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Solution to the problems of acellular hemoglobins by encapsulation and the intrinsic issues of hemoglobin vesicles as a molecular assembly

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Publication data
Received: 8 August 2007
Revision received: 5 September 2007
Accepted: 13 September 2007

Keywords
- Artificial red cells
- Blood substitutes
- Complement activation
- Liposome
- PEG
- Suspension rheology

SUMMARY

Hemoglobin vesicles (HbV), or liposome-encapsulated hemoglobins, are developed as artificial oxygen carriers for the use as a transfusion alternative. The safety and efficacy of HbV have been clarified in detail: HbV can overcome the side effects of hemoglobin (Hb) molecules (stroma-free, and intra- or intermolecularly crosslinked) such as vasoconstriction, hypertension and possible vascular damage induced by direct contact of the vascular surface with Hb. On the other hand, intrinsic issues related to the suspension of HbV as a molecular assembly have to be considered: blood compatibility, structural and dispersion stabilities of the vesicles, and the requirement of prompt degradation in the reticuloendothelial system. Having overcome these issues, the results make us confident in advancing further development of HbV. Easy manipulation of physicochemical parameters of HbV provides possibilities for various clinical applications in addition to their use as a transfusion alternative.

PHYSIOLOGICAL IMPORTANCE OF THE RED BLOOD CELL CELLULAR STRUCTURE FOR ENCAPSULATED HEMOGLOBIN DESIGN

It has been well documented during the long history of the development of hemoglobin (Hb)-based oxygen carriers (HBOCs, Figure 1) that many side effects of stroma-free Hb and chemically modified Hbs exist: renal toxicity; entrapment of gaseous messenger molecules [nitric oxide (NO) and carbon monoxide (CO)] inducing vasoconstriction, hypertension, reduced blood flow, and reduced tissue oxygenation at microcirculatory levels[1-4]; neurological disturbances; malfunction of esophageal motor function[5]; and myocardial lesions.[6,7] These side effects of Hb molecules imply the importance of the cellular structure of red blood cells (RBCs). From the retrospective and recent observations, the main justifications for Hb encapsulation in RBCs are: (i) a decreased high colloidal osmotic pressure[8]; (ii) prevention of the removal of Hb from blood circulation; (iii) prevention of direct contact of toxic Hb molecules and endothelial lining[9]; (iv) preservation of the chemical environment in

doi: 10.1111/j.1778-428X.2007.00089.x

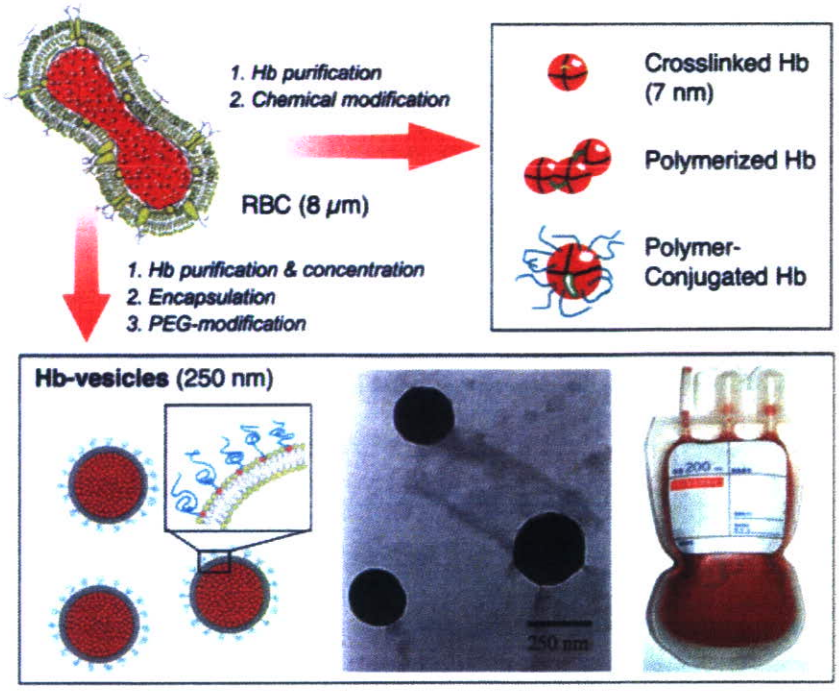

Figure 1. Schematic representation of a series of Hb-based oxygen carriers. In the case of Hb-vesicles (Hbv), the purified and concentrated Hb solution (35 g/dL) is encapsulated in phospholipid vesicles and the surface is modified using PEG chains. The particle size is well regulated at 250 nm, which is apparent from the transmission electron micrograph. The color of HbV is like a mixture of red wine and milk, due to the light scattering effect of small particles. Hb, hemoglobin; PEG, polyethyleneglycol; RBC, red blood cell.

cells, such as the concentration of phosphates [2,3-diphosphoglycerate (2,3-DPG), adenosine triphosphate, etc.] and other electrolytes; (v) RBCs are the major component that renders blood as non-Newtonian and viscous, which is necessary to pressurize the peripheral artery for homogeneous blood distribution and for maintenance of blood circulation[10]; and (vi) the cellular structure of RBCs retards oxygen release in comparison to acellular Hb solutions,[11,12] thereby retaining oxygen to peripheral tissues where oxygen is required.

For those reasons, the optimal structure of HBOCs might be to mimic the RBC cellular structure. The pioneering work of Hb encapsulation to mimic the cellular structure of RBCs was performed in 1957 by Chang,[13] who prepared microcapsules (5 μm) made of nylon, collodion and other materials. Toyoda[14] in 1965 and the Kambara-Kimoto group[15] in 1968 also investigated encapsulation of Hbs with gelatin, gum arabic, silicone, etc. Nevertheless, results emphasized the extreme difficulty in regulating the particle size to be appropriate for blood flow in the capillaries and to obtain sufficient biocompatibility. After Bangham and Horne[16] reported in 1964 that phospholipids assemble to form vesicles in aqueous media, and that they encapsulate water-soluble materials in their inner aqueous interior, it seemed reasonable to use such vesicles for Hb encapsulation. Djordjevich and Miller[17] in 1977 prepared liposome-encapsulated Hb (LEH) composed of phospholipids, cholesterol, fatty acids, etc. The US Naval Research Laboratories showed remarkable progress in the use of LEH.[18–20] Terumo Corporation (Tokyo) developed a different LEH called Neo Red Cells (Table 1).[21,22]

However, some intrinsic issues of encapsulated Hbs remained, which were mainly related to the nature of molecular assembly and particle dispersion. What we call HbV, with their high-efficiency production processes and improved properties, were established by our group based on technologies of molecular assembly in concert with precise analyses of pharmacological and physiological aspects (Table 2).[23–25] We use stable carbonylhemoglobin (HbCO) for purification with pasteurization at 60°C for 10 hours. The purity of the obtained Hb solution is extremely high.[26,27] Utilization of the stable and purified HbCO enables higher concentrations than 40 g/dL using ultrafiltration and easy handling of encapsulation by the extrusion method without causing protein denaturation. It has been confirmed that HbV encapsulates nearly 35 g/dL with a thin bilayer membrane. In the final process, HbCO in HbV is photodissociated by irradiation of visible light under an oxygen atmosphere to convert HbO_2.[28]

Table 1. A list of representative LEH extensively studied aiming at industrialization

