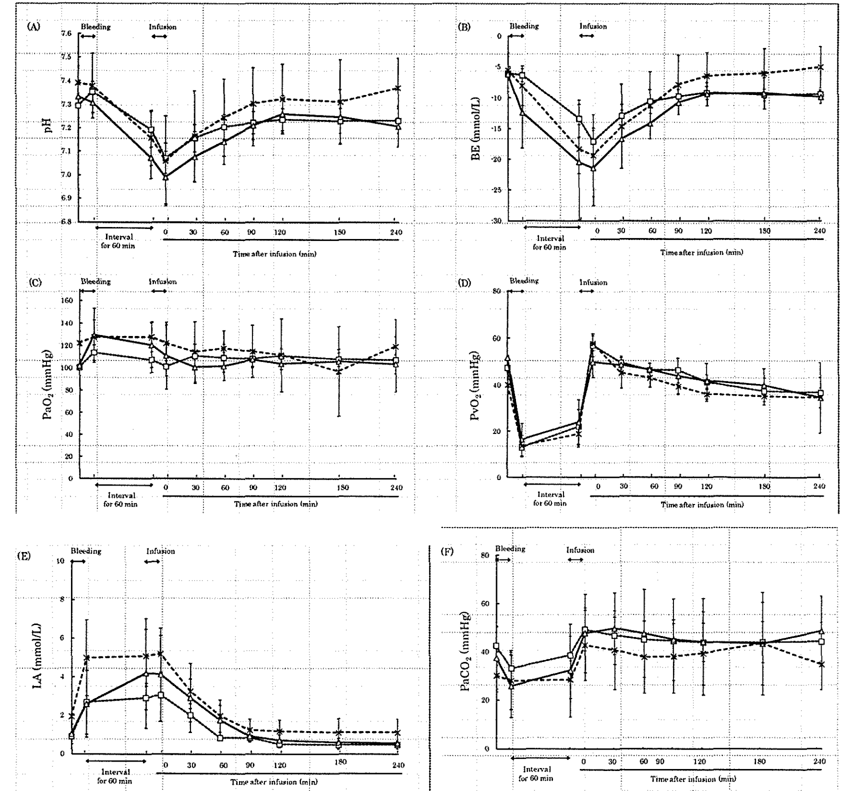
Figure 9. Histology of kidneys (A), heart (B), pancreas (C), and lungs (D) of HbV/rHSA group at 365 days after resuscitation. No significant change is noted in these organs. Scale bar, 100 μm. Hematoxylin and eosin stains.

Figure 1. ×SAB, △HbV/HSA, □rHSA



#,§,†¶: significant differences between HbV/rHSA group

Figure 2. ×SAB, △HbV/HSA, □rHSA



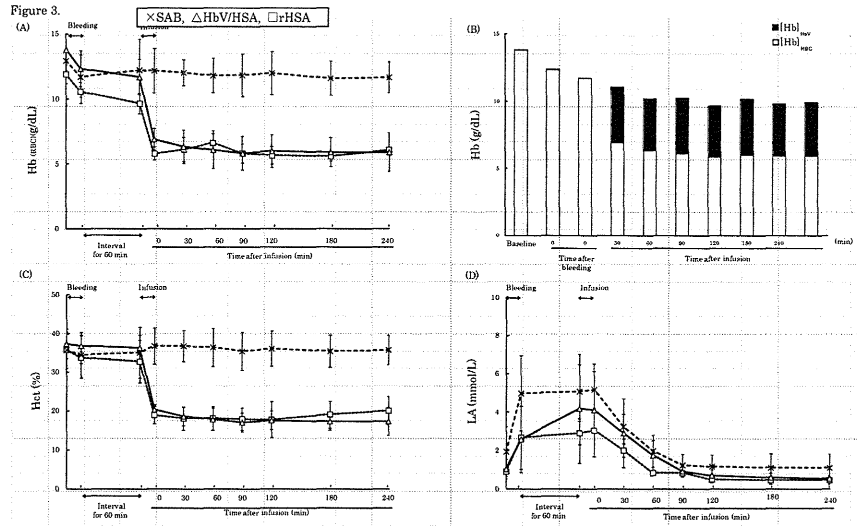


Figure 3.
×SAB, △HbV/HSA, □rHSA
(A)
Bleeding
Infusion
Hb (g/dL)
Interval for 60 min
Time after infusion (min)
(B)
[Hb]HbV
[Hb]RBC
Hb (g/dL)
Baseline
Time after bleeding
Time after infusion
(min)
(C)
Bleeding
Infusion
Hct (%)
Interval for 60 min
Time after infusion (min)
(D)
Bleeding
Infusion
LA (mmol/L)
Interval for 60 min
Time after infusion (min)

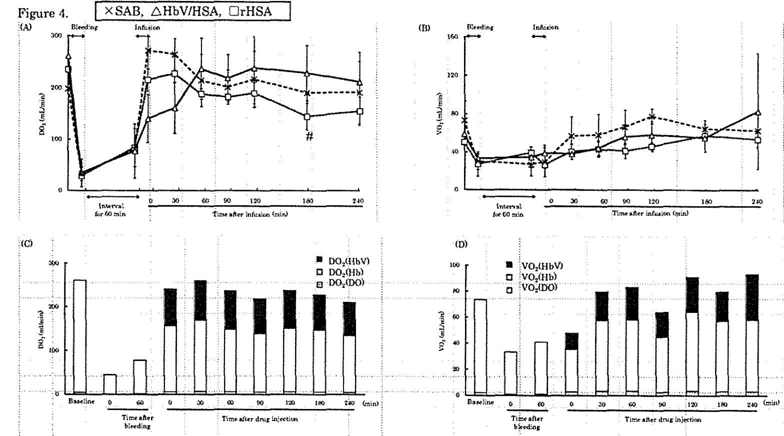


Figure 4.
×SAB, △HbV/HSA, □rHSA
(A)
Bleeding
Infusion
Interval for 60 min
Time after infusion (min)
(B)
Bleeding
Infusion
Interval for 60 min
Time after infusion (min)
(C)
DO2(HbV)
DO2(Hb)
DO2(DO)
Baseline
Time after bleeding
Time after drug injection
(min)
(D)
VO2(HbV)
VO2(Hb)
VO2(DO)
Baseline
Time after bleeding
Time after drug injection
(min)