Product Name	Group	Characteristics	Current status
Hb-vesicles (HbV)	Waseda University and Keio University	1. Pasteurization of HbCO at 60°C for virus inactivation, and high purity and concentration of encapsulated Hb 2. Lipid composition to improve blood compatibility 3. PEG modification and deoxygenation for 2 years storage 4. (Hb) = 10 g/dL	Preclinical
Neo Red Cells (NRC)	Terumo Corporation	1. Inositol hexaphosphate to regulate P_{50} (= 40–50 torr) 2. Lipids: HSPC/cholesterol/fatty acid/PEG-lipid 3. Storage in a refrigerator for 6 months 4. (Hb) = 6 g/dL	Preclinical
Artificial Red Cells (ARC)	NOF Corporation and Waseda University	1. Polymerized lipids (DODPC) for stabilization 2. Storage in powdered or frozen state 3. Difficulty in degradation in RES	Suspended
LEH	US Naval Research Laboratory	1. Freeze-dried powder with trehalose 2. Low Hb encapsulation efficiency 3. Thrombocytopenia, complement activation	Suspended
Synthetic erythrocytes	Rush-Presbyterian-St. Luke's Medical Center, University Illinois	1. The first attempt of LEH	Suspended

DODPC, 1,2-dioctadecadienoyl-*sn*-glycero-3-phosphatidylcholine; Hb, hemoglobin; HbCO, carbonylhemoglobin; HSPC, hydrogenated soy phosphatidylcholine; LEH, liposome-encapsulated Hbs.

Table 2. Characteristics of Hb-vesicles developed in Waseda University

Parameter	
Particle diameter	240–280 nm
P_{50}	25–28 torr
(Hb)	10 g/dL
Suspending medium	Physiologic saline solution (0.9% NaCl)
Colloid osmotic pressure	0 torr
Intracellular Hb concentration	ca. 35 g/dL
Lipid composition	DPPC/cholesterol/DHSG/DSPE-PEG_{5000}
Weight ratio of Hb to lipids	1.6–1.9 (w/w)
Stability for storage at room temperature	2 years
Circulation half-life	32 hours (rats)

DHSG, 1,5-*O*-dihexadecyl-N-succinyl-L-glutamate; DPPC, 1,2.dipalmitoyl-*sn*-glycero-3-phosphatidylcholine; DSPE-PEG, 1,2-distearoyl-*sn*-glycero-3-phosphatidyethanolamine-*N*-polyethyleneglycol; Hb, hemoglobin.

The oxygen-bound HbV can provide oxygen-transport capacity that is both sufficient and comparable to that of RBCs in experiments related to extreme blood exchange[29-34] and fluid resuscitation from hemorrhagic shock.[35-38] A recent experiment of HbV as a priming solution for cardiopulmonary bypass in a rat model showed that HbV protects neurocognitive function by transporting oxygen to brain tissue even when the hematocrit is markedly reduced.[39] Other studies investigating HbV suspension as a possible perfusate for organ transplantation are also underway for the heart, liver, intestine, etc.

In fact, Hb encapsulation provides a unique opportunity to add new functions to particles. Other regulators, such as antioxidants and enzymes, can be embedded on the capsule or coencapsulated to reduce methemoglobin (metHb),[40-42] as can allosteric effectors to modulate oxygen affinity (P_{50}).[33,43] The P_{50} of HbV is regulated by coencapsulation of pyridoxal 5′-phosphate (PLP) in place of 2,3-DPG. The present HbV, being developed by Waseda University, contains PLP at PLP/Hb = 2.5 by mol; the resulting P_{50} is about 25–28 torr, which shows sufficient oxygen transporting capacity as a transfusion alternative. The P_{50} of HbV without PLP and Cl^- is 8–9 torr. This formulation is effective for targeted oxygen delivery to anoxic tissues caused by reduced blood flow.[34,44,45]

In addition to HbV, new encapsulated Hbs without liposomes have emerged with the use of recent advanced nanotechnologies, such as polymersome,[46] polyethyleneglycol (PEG)-poly(ε-caprolactone) copolymer nanoparticles,[47] and *in vivo* evaluation of oxygen-carrying capacities of these new materials is anticipated. Encapsulation of Hb can reduce the toxicity of cell-free Hbs. However, many hurdles must be surmounted to realize encapsulated Hbs because of the components of the capsules themselves and their structural complexity as a molecular assembly. It is also important to consider the larger dosage requirement of encapsulated Hb for blood substitution in comparison with those available with conventional drug delivery systems, which require no large dosage.

STRUCTURAL STABILIZATION OF ENCAPSULATED HEMOGLOBIN FOR STOCKPILING

Hb autoxidizes to form metHb and loses its oxygen-binding ability during storage, as well as during blood circulation. Therefore, prevention of metHb formation is necessary. A method exists to preserve deoxygenated Hbs in a liquid state using well-known intrinsic characteristics of Hb: the Hb oxidation rate in a solution is dependent on the oxygen partial pressure; also, deoxyHb is not autoxidized at ambient temperatures.[48] In the case of HbV, not only the encapsulated Hb but also the capsular structure (liposome) must be physically stabilized to prevent irreversible intervesicular aggregation, fusion and leakage of the encapsulated Hb.

Liposomes, as molecular assemblies, have been generally inferred to be structurally unstable. The US Naval Research Laboratory tested the addition of cryoprotectants and lyoprotectants, such as trehalose, to LEH for its preservation as a powder without causing hemolysis after rehydration.[49,50] In addition, many researchers have developed stabilization methods for liposomes that use polymer chains.[51-54] Polymerization of phospholipids that contain two dienoyl groups (DODPC) was studied extensively in our group. For example, gamma-ray irradiation induces radiolysis of water molecules and generates OH radicals that initiate intermolecular polymerization of dienoyl groups in DODPC. This method produces enormously stable liposomes, resembling rubber balls, which are resistant to freeze-thawing, freeze-drying and rehydration.[55,56] However, the polymerized liposomes were so stable that they were not degraded easily in the macrophages, even 30 days after injection.[57] It became widely believed that polymerized lipids are inappropriate for intravenous injection. Subsequently, it was clarified that the selection of appropriate lipids (phospholipid/cholesterol/negatively charged lipid/PEG-lipid) and their composition are important to enhance the stability of nonpolymerized liposomes.[31,58] Surface modification of liposomes with PEG chains is sufficient for dispersion stability.[32] In fact, in comparison to RBCs, HbV is highly resistant to hypotonic shock, freeze-thawing and enzymatic attack by phospholipase A_2.

We investigated the possibility of long-term preservation of HbV during storage for 2 years through a combination of two techniques: deoxygenation and PEG modification.[59] The PEG chains on the vesicular surface stabilize the dispersion state and prevent aggregation and fusion for 2 years because of their steric hindrance.[60] The original metHb content (ca. 3%) before preservation decreased gradually to less than 1% in all samples after 1 month because of the presence of a

reductant, such as homocysteine, inside the vesicles that consumed the residual oxygen and gradually reduced the trace amount of metHb. The rate of metHb formation was strongly dependent on the oxygen partial pressure: a lack of increase in the metHb formation was observed because of the intrinsic stability of the deoxygenated Hb. In fact, the metHb content did not increase for 2 years. These results indicate the possibility that the HbV suspension can be stored at room temperature for at least 2 years, which would enable stockpiling of HbV for any emergency.

BLOOD COMPATIBILITY OF LIPOSOMES AND HEMOGLOBIN VESICLES

Liposome is not a solute but a particle in a suspension. The surface of the particle may be recognized, interact with blood components, including complements. The so-called *injection reaction*, or pseudoallergy, is caused by complement activation, giving rise to anaphylatoxins that trigger various hypersensitivity reactions. This reaction is sometimes observed not only with liposomal products,[61] but also with fat emulsions[62] and a perfluorocarbon emulsion.[63] Therefore, the examination of blood compatibility of encapsulated Hbs is important for clinical use. Transient thrombocytopenia and pulmonary hypertension in relation to complement activation is an extremely important hematologic effect observed in rodent models after infusion of LEH (containing DPPG: 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidyl glycerol) developed by the US Naval Research Laboratory[64,65] and of other products. In our group, exchange transfusion with the proto-type HbV (containing DPPG, no PEG modification) in anesthetized rats engendered transient thrombocytopenia and slight hypertension.[30] Similar effects were also observed for administration of negatively charged liposomes.[66,67] The transient reduction in platelet counts caused by complement-bound liposomes was also associated with sequestration of platelets in the lung and liver. Such nonphysiological platelet activation probably leads to initiation and modulation of inflammatory responses as platelets contain an array of potent proinflammatory substances. However, it must be emphasized that the present HbV formulation apparently does not induce thrombocytopenia in animal experiments, probably because the present HbV contains PEG-modification and a different type of negatively charged lipid (DHSG: 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate), not DPPG or a fatty acid.[68-70]

Detailed blood compatibility of HbV in relation to negatively charged lipid was examined by Dr H. Ikeda at the Hokkaido Red Cross Blood Center (Sapporo) and his colleagues.[69-72] The present PEG-modified HbV containing DHSG did not affect the extrinsic or intrinsic coagulation activities of human plasma, although HbV containing DPPG and no PEG modification tended to shorten the intrinsic coagulation time. The kallikrein-kinin cascade of the plasma was activated slightly by the proto-type DPPG-HbV, but not by the present PEG-DHSG-HbV. Moreover, the complement consumption in the plasma was detected by incubation with DPPG-HbV, but not with the present PEG-DHSG-HbV.[71] The exposure of human platelets to high concentrations of the present HbV (up to 40%) *in vitro* did not cause platelet activation and did not adversely affect the formation and secretion of prothrombotic substances or proinflammatory substances that are triggered by platelet agonists. These results imply that HbV, at concentrations of up to 40%, has no aberrant interactions with either unstimulated or agonist-induced platelets. It can be concluded that the present PEG-DHSG-HbV has a higher blood compatibility.

BIODISTRIBUTION, METABOLISM AND EXCRETION OF HEMOGLOBIN VESICLES

The dosage of blood substitutes should be considerably larger than those of other drugs, while their circulation time is considerably shorter than that of RBCs. Therefore, their biodistribution, metabolism, excretion and side effects must be characterized in detail, especially in relation to the reticuloendothelial system, RES (or termed the mononuclear phagocytic system).

Normally, free Hb released from RBC is bound rapidly to haptoglobin and is consequently removed from circulation by hepatocytes. However, when the Hb concentration is greater than the haptoglobin binding capacity, unbound Hb is filtered through the kidney, where it is actively absorbed. Hemoglobinuria and eventual renal failure occur when the reabsorption capacity of the kidney is exceeded. The encapsulation of Hb in vesicles completely suppresses renal excretion. However, HbV in the bloodstream is ultimately captured by phagocytes in the RES in much the same manner as senescent RBCs are, as confirmed by radioisotope ^{99m}Tc-labelled HbV

injection.[19,68] The HbV are finally distributed mainly in the liver, spleen and bone marrow. The circulation half-life is dose-dependent; when the dose rate was 14 mL/kg, the circulation half-life was 32 hours in rats. The circulation time in the case of the human body can be estimated as two or three times longer; or about 2 or 3 days at the same dose rate.

It is generally accepted that the liposome clearance by RES at a small dosage is accelerated by opsonization (absorption of plasma proteins such as antibodies and complements on the liposomal surface); PEG-modification prevents opsonization for prolonged circulation times.[73] However, considering the condition that the dosage of HbV is extremely high and requires a considerable amount of opsonins, and that HbV does not induce complement activation,[71] then the opsonin-dependent phagocytosis would not be a major component in the case of HbV with a large dosage. Actually, opsonin-independent phagocytosis, a direct recognition by macrophages, has been clarified in some studies.[74,75]

Transmission electron microscopic analysis of the spleen 1 day after infusion of HbV revealed the presence of HbV particles in the phagosomes of macrophages.[76] However, after 7 days, the HbV structure cannot be observed. We confirmed transient splenomegaly with no irreversible damage to the organs and complete metabolism within a week. Immunochemical staining with a polyclonal antihuman Hb antibody was used as the marker of Hb in the HbV, clarifying that HbV almost disappeared after 7 days in both the spleen and liver.

Bilirubin and iron are believed to be released during metabolism of Hb, but our animal experiments of topload infusion, daily repeated infusions and 40% blood exchange showed that neither of those products increased in the plasma within 14 days.[77-79] The released heme from Hb in HbV might be metabolized by the inducible form of heme oxygenase-1 in the Kupffer cells of the liver and the spleen macrophages. Bilirubin would normally be excreted in the bile as a normal pathway; no obstruction or stasis of the bile is expected to occur in the biliary tree. Berlin blue staining revealed considerable deposition of hemosiderin in the liver and spleen, even after 14 days. Hemosiderosis often occurs in patients who have received repeated blood transfusions because of the shorter half-life of the stored RBCs. Moderate splenomegaly and hemosiderin deposition were also confirmed in the spleen after injection of stored RBCs, partly because of the accumulation and degradation of stored RBCs with lowered membrane deformability and shortened circulation half-life.[79]

As for membrane components of Hb-vesicles, phospholipids are metabolized and reused as a component of the cell membrane, or excreted in bile, especially as fatty acids and CO_2 in exhaled air. The plasma cholesterol level elevated transiently 3 days after injection, that was released from macrophages after degradation of HbV in the phagosomes.[77,79] However, the plasma phospholipid level did not increase significantly. It was recently clarified using ^{3}H-cholesterol that the cholesterol of HbV is released from macrophages to blood; it is ultimately excreted in the feces. The PEG chain is widely used for surface modification of liposomal products. The chemical crosslinker of PEG-lipid is susceptible to hydrolysis to release PEG chains during metabolism. The released PEG chains, which are known as inert macromolecules, should be excreted in urine through the kidneys.[80]

In order to know the physiological capacity of RES for degradation of HbV, we tested massive intravenous doses by daily repeated infusion of 10 mL/kg/day into Wistar rats for 14 days. The cumulative dosage was 140 mL/kg (Hb and lipids, 20,689 mg/kg). The total volume was equal to 2.5 times of whole blood volume (56 mL/kg).[78] Even though the splenohepatomegaly was significant, all rats tolerated the infusions, and the body weight increased until the intentional sacrifice for the succeeding 14 days. The phagocyted HbV disappeared though significant hemosiderin deposition and was confirmed in the spleen, liver, kidney, adrenal grand and bone marrow. We could not define a lethal dose of HbV in this experiment.

The profile of liposome clearance is species-dependent. More precise data are necessary to extrapolate the phenomena observed in animal experiments to humans. However, these results imply that the metabolism and excretion of HbV are within the physiological capacity that has been well characterized for the metabolism of senescent RBCs and conventional liposomal products.