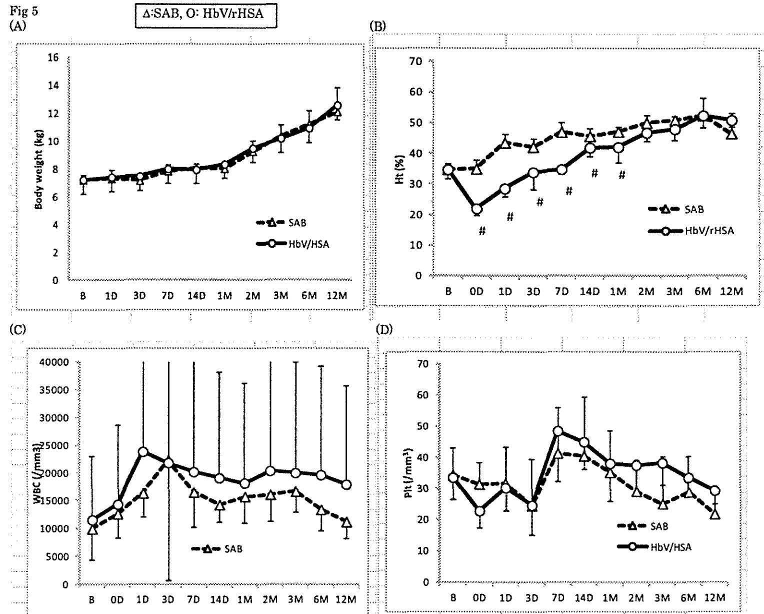
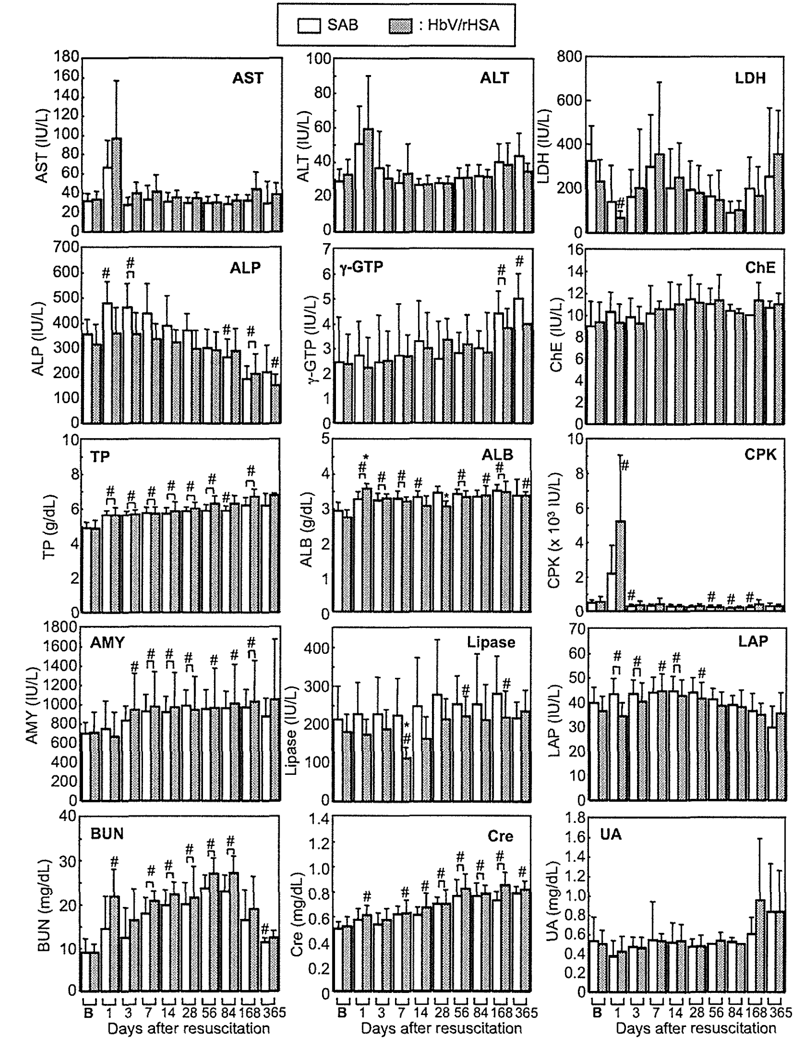


Fig 5
(A)
Δ:SAB, O: HbV/rHSA
Body weight (kg)
B 1D 3D 7D 14D 1M 2M 3M 6M 12M
SAB
HbV/HSA
(B)
Ht (%)
B 0D 1D 3D 7D 14D 1M 2M 3M 6M 12M
SAB
HbV/rHSA
(C)
WBC (/mm3)
B 0D 1D 3D 7D 14D 1M 2M 3M 6M 12M
SAB
(D)
Plt (/mm³)
B 0D 1D 3D 7D 14D 1M 2M 3M 6M 12M
SAB
HbV/HSA


SAB
: HbV/rHSA
AST
AST (IU/L)
ALT
ALT (IU/L)
LDH
LDH (IU/L)
ALP
ALP (IU/L)
γ-GTP
γ-GTP (IU/L)
ChE
ChE (IU/L)
TP
TP (g/dL)
ALB
ALB (g/dL)
CPK
CPK (x 10³ IU/L)
AMY
AMY (IU/L)
Lipase
Lipase (IU/L)
LAP
LAP (IU/L)
BUN
BUN (mg/dL)
Cre
Cre (mg/dL)
UA
UA (mg/dL)
B 1 3 7 14 28 56 84 168 365
Days after resuscitation