UNIQUE RHEOLOGICAL PROPERTY OF HEMOGLOBIN VESICLES SUSPENSION

The extremely high concentration of the HbV suspension [(Hb) = 10 g/dL; (lipids) = 6 g/dL, volume fraction, ca. 40 vol%] imparts an oxygen-carrying capacity that is comparable to that of blood. The HbV suspension does

not possess a colloid osmotic pressure (COP) because one HbV particle (ca. 250 nm diameter) contains about 30,000 Hb molecules, and HbV acts as a particle, not as a solute. Therefore, HbV must be suspended in or coinjected with an aqueous solution of a plasma substitute. This requirement is identical to that for emulsified perfluorocarbon, which does not possess COP;[81,82] it contrasts to characteristics of other HBOCs, intramolecular crosslinked Hbs, polymerized Hbs and polymer conjugated Hbs, which all possess very high COP as protein solutions.[8,83]

Animal tests of HbV suspended in plasma-derived human serum albumin (HSA) or recombinant HSA (rHSA) showed an oxygen-transporting capacity that is comparable to that of blood.[36,39] We reported previously that HbV suspended in plasma-derived HSA or rHSA was almost Newtonian: no aggregation was detected microscopically.[31,32] In Japan, rHSA will be approved for clinical use in 2007,[84] but various plasma substitutes are used worldwide, such as hydroxylethyl starch (HES), dextran (DEX), and modified fluid gelatin (MFG). The selection among these plasma substitutes should be determined not only according to their safety and efficacy, but also by the related price, experience of clinicians and customs of respective countries. Water-soluble polymers generally interact with particles such as polystyrene beads, liposomes and RBCs to induce aggregation or flocculation.[85,86] For that reason, it is important to determine the compatibility of HbV with these plasma substitutes. With that background, we studied rheological properties of HbV suspended in these plasma substitute solutions using a complex rheometer and a microchannel array.[87] The rheological property of an HBOC is important because the infusion amount should be considerably large, which might affect the blood viscosity and hemodynamics.

The HbV suspended in rHSA was nearly Newtonian. Its viscosity was similar to that of blood, and the mixtures with RBCs at various mixing ratios showed viscosities of 3–4 cP. Other polymers, HES, DEX and MFG, induced flocculation of HbV, possibly by depletion interaction, and rendered the suspensions as non-Newtonian with the *shear-thinning* profile.[87] These HbV suspensions showed a high viscosity and a high storage modulus (G') because of the presence of flocculated HbV. On the other hand, HbV suspended in rHSA exhibited a very low G'. The viscosities of HbV suspended in DEX, MFG and high molecular weight HES solutions responded quickly to rapid step changes of shear rates of 0.1–100 s^{-1} and a return to 0.1 s^{-1}, indicating that flocculation formation is both rapid and reversible. Microscopically, the flow pattern of the flocculated HbV perfused through microchannels (4.5 μm deep, 7 μm wide, 20 cmH_2O applied pressure) showed no plugging. Furthermore, the time required for passage was simply proportional to the viscosity.

It has been regarded that lower blood viscosity after hemodilution is effective for tissue perfusion. However, microcirculatory observation shows that, in some cases, lower viscosity decreases shear stress on the vascular wall, causing vasoconstriction and reduced functional capillary density.[88] Therefore, an appropriate viscosity might exist, which maintains the normal tissue perfusion level. The large molecular dimension of HbV result in a transfusion fluid with high viscosity. A large molecular dimension is also effective to reduce vascular permeability and to minimize the reaction with NO and CO as vasorelaxation factors. These new concepts suggest reconsideration of the design of artificial oxygen carriers.[89] Actually, new products are appearing, although they are in the preclinical stage, not only HbV, but also zero-link polymerized Hb[90] and others with larger molecular dimensions and higher oxygen affinities.[91] Erni *et al.* clarified that HbV with a high O_2 affinity (low P_{50}, such as 8–15 torr) and high viscosity (such as 11 cP) suspended in a high-molecular-weight HES solution was effective for oxygenation of an ischemic skin flap.[45,92,93] That study showed that HbV would retain O_2 in the upper arterioles, then perfuse through collateral arteries and deliver oxygen to the targeted ischemic tissues, a concept of targeted oxygen delivery by an HBOC.[44] Some plasma substitutes cause flocculation of HbV and hyperviscosity. However, reports show that hyperviscosity would not necessarily be deteriorative and might be, in some cases, advantageous in the body.[10] The combination of HbV and plasma substitute solutions provides a unique opportunity to manipulate the suspension rheology, not only as a transfusion alternative, but also for other clinical applications, such as oxygenation of ischemic tissues and *ex vivo* perfusion systems.

CONCLUSION

Other related issues for HbV in a clinical situation include the interference effect of HbV on spectrophotometric measurements in routine clinical laboratory tests

and noninvasive pulse oximetry monitoring of arterial blood oxygen saturation. Such interference is caused by strong light scattering resulting from the small HbV particles in blood.[94] We clarified that HbV can be removed easily from a blood specimen by the addition of high molecular weight dextran and centrifugation. Pulse oximetry can be improved by some modifications of the detection wavelength and software.

Encapsulation of Hb was initiated with the simple idea of duplicating the structure and function of RBCs. However, we are convinced that obstacles remain for the approach to realize the sophisticated function of RBCs; for example, it is impossible to mimic the flexibility of the unfilled biconcave structure of RBCs. The present HbV lacks ionophores in the bilayer membrane which facilitate the transport of small functional molecules from the outer medium, such as ascorbic acid or glutathione, to reduce metHb in HbV that does not contain enzymatic metHb reducing system, because the unstable enzymes are removed during the virus inactivation process of Hb purification.[26,27] On the other hand, clear advantages of simplified HBOCs exist, such as the absence of blood-type antigens and infectious viruses, along with stability for a long-term storage at room temperature for any emergency, all of which might overwhelm the functions of RBCs. The shorter half-life of the HBOCs in the bloodstream (2–3 days) limits their use, but they are applicable as a transfusion alternative for shorter periods of use. Easy manipulation of physicochemical properties of HbV such as P_{50} and viscosity supports the possible development of tailor-made oxygen carriers that suit various clinical indications. The achievements of ongoing HbV research described above give us confidence in advancing further development of HbV, with the expectation of its eventual realization.

ACKNOWLEDGMENTS

The authors acknowledge Professor Marcos Intaglietta (University of California, San Diego) for cooperation in this work and inviting us to participate in this special issue of *Transfusion Alternatives in Transfusion Medicine*. The authors acknowledge Professor Koichi Kobayashi (Keio Univ.), Professor S. Takeoka (Waseda Univ.), Dr H. Ikeda (Hokkaido Red Cross Blood Center), Dr M. Takaori (Higashitakarazuka Satoh Hospital), Professor D. Erni (Inselspital Hospital, University of Berne), Professor W.T. Phillips (University of Texas, San Antonio), Professor M. Otagiri (Kumamoto University), and their active colleagues for meaningful discussions and contributions to this research.

DISCLOSURE

This work was partly supported by Health Sciences Research Grants (Research on Regulatory Science) from the Ministry of Health, Labour and Welfare, Japan. The authors are the holders of patents related to the production and utilization of HbV.