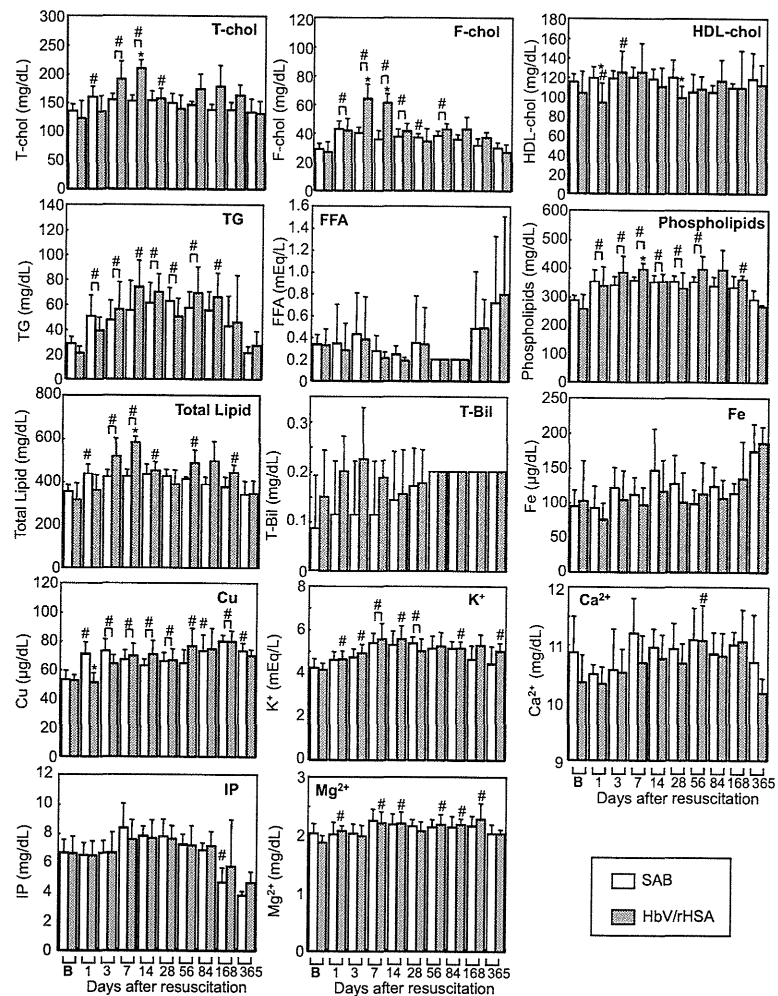


Figure 7

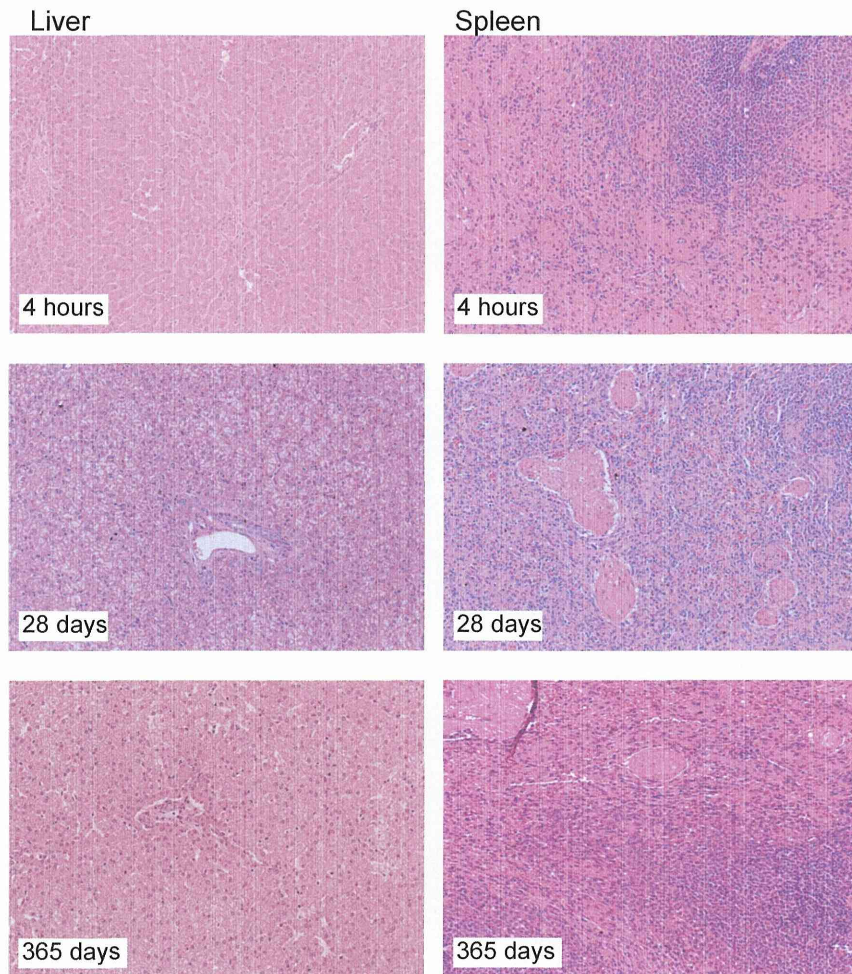


Figure 8

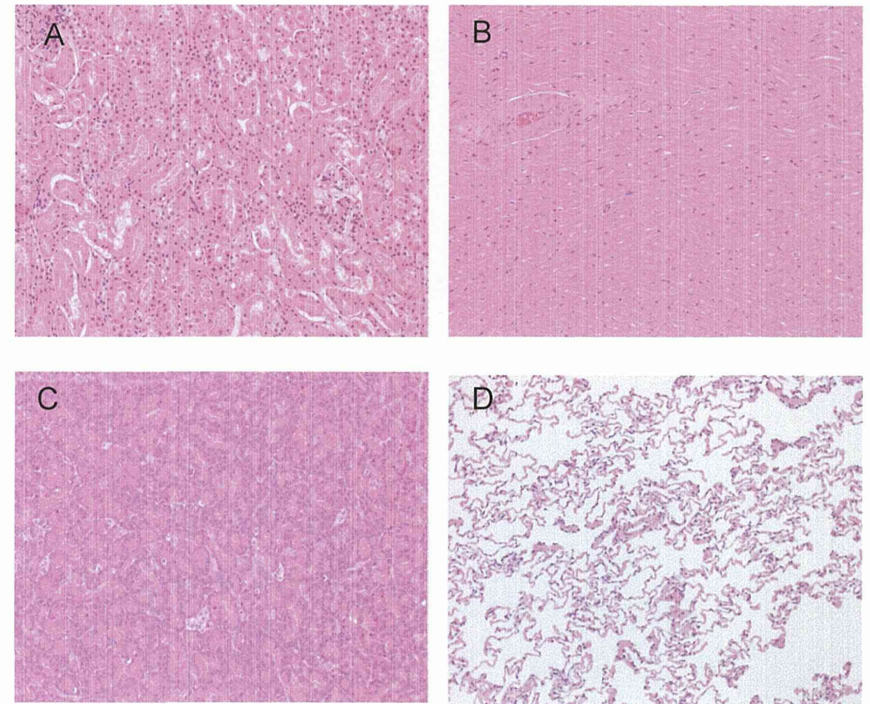


Figure 9

Chapter 21. Hemoglobin vesicles (HbV)

Biocompatibility of hemoglobin vesicles, a cellular-type artificial oxygen carrier, on blood cells and plasma proteins in vitro and in vivo

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Abstract

Hemoglobin vesicles (HbV), cellular-type artificial oxygen carriers, are human hemoglobin encapsulated within a phospholipid bilayer membrane. Because HbV are injected intravenously, the biocompatibility of the HbV with blood components is extremely important to ensure their safety for clinical use. We evaluated this biocompatibility by particularly examining the influence of HbV on human blood cells and on plasma proteins in vitro. 1) Regarding influences to platelets that are involved not only in hemostasis but also in inflammation, HbV themselves neither activate platelets nor show aberrant effect on agonist-induced platelet activation. 2) HbV do not affect the agonist-induced activation of neutrophil functions that play important roles in innate immunity. 3) HbV do not affect hematopoietic progenitor activity. 4) The HbV examined herein (containing DHSG) do not activate the complement system, but old-type HbV (containing DPPG, no PEG modification) do so to a marked degree. 5) Neither coagulation nor the kallikrein–kinin cascade was affected by the current type of

HbV. 6) Multiple HbV infusions in rats cause a transiently slight decrease in the complement titer only after first infusion, with no allergic reaction. No anaphylactic shock was observed in rats administered multiple empty vesicles without hemoglobin. 7) Infusion of HbV can "transiently" render HbV phagocytized-splenic cells immunosuppressive in ex vivo culture condition.

Results of our in vitro and in vivo investigations show that HbV are highly biocompatible with human blood cells, human plasma proteins, and rat immune systems.

21.1 Introduction

Vigorous efforts have been undertaken to develop hemoglobin (Hb)-based oxygen carriers (HBOCs) for use as red blood cell substitutes [1]. HBOCs present several potential benefits for red blood cell transfusion applications, including the absence of blood-type antigens and infectious viruses and the ability to be stored stably for long time periods. HBOCs are expected to satisfy emergency purposes until allogeneic transfusion of compatible red cells. Moreover, their use can satisfy requirements for huge amounts of red cells in times of catastrophe. Consequently, HBOCs can contribute to construction of an ideal blood program when used in conjunction with present allogeneic transfusion capabilities.