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シンポジウム：重症病態と輸血ヘモグロビンはどのレベルに保つべきか

人工赤血球（HbV）と酸素運搬機能および組織酸素代謝

至適 Hb レベルならびに人工赤血球の展望

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キーワード
Hemoglobin vesicle, Hemorrhagic shock

はじめに

20 世紀初頭，Landsteiner らによる ABO 式血液型の発見以来，現在に至るまで輸血療法が研究され進歩してきた．特に 1980 年代から成分輸血が開始され，現在に至るまで頻繁に使用されている．一方で，多くの合併症の報告がなされてきた．合併症に対し様々な対策が施されてきたが，根本的な解決には至っていない．輸血に関する報告を基に，日本赤十字社が輸血療法の実施に関する指針及び血液製剤の使用指針を発表し，改訂を重ねてきた．しかし輸血を開始する明確な基準は存在せず，現在も議論がつきないところである．平行して人工酸素運搬体も 20 世紀半ばから盛んに行われてきた．我々は，臨床応用の可能性が高い Hemoglobin Vesicle(HbV)の安全性及び効果についての研究に関わる機会を得た．輸血指針に関して文献的考察を加えた上で，HbV の安全性及び効果についての研究の報告をする．

輸血適応の現状

平成 17 年 9 月，厚生労働省は「輸血療法の実施に関する指針」及び「血液製剤の使用指針」（改訂版）を通知した．表 1 に大まかな輸血基準を示す[1]．しかし，厳密にこの基準に従っている医療従事者はいないであろう．実際，輸血の適応を決定する場合には循環動態を注意深く観察し，虚血性心疾患などの基礎疾患などにも注意を払う必要がある．

表 1　急性出血，周術期における輸血基準（RCC）

1．外科的適応として Hb 値が 10g/dL を超える場合は不必要，6g/dL 以下ではほぼ必須
2．周術期は Hb 値が 7～8g/dL あれば十分な酸素供給が可能
「輸血療法の実施に関する指針」及び「血液製剤の使用指針」，平成 17 年 9 月，日本赤十字より

表 2　Indications for RBC transfusions

Parameter	Evidence based: Intra-operatively and ICU/Postoperatively general ward		
Physiologic transfusion trigger			
Relative hypotension	Yes	Yes	Yes
Relative tachycardia	Yes	Yes	Yes
New ST segment depression > 0.1mV	Yes	Yes	Yes
New ST segment elevation > 0.2mV	Yes	Yes	Yes
New wall motion abnormality (TEE/TTE)	Yes	Yes	Not applicable
PvO2 (mmHg)	< 25	< 32	Not applicable
O2 extraction rate (%)	> 50	> 40	Not applicable
SvO2	< 50	< 60	Not applicable
Decrease in VO2 (%)	> 10-50	> 10	Not applicable
Hemoglobin based transfusion trigger			
In all patients	< 6g/dL	7 g/dL	7-8g/dL
In patients > 60 years		7-8g/dL	8-9g/dL
In patients with sever COPD or CHF		8 g/dL	8-9g/dL
In patients with SaO2 < 90%		8-9g/dL	9 g/dL
With fever/hypermetabolism		7-8g/dL	8-9g/dL

（文献 2）より引用）

表 2 に最近の文献から輸血の基準を示す[2]．この基準でもヘモグロビン値が 6 g/dL 以下の場合にはほぼ輸血は必須であるとなっている．しかし臨床報告などでは Hb 値が 6 g/dL 以下でも死亡率は変わらなかったとしている[3)-5)]．

逆に健常成人ボランティアを使用した研究で，isovolemic anemia（Hb 5-6g/dL）の状態で認知機能テストをしたところ，有意に認知機能が障害されたという報告もある[6]．これらのことから，Hb 値 6～7g/dL という値は現在において安全最下限値ではないかと思われる．また冠動脈疾患や脳循環障害のある患者ではやや高い Hb 値 8～10g/dL 前後に保つことが推奨されているが，明確なエビデンスがなく現在も議論が絶えない．

表３　同種血輸血の問題点

- 極めて短い保存期間（3週間）
- 近未来における献血量の不足
- 血液交差試験の必要性
- Infections（HIVウィルス、肝炎ウィルス、未知のウィルスなど）
- Immunological reactions（GVHD、TRALI,異型輸血など）

輸血を取り巻く諸問題

表３に輸血の問題点を示す．保存期間に関しては，それまで保存期間が６週間であったが，エルシニア菌による輸血製剤の汚染が問題になり保存期間が３週間に短縮された．献血量の不足も問題である．実際に平成18年度，全国で使用されたMAP血だけでも572万単位にもおよび，前年に比較して１%増加している．高齢者の増加，若年者の減少といった社会的な問題も献血量不足を悪化していくだろう．

血液交差試験などのような輸血前検査は緊急輸血が必要な時には時間的コストがかかり，臨床に携わるものにとっては問題となることがある．

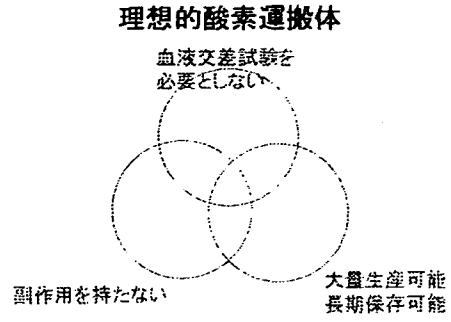
C型肝炎ウイルスやHIVウイルスなど，感染の危険性もある．ウィンドウピリオドにある供血者からの血液は偽陰性となる．過去の輸血に関連したHCV感染のように，未知の感染症に対しては防ぎようもない．近年，日本でも大きな社会問題になり，医療の信頼性に関わる大きな問題に発展したのは記憶に新しい．

同種血輸血自体にも問題があり，GVHDやTRALI等である．これらを防ぐために平成18年度から日本赤十字社では白血球を除去したRCCが供給されている．

理想的な酸素運搬体を考える

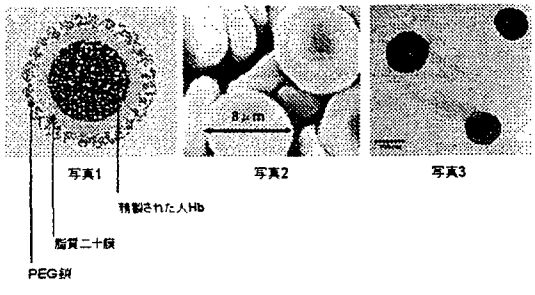
理想的な酸素運搬体とはどのようなものであろうか？　表４に理想的酸素運搬体の条件を示す．理想的酸素運搬体は，医療現場だけでなく，大規模災害における危機対応としての役割も担うことが期待できる．

表４　理想的酸素運搬体



現在まで多くの人工酸素運搬体の研究がなされてきたが，最近10年の進歩は目覚しく，一部の国では臨床応用や臨床治験されるまでになった．今回われわれは，早稲田大学理工学部，土田英俊先生の協力を得て，同大学で開発された人工赤血球（Hemoglobin-Vesicle；HbV）の研究に携わる機会を得たのでここで紹介させていただく．

写真１，２，３
Hemoglobin-Vesiclesとは



HbVとは？

HbVは，保存期間の過ぎたヒトHbを精製し，人工脂質二重膜で封入し表面にポリエチレングリコール鎖を配したものである（写真１，２，３）．特徴としては表面抗原を持たない，ヒト赤血球より径が小さい（1/40）が変形能はないことなどが挙げられる[7]．次に表５に人工酸素運搬体の問題点を挙げる．まず，酸素運搬能はヒト赤血球と同じかどうか？“白い血液”のように酸素解離曲線を持たないと臨床応用は難しい．次に，HbVによる血管収縮作