HBOCs are categorized into two types: acellular modified Hb molecules and cellular liposome-encapsulated Hb. Actually, hemoglobin-vesicles(HbV, developed by Waseda University) are of the latter type. They have phosphatidylcholine, cholesterol,

PEG-conjugated lipid, a negative charged lipid, and concentrated Hb molecules, as do actual red blood cells [2]. Their sufficient O_2 transport capability, comparable with that of blood, has been established in several animal models [1]. The distribution of HbV after administration and the prompt metabolism of HbV in the reticuloendothelial system have been demonstrated [3, 4].

The biocompatibility of HbV is an important issue for the clinical use of these materials. Several indexes of biocompatibility have been proposed. In this chapter, we present biocompatibility of HbV for human blood cells and human plasma proteins in vitro, and in immune systems in rat models.

21-2-1 Effect of HbV on human platelet function

Circulating platelets bind to the subendothelial matrix of injured vessels and subsequently become activated, causing the release or the expression of components in their intracellular granules and the formation of metabolic products. These products include prothrombotic substances (e.g., adenine nucleotides, thromboxane A_2 [TXA_2], serotonin, and CD62P) [5] and an array of potent proinflammatory chemokines (e.g., RANTES, MIP-1) [6]. Prothrombotic substances function as agonists for the recruitment of additional platelets into the evolving thrombus. Chemokines released from the activated platelets trigger the recruitment of leukocytes into the evolving thrombus and play a large role in the initiation and perpetuation of inflammatory responses [7].

Platelet activation is apparently necessary to prevent bleeding in vivo. However,

nonphysiological activation engenders pathological thrombosis and the modulation of inflammatory responses. The biocompatibility of HbV and human platelets was evaluated by examining the effects of HbV on the most frequently used platelet activation markers (i.e., CD62P expression and the binding of activation-dependent $\alpha_{IIb}\beta_3$ antibody PAC-1 to platelets) in the presence or absence of agonists in vitro. We also investigated the effects of high concentrations of HbV (up to 40%) on the secretion of other substances (i.e., serotonin, RANTES, and β-thromboglobulin [β-TG]) and the formation of thromboxane B_2 (TXB_2), a metabolite of TXA_2.

Table 1. Effect of HbV on human platelets

Index	Stimulant	Type of HbV	Conc. of HbV	Effect
RANTES release	Collagen (+)	DPPG-HbV	≤ 20%	No effect
	(-)	DPPG-HbV	≤ 20%	No effect
RANTES release	Collagen (+)	DHSG-HbV	≤ 40%	No effect
	(-)	DHSG-HbV	≤ 40%	Marginal reduction
β-TG release	Collagen (+)	DHSG-HbV	≤ 40%	No effect
	(-)	DHSG-HbV	≤ 40%	No effect
Serotonin release	Collagen (+)	DHSG-HbV	≤ 40%	No effect
	(-)	DHSG-HbV	≤ 40%	No effect
TXB_2 production	Collagen (+)	DHSG-HbV	≤ 40%	No effect
	(-)	DHSG-HbV	≤ 40%	No effect
CD62 expression	ADP (+)	DHSG-HbV	≤ 40%	No effect
	(-)	DHSG-HbV	≤ 40%	No effect
PAC-1 binding	ADP (+)	DHSG-HbV	≤ 40%	Slight potentiation
	(-)	DHSG-HbV	≤ 40%	No effect

In this series of experiments, our earlier formulation of HbV containing DPPG (DPPG-HbV) and the present formulation of HbV containing a different type of negative charged lipid, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (DHSG) (DHSG-HbV) were used. Table 1 presents our results, demonstrating that incubation of human platelets to high concentrations of HbV in vitro did not cause platelet activation. Moreover, it did not adversely affect the formation or secretion of prothrombotic substances or proinflammatory substances in response to platelet agonists. Although a marginal

reduction of spontaneous release of RANTES by HbV and a slight potentiation of ADP-triggered PAC-binding in the presence of HbV were noted, these effects were regarded as less meaningful from a clinical perspective [8, 9]. Results show that HbV has superior biocompatibility to human platelets.

21-2-2 Effects of HbV on neutrophil functions

Neutrophils play important roles on host defense against various infectious agents. Neutrophils perform various functions (e.g., chemotaxis, superoxide generation) in response to zymosan as well as bacterially derived peptides such as N-formyl-methionyl-leucyl-phenylalanine (fMLP) (10). A certain type of liposome modified with PEG-distearoyl-phosphatidylethanolamine (PEG-DSPE) was reported to reduce chemotaxis in response to these agents [11]. In contrast, liposomes composed of phosphatidylcholine and phosphatidylserine have been shown to recruit neutrophil in the lungs of allergic-model mice [12]. The interaction of HbV and neutrophils is apparently important in terms of the biocompatibility of HbV. With this in mind, we evaluated effects of HbV on four major functions of human neutrophils in response to fMLP (Table 2).

The results of the earlier formulation of HbV containing DPPG (DPPG-HbV) are shown in Table 2. Pre-incubation with HbV did not affect fMLP-triggered chemotaxis, upregulation of CD11b expression, degranulation of gelatinase granules (gelatinase-B), or superoxide generation under those experimental conditions [13]. Consequently, the composition of phospholipid in HbV neither suppresses nor activates the fMLP response of human neutrophils, which makes it highly biocompatible with neutrophils.

Table 2. Effect of DPPG-HbV on fMLP-induced neutrophil functions

Function	Conc. of HbV	Effect
Chemotaxis	≤0.6%	No effect
Upregulation of CD11b expression	≤ 0.6%	No effect
Degranulation of Gelatinase B	≤ 6%	No effect
Superoxide production	≤ 6%	No effect

21-2-3 Effects of HbV on human hematopoietic stem/progenitor cells

The large amounts of liposomes infused intravenously have been shown to distribute into the mononuclear phagocytic system (MPS) including Kupffer cells in the liver and macrophages in the spleen and bone marrow (14). A radiolabeling study revealed that HbV administered intravenously distribute mainly to the liver, spleen, and bone marrow (4). Concern has arisen over whether the HbV which are distributed into bone marrow might adversely affect hematopoiesis because the bone marrow is the major site of hematopoiesis. From this perspective, rats that received an acute 40% exchange-transfusion with HbV showed complete recovery of hematocrit within 7 days

because of elevated erythropoietic activity [15]. Furthermore, the number of red blood cells, leukocytes, and platelets remained unchanged for 1 week after the infusion of HbV at 20% of the whole blood volume. Findings obtained in these animal models strongly suggest the absence of inhibitory activity of HbV against hematopoiesis. However, the influence of HbV on human hematopoietic stem/progenitor cells has not yet been studied. We sought to evaluate the effect of HbV on the proliferation and differentiation of both the erythroid and myeloid lineages of cord blood (CB)-derived hematopoietic cells in liquid culture [16].

Table 3. Effect of DHSG-HbV on the proliferation of erythroid and myeloid lineage cells in liquid culture.

Exposure period to HbV	$CD235a^+$ cells			$CD15^+$ cells		
	HbV conc.(%)			HbV conc. (%)		
	0.75	1.5	3.0	0.75	1.5	3.0
20h	93.7 ± 10.0	94.9 ± 1.2	92.2 ± 8.8	100.8 ±14.3	96.3 ±7.9	96.6 ±13.3
3 days	85.2 ± 22.3	92.6 ± 11.5	89.0 ± 14.5	92.9 ± 6.1	95.8 ± 5.4	91.7 ± 4.7

Various concentrations of HbVs were added to the medium containing the cord blood-derived $CD34^+$ cells. After 10 days' incubation, $CD235a^+$ cells for erythroid lineage and $CD15^+$ cells for myeloid lineage, respectively, were analyzed by flow cytometry. The number of $CD235a^+$ cells or $CD15^+$ cells at each concentration of DHSG-HbV is expressed as a percentage of the number in the control (HbV 0%). Data are represented as the mean ± SD from three experiments performed on three separate cord blood donors.