用はあるのか？　これは修飾 Hb4 量体の臨床試験の失敗[8]に見られるように，血管内皮細胞の間隙に浸透し NO 補足による副作用としての血管収縮，高血圧が知られているからである．3 番目に，凝固系への影響はどうか？代用血漿の大量投与が戦争による負傷者の死亡率を増加させた原因のひとつには出血量の増加かもしれない．4 番目に，HbV 自体の生体への影響はどうか？　これは血液製剤については，保存中に白血球や血小板から放出されるサイトカインによる副作用が懸念されるが，HbV では製剤自体からの産生がないことは有利である．しかし異物である HbV 投与に際して血中の IL-6 や TNF-α の増加の可能性がある．5 番目に，外傷後多臓器不全と肺への影響はどうか？ これは，ISS15 以上の患者に 12 時間以内 6 単位以上の輸血は多臓器不全の独立危険因子であるという報告や実験的に輸血による肺障害の報告があることなどから，HbV に関してもこれらのことが検証されなければならない．つまり，先行する外傷やショックによる侵襲に加え，HbV の投与自体が二次的侵襲とならないかということである．

表 5　人工酸素運搬体の問題点

人工酸素運搬体の問題点

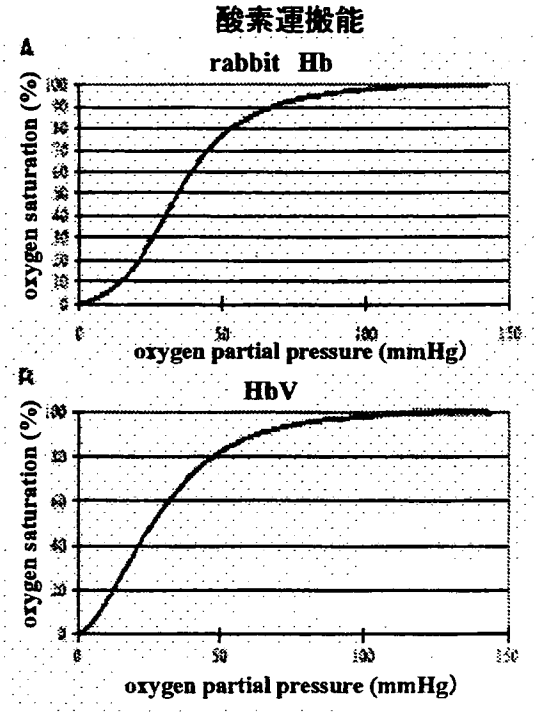
- 酸素運搬能
  - 生体の赤血球と相違は？
- 血管収縮作用(NO scavenger)
  - 修飾Hb4量体の臨床治験の失敗(*J Am Med Assoc* 1999)
- (抗)凝固作用の評価
- 炎症反応
  - 異物としての生体内反応
- 外傷後多臓器不全と肺障害
  - ISS15以上の患者12時間以内6単位以上の輸血はMOFの独立危険因子(*Arch Surg* 1997)
  - 実験的に輸血は肺障害を来たす(*J Clin Invest* 1998)

現在までの研究結果

1）酸素親和性

図 1 に人工赤血球の酸素解離曲線とウサギ Hb の酸素解離曲線を示す．ほぼ同じシグモイドカーブを示しており，P50 の値も 27～30 とヒト Hb に近似した．

図 1　酸素運搬能



2）出血性ショックに対する蘇生

図 2 に，ウサギ出血性ショックモデルを作成し，HbV，リコンビナント 5%アルブミン (rHSA) 溶液，乳酸リンゲル液 (LR)，3 倍量の乳酸リンゲル液 (3×LR)，リコンビナント 5%アルブミン加 HbV 溶液で蘇生したデータを示す．平均動脈圧，中心静脈圧，心係数において脱血時にはそれぞれの群間で有意差はなく，蘇生後 2 時間後において HbV 群と LR 群で有意差が見られた．HbV 投与により，base excess や乳酸値の早期回復が見られた[9]．

3）臓器酸素運搬能

図 3 にウサギ出血性ショックモデルの蘇生実験の際に酸素電極を脳，腎臓，肝臓，筋肉に装着し，蘇生後 15 分後，1 時間後，2 時間後に酸素分圧を計測した．その結果，脳と腎臓において蘇生後 HbV 群で酸素分圧がベースラインに回復し，2 時間以上維持された．肝臓や筋肉では脳や腎臓に比べ，ベースラインへの回復が遅いものの，1 時間後，2 時間後で LR 群に比べ酸素化の改善が認められた．

図2　ウサギ出血性ショックモデルにおける平均動脈圧,中心静脈圧,心係数の経時的変化

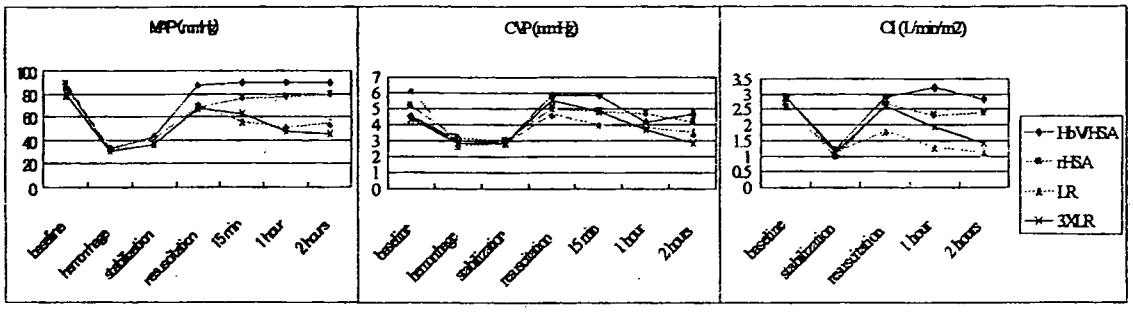
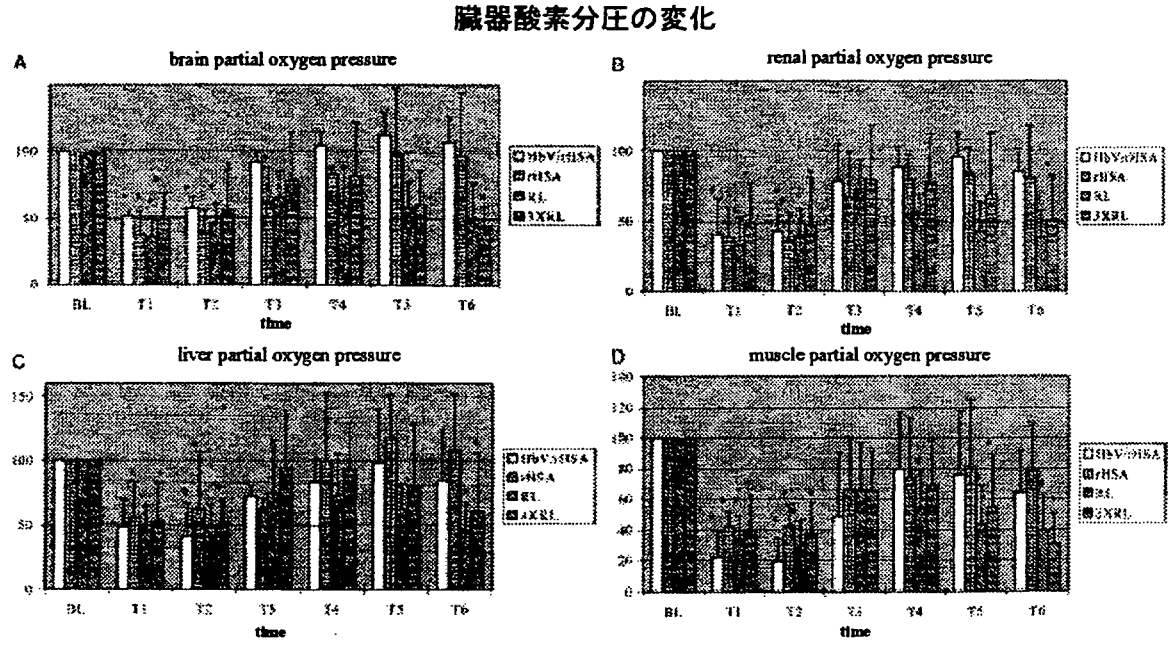


図3　主要臓器の組織酸素分圧：Base line (BL). Hemorrhagic shock (T1).　After stabilization for 30 min (T2). Resuscitation over 15 min (T3). Further measurements for 15 min (T4), 1h (T5), and 2h (T6) after fluid resuscitation.

*Siginificant different from BL($P<0.05$). +Significant difference from the RL group($P<0.05$)

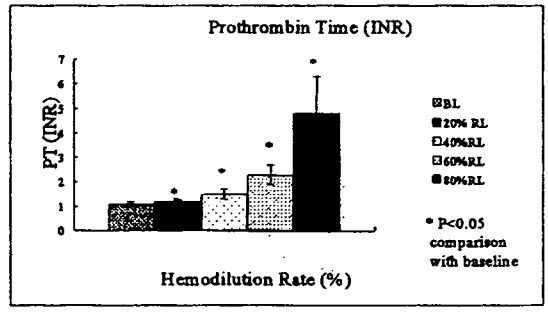


4）凝固機能への影響

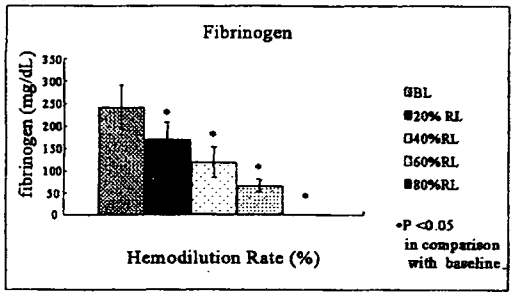
図4に凝固能への影響を調べた結果を示す．健康成人ボランティアの採血血液に対し，乳酸リンゲル液でそれぞれ20%，40%，60%，80%希釈を行った．それぞれのフィブリノゲン濃度，プロトロンビン時間，活性化部分トロンボプラスチン時間を測定した．乳酸リンゲル液の希釈では，希釈率が高くなるにつれてPT，APTT値は延長し，フィブリノゲン値は低下した．図5に同様に乳酸リンゲル液またはHbV溶液で20%，40%，60%，80%の希釈を行いソノクロットで測定した結果を示す．結果から80%希釈においてHbV溶液群はLR群に比べて凝固抑制を示した．60%希釈までは両群間に有意差は認めなかった．コントロールのついていない出血性ショックに対する凝固因子や血小板を含まないHbV単独での蘇生は危険かもしれない．

図4　ヒト全血に対する希釈

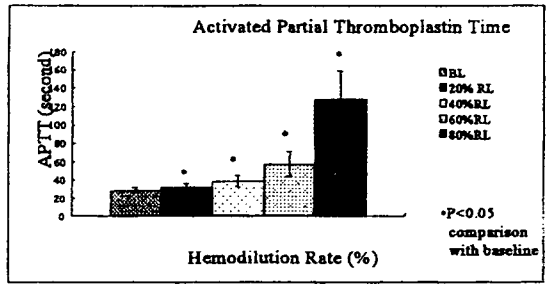
健康成人ボランティアの採血に対する乳酸リンゲル液(RL)と10%HbV溶液による血液希釈
RL溶液ではフィブリノーゲン濃度、プロトロンビン時間、活性化トロンボプラスチン時間を測定
また、両群のソノクロット測定値を比較



PT (INR)

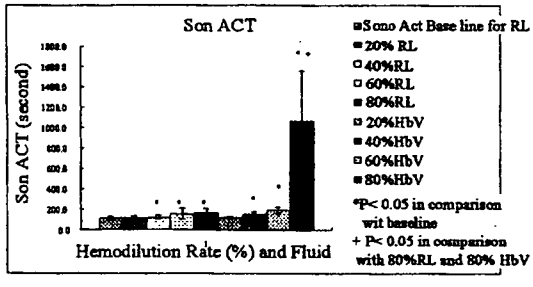


Fibrinogen (mg/dL)

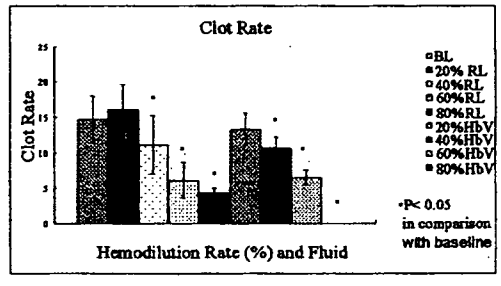


APTT (seconds)

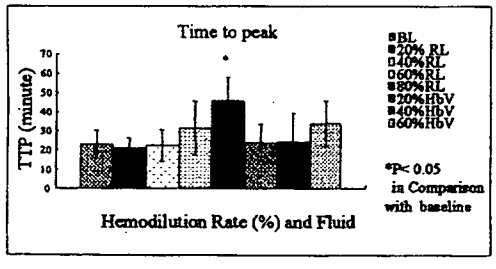
図5　ソノクロットデータ比較



Son-ACT



clot rate



time to peak

80%血液希釈では、HbVのソノクロットデータは、LR溶液に比べ凝固抑制を示す
60%血液希釈までは、両群間に有意差が見られない

5）単純投与による生態への影響

次に，HbV 溶液が生体にどのような影響を与えるか調べた．S-D ラットを使用し，推定循環血液量の 20%の HbV 溶液を投与し，肺の TNF-α，HO-1，HIF1-α，iNOS，ICAM の遺伝子発現を 2 時間後，24 時間後，72 時間後にそれぞれ計測した．測定方法は RT-PCR 法，比較 CT 法を用いた．結果を図 6 に示す．2 時間後の TNF-α にのみ有意差が認められた．