As shown in Table 3, the incubation of HbV with CB-derived $CD34^+$ cells for up to 3 days had less effect on the proliferation of erythroid lineage ($CD235a^+$ cells) and myeloid lineage cells ($CD15^+$ cells). Furthermore, the incubation of HbV with CB-derived CD34+ cells for up to 3 days had no adverse effect on the clonogenic activity of CB-derived hematopoietic cells

(data not shown).

We previously established a coculture system of human telomerase catalytic subunit-transfected bone marrow stromal cells and $CD34^+$ cells in vitro, by which the expansion of human hematopoietic stem/ progenitor cells is visible. Using this in vitro expansion system, we found that the incubation of HbV with CB-derived $CD34^+$ cells up to 3 days had no adverse effect on the expansion of CB-derived hematopoietic stem/ progenitor cells (data not shown) (17). Taken together, the evidence shows that HbV are apparently biocompatible with human CB-derived hematopoietic stem/progenitor cells.

21-2-4 Effects of HbV on complement systems, coagulation, and the kallikrein–kinin pathway in human plasma

Negatively charged liposomes activate complements via both classical and alternative pathways in rat and human models (18–20). Consequently, the reticuloendothelial system rapidly removes opsonized liposomes from blood circulation. Furthermore, complement activation can engender cardiovascular and pulmonary adverse responses, called complement activation-related pseudoallergy

Table 4. Consumption of complement by HbV and Liposome

Additive	CH50 (U/mL) (additive:serum) 20:80	40:60
Saline	33.4 ± 2.8	21.4 ± 1.7
DHSG-HbV	33.5 ± 2.9	22.9 ± 2.4
EL-A	25.1 ± 2.7*	5.9 ± 0.7*

The complement titer (CH50) was measured using a 50% hemolysis assay with a commercial kit. DHSG-HbV, saline or Coatsome EL-A (a negative-charged liposome) were mixed with serum at the indicated ratio (v/v) at 37°C for 24 hr. The lipid composition (mol%) of Coatsome EL-A was DPPC:CHOL:DPPG=30:40:30. Data are presented as mean ± SD using sera from five individuals. The CH50 of 100% serum was 38 ± 3.2 U/mL. *$p < 0.05$ vs. saline.

(CARPA) (21, 22). Indeed, certain types of liposome-encapsulated hemoglobin cause CARPA in pigs (23).

A negatively charged surface also triggers intrinsic coagulation pathway and the kallikrein–kinin cascade by activating coagulation factor XII (24, 25). PEGylation was regarded as effective for prevention of complement activation by liposomes (26–28).

We evaluated the interaction of HbV between human plasma using HbV of three types: PEGylated HbV having DHSG (DHSG-HbV), PEGylated HbV having DPPG (DPPG-HbV), and DPPG-HbV without PEGylation (DPPG-HbV (no PEG)) (29). Coatsome EL-A was used as a highly negative-charged liposome without PEGylation. The EL-A greatly reduced the complement titer, but DHSG-HbV had no effect (Table 4).

Among the three types of HbV, DHSG-HbV and DPPG-HbV show no reduction of the complement titer, although DPPG-HbV (no PEG) showed drastic reduction (Table 5).

Table 5. Consumption of complement by various types of HbV

Additive	CH50 (U/mL) (additive:serum) 20:80	40:60
Saline	36.4	27.9
DHSG-HbV	37.6	31.4
DPPG-HbV	35.9	28.4
DPPG-HbV (no PEG)	29.9	Under detection limit

Complement titer (CH50) was measured using a 50% hemolysis assay using a commercial kit. DHSG-HbV, DPPG-HbV, DPPG-HbV (no PEGylation) or saline was mixed with serum as indicated ratio (V/V) at 37°C for 24 hr. The CH50 of 100% serum was 45.1 U/mL.

In terms of coagulation activity, DHSG-HbV had no effect on the prothrombin time (PT) or on the activated partial

thromboplastin time (APTT), but DPPG-HbV and DPPG-HbV (no PEG) tended to shorten APTT (data not shown). Furthermore, DHSG-HbV did not cause activation of the kallikrein–kinin cascade even when DHSG-HbV was mixed with plasma at 60%, whereas DPPG-HbV (no PEG) and DPPG-HbV caused activation of the kallikrein–kinin cascade, producing a digested product. Collectively, DHSG-HbV, which is PEGylated HbV of the most advanced type, is highly biocompatible with human plasma protein.

21-2-5 Effects of HbV on complement and anaphylactic reactions in rats

CARPA represents a novel subcategory of acute (type I) hypersensitivity reactions (HSR), which are mostly mild, transient, and preventable using appropriate precautions (30). However, in an occasional patient, it can be severe or even lethal. Because a main manifestation of complement activation is cardiopulmonary distress, CARPA might be a safety issue primarily in cardiac patients. Although PEGylation is regarded as effective for prevention of complement activation by liposome, clinical experience shows that even PEGylated liposomal anti-cancer drug caused CAPRA, suggesting that PEGylation is insufficient to escape from the complement system in vivo (31–33). Therefore, we evaluated whether the infusion of HbV into rats affects the complement titer in vivo (34).

A transient decrease of the complement titer of the rat serum was apparent three days after the infusion of HbV or empty vesicle without hemoglobin (EV) (Fig. 1).

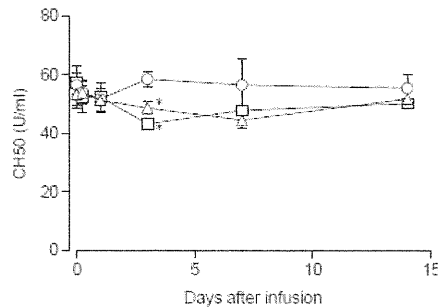

Figure 1. Changes of the complement titer in the rat serum after HbV infusion. HbV (triangles), EV (squares), or saline (circles) was infused into the rats at top-load from the tail vein. The complement titer in the rat serum was measured. It is shown as CH50.