図6　HbV の生体への影響

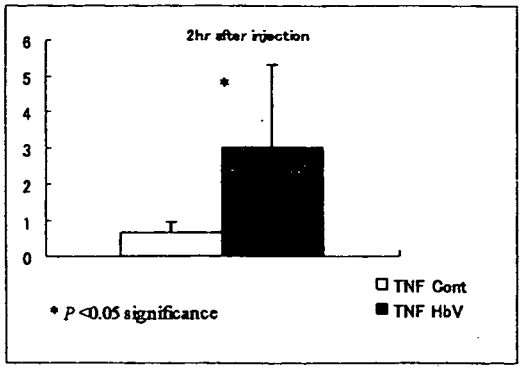
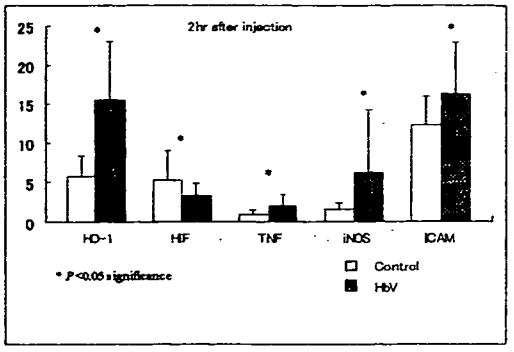


図8　肺における mRNA 発現



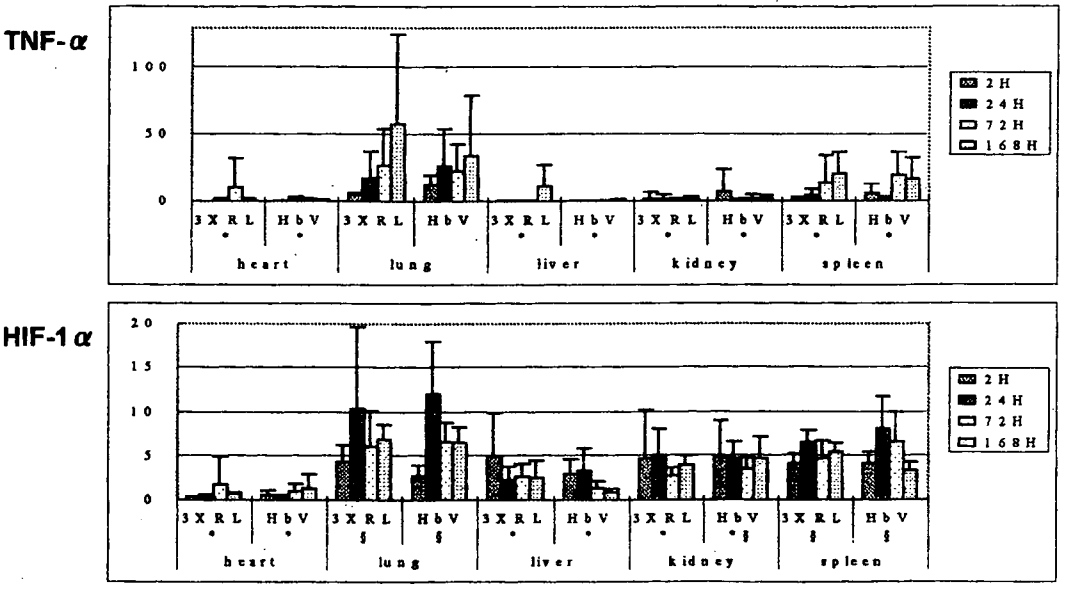
6）出血性ショック後の主要臓器への影響

さらに推定循環血液量の 50%出血性ショックモデルを S-D ラットを用い作製し，蘇生後，TNF-α，HIF1-α の臓器遺伝子発現を 2 時間後，24 時間後，72 時間後，168 時間後に計測した．結果を図 7 に示す．明らかに他臓器に比べ，肺において有意に発現しているのがわかる．しかし，HbV 群と LR 群の両群間で有意差は認められなかった．このことより，出血性ショックが生体にとって大きな侵襲であり，HbV 投与が二次的侵襲とならないことを示唆している．

7）輸血後肺障害への影響

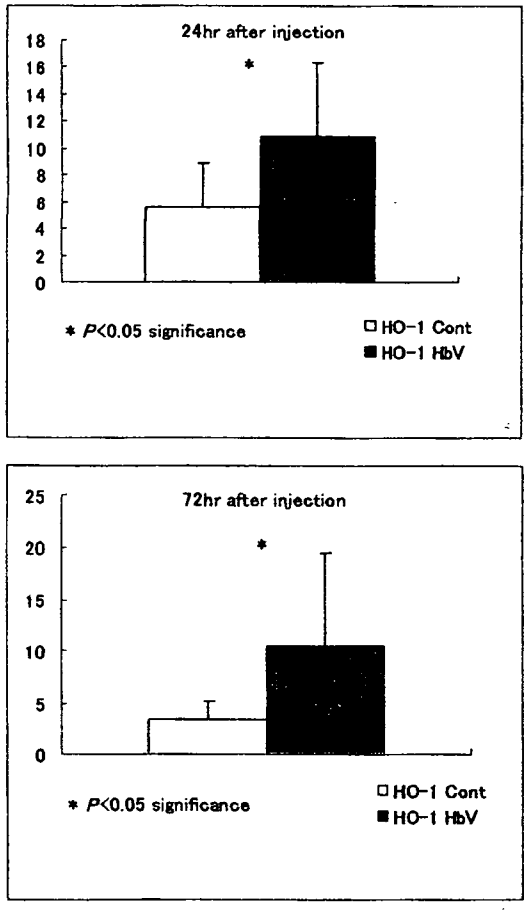
そこで肺において同様に出血性ショックモデルを作製し，蘇生 2 時間後，24 時間後，72 時間後の TNF-α，HO-1，HIF1-α，iNOS，ICAM の遺伝子発現を計測した．結果を図 8 に示す．蘇生開始 2 時間後で HIF1-α を除く全ての発現が増加しており，HbV 群で発現増強していた．しかし 24 時間後，72 時間後では両群間で有意差は認められなかった．逆に HIF1-α は HbV 群で発現が抑えられていた．これは HbV が出血性ショック下生体に対して二次的侵襲を惹起したのではないかと考えられる．しかし，24 時間後，72 時間後で有意差がないことよりその程度が軽度のものではないかと推測される．また，HbV の酸素運搬能により酸素化が改善されたと推測される．

図7　出血性ショック後の主要臓器への影響，TNF-α および HIF1-α



HO-1 では 24 時間後，72 時間後，HbV 群で有意に発現していた．図 9 に示す．HbV は，生体の Hb のように HO-1 ドナーとして臓器保護作用があるのではないかと推測される．

図 9　肺における HO-1 の mRNA 発現



8）まとめ

今回の研究の考察として，酸素運搬能は生体赤血球とほぼ同じであり，大量出血後の速やかな臓器酸素化能の改善効果が期待でき，HbV 溶液単独による 60%までの希釈は乳酸リンゲル液のそれと変わらない．しかし HbV 自体に若干の凝固抑制作用がある．

HbV の生体への投与における影響では，大量出血後の HbV 投与による臓器の炎症反応は軽度である．その炎症反応は肺で強い．HbV も HO-1 ドナーとしての効果があり臓器保護的作用を有する可能性がある．

終わりに

今回，重症病態と輸血ーHb はどのレベルに保つべきかーということで，特に救急や周術期における輸血に関して，私見を述べさせていただいた．また，人工赤血球ーHbV（早稲田型）に関しての研究結果を発表した．今後，さらに実験を進め人工赤血球の実現に向け努力していきたい．

謝辞

HbV を供与頂いた早稲田大学理工学部，土田英俊教授ならびに酒井宏水准教授に深く感謝いたします．また，今回体液代謝研究会で発表の場を頂いたことにつき今井孝祐先生に感謝申し上げます．

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