Neither HbV nor EV caused the consumption of the complement in rat serum in vitro (data not shown). It is particularly interesting that a repeated-infusion study showed that only first infusion of HbV reduced the complement titer. Despite additional infusions of HbV, gradual recovery of the complement titer occurred, suggesting that additional infusions of HbV did not cause the complement consumption (Fig. 2).

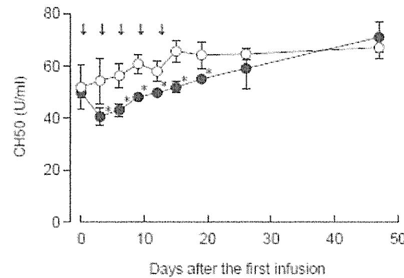

Figure 2. Effects of repeated infusion of HbV on the rat serum complement. HbV (closed circles) or saline (open circles) was infused into rats at top-load from the tail vein at the time points indicated by arrows. *N*=3--5, mean±SD. *p<0.05

Furthermore, multiple administration of EV caused no anaphylactic shock, although

ovalbumin-sensitized rats died with symptoms of respiratory distress after the second ovalbumin administration (data not shown). Regarding the evidence collectively, the administration of HbV is apparently safe, without allergic or anaphylactic reactions.

Intravenous injection of liposomes into pigs reportedly induces anaphylactoid reactions at small doses, resulting in circulatory disorder. Therefore, the pig model is regarded as useful for the safety evaluation of liposome drugs. HbV did not cause a significant anaphylactoid reaction in pigs, thereby reconfirming the high biocompatibility of HbV [35].

21-2-6 Effect of HbV on immune response of rat splenocytes

Large amounts of HbV must be transfused to substitute for allogeneic red blood cell transfusion in a clinical setting. Therefore, a considerable number of liposome particles must accumulate in the MPS after HbV infusion, mainly in the spleen and liver (14). Consequently, it is possible that the immune response fluctuates because of phagocytic cells, which phagocytize HbV, because those cells can become not only positive regulators of immune response

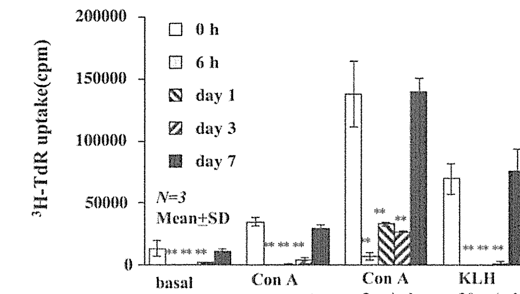

Figure 3. Effect of HbV and empty vesicles on proliferation of Con A-stimulated rat splenic T cells.
Rats were immunized with KLH. After 7 days, they were injected with HbV. Spleens were excised at 6 h, 1 day, 3 days, and 7 days later. Bulk splenocytes were stimulated with Con A or KLH. The proliferative response of splenic T cells to Con A and KLH was inhibited from 6 h to 3 days after injection of HbV compared to control (**:p<0.01). No suppression was observed after 7 days. Data are representative of at least three independent experiments and are expressed as the mean±SD (cited from reference 39).

as an antigen presenting cells but also negative regulators designated as suppressor macrophages. Reportedly, the production of nitric oxide was involved in its suppressive effect [36–38]. However, the latter effect has been of little concern, possibly because the amount of liposome used as a drug vehicle is so small that it has no notable negative effect on the immune system in an experimental animal model. This possibility has been addressed recently by our colleagues with the infusion of large numbers of liposomal particles (HbV) [39]. Normal rat splenocytes proliferate well in response to Concanavalin A (Con A) stimulation. However, when the rat splenocytes were taken out 24 h after infusion of HbV (20% of total blood volume), they failed to proliferate in response to Con A stimulation. When the splenocytes were taken at seven days after HbV injection, this immune suppression was no longer observed (Fig. 3). These results show a transient effect of HbV infusion on immune response. The time course of the suppression appeared to be correlated with that of accumulation and disappearance of HbV from the spleen

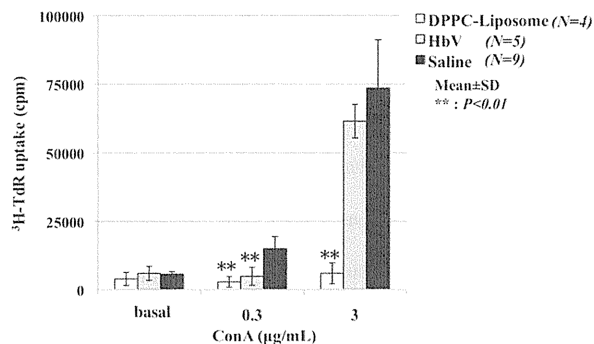

Figure 4. Effect of DPPC-liposomes on immune suppression. HbV, EV, DPPC-liposome or saline was injected intravenously. The spleen was excised 18 h later. DPPC-liposome induced immune suppression. Data from 2--3 independent experiments are collected and expressed as the mean±SD (cited from reference 39).

evaluated based on a histochemical analysis (3, 4). The suppressive effect can also be induced by injection of empty liposome particles composed of DPPC only (Fig. 4), indicating that transient immune suppression is unavoidable as long as the current liposome particle is used as a vehicle for Hb molecules.

Extensive analyses were performed to elucidate the mechanism underlying this phenomenon. Results obtained so far are the following: 1) T cells were activated and express IL2 receptor (CD25) but were unable to proliferate. 2) T-cell proliferation specific to keyhole limpet hemocyanin (KLH) was also inhibited from 6 h to 3 days after the injection of liposomes (Fig. 1). 3) Direct cell-to-cell contact was necessary for the suppression. 4) Both iNOS and arginase inhibitors restored T cell proliferation to some degree. 5) Cells that trapped vesicles were responsible for suppression. 6) Most of them expressed CD11b/c, but lacked class II molecules. To

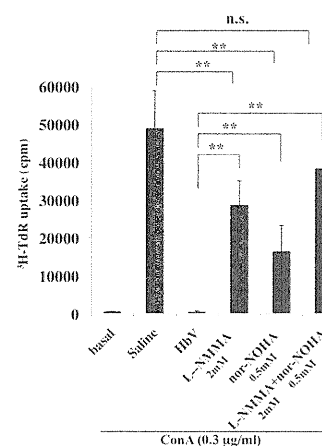

Figure 5. Effect of L-NMMA and nor-NOHA on suppression of T cell proliferation. Each rat was injected with HbV or saline. Splenocytes were stimulated with Con A (0.3 μg/ml) in the presence or absence of iNOS inhibitor (L-NMMA, 2 mM) or arginase inhibitor (nor-NOHA, 0.5 mM) or both. T cell proliferation was restored in the presence of each inhibitor to a certain degree. Significant inhibition disappeared in the presence of both inhibitors, suggesting that both iNOS and arginase were involved in the suppression. Data from two independent experiments were collected and expressed as the mean±SD ($N=5$) (cited from reference 39). **: p<0.01. n.s. : not significant.

summarize these results, the phagocytosis of a large load of liposomal particles by rat CD11b/c+, class II- immature monocytes temporarily renders them highly immunosuppressive. In addition, nitric oxide, possibly produced from cells that phagocytized HbV, is involved in immune suppression. It is noteworthy that the results from an additional experiment showed that HbV infusion did not interfere in the in vivo production of KLH-specific antibody (unpublished data), suggesting that the observed immune suppression is restricted in spleen and not systemic phenomenon.

These data and the effects observed on other blood components revealed the excellent and satisfactory bioavailability of HbV and are expected to guarantee the application of HbV to human as a blood substitute in the near future. Finally, from a different perspective, the observed immunosuppressive effect induced by liposomes might open new fields for the clinical application of liposomes themselves.

21-3 Conclusion

In this chapter, we presented the excellent biocompatibility of HbV with human blood cells, human plasma proteins in vitro, and rat immune systems in vivo. Along with several lines of evidence, our data demonstrate that HbV are promising candidates for use as artificial oxygen carriers.

Acknowledgement

The work presented here in was supported in part by Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan)

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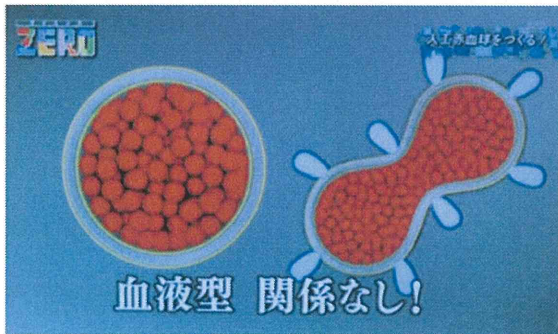
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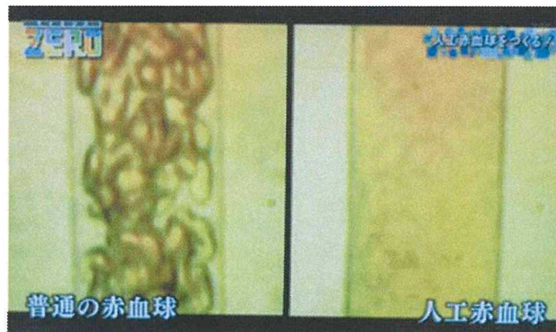
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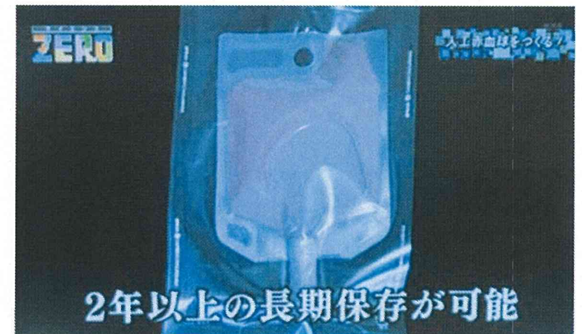
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今年度のノーベル医学生理学賞が山中伸弥・京都大学iPS細胞研究所長に決まり、科学界は沸いている。数個の遺伝子の操作で、どんな細胞にも育ちうる万能のiPS細胞（人工多能性幹細胞、新型万能細胞）は間違いなく近年の日本技術の頂点だが、実はそれに匹敵するような目ざましい技術がないわけではない。

輸血用として長期保存が可能

2012年9月27、28日に東京国際フォーラムで開かれた大学見本市「イノベーション・ジャパン2012」で、早稲田大学重点領域研究機構の酒井宏水・上級研究員グループが発表した人工赤血球の開発がその一つだ。

赤血球は 3週間程度しか保存できないが、グループは期限切れの赤血球から主成分のヘモグロビンを精製、脂質膜で包んだ直径250ナノメートル (ナノは10億分の1) のヘモグロビン小胞体に再生する技術を確立した。途中で摂氏60度10時間の加熱処理が可能になったため、感染源を排除して長期間保存でき、血液型もない。空気に触れるとすぐに酸素と結合する。輸血用人工血液としては、病院で長期保存できるので、緊急事態や不測の災害にも対応でき、血液型の間違いもなくせる。また、濃厚酸素液として脳梗塞や心筋梗塞直後の治療、臓器保存などの用途も考えられる。脳梗塞治療に有効なことはネズミ実験で確認ずみ。実験室では数リットル規模の製造法が完成している。

大学見本市は大学の研究者と企業を結びつける場。土田英俊・理工学部教授 (故人) らが約20年前に合成に成功して以来、早稲田大学は人工血液研究で世界をリード、すでに実用化段階に達したと見られる。しかし、これまで強い関心を示したのは米国やイスラエルなどの軍関係企業で、血液は日本赤十字社の独占事業になっていて制約の多い日本では、企業の関心は高くない。「厚生労働省など国の研究費が投入された人工血液を日本で生産し、世界中に輸出して役立てたいのだが...」と、グループの研究者は訴えていた。

（医療ジャーナリスト・田辺功）

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