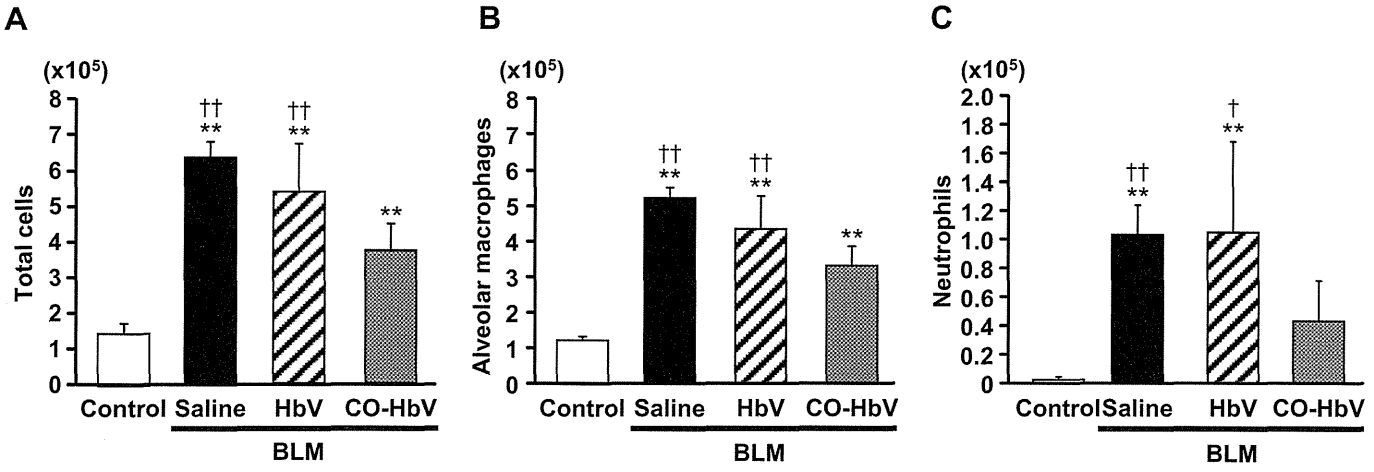


Fig. 4. Effect of CO-HbV on cells in bronchoalveolar lavage fluid in bleomycin-induced pulmonary fibrosis mice. The number of inflammatory cells including (A) total cells, (B) alveolar macrophages and (C) neutrophils in bronchoalveolar lavage fluid on day 3. These inflammatory cells were determined on day 3 as described in the "Materials and methods" section. Each value represents the mean ± s.d. ($n = 3$–6). $^{**}P < 0.01$ versus control. $\dagger\dagger P < 0.01$ versus CO-HbV. $\dagger P < 0.05$ versus CO-HbV.

administration, compared to saline treatment in BLM-induced pulmonary fibrosis mice (Table 1). However, cholesterol levels were significantly elevated at 7 days after the administration of CO-HbV. This is likely derived from metabolites contained by the HbV particles because they contain a large amount of cholesterol for structural stabilization and efficient Hb encapsulation. In a study using healthy mice and rats, we demonstrated that the added cholesterol was completely eliminated in the feces *via* biliary excretion within 14 days after the administration of HbV [41]. In fact, the serum cholesterol levels at 14 days after CO-HbV administration was not different compared to that in saline administration in this study. These results indicate that CO-HbV could suppress the progression of pulmonary fibrosis and the decline of lung mechanics without any severe side effects, thus, represents a promising candidate agent for novel IPF treatment.

Although the pathogenic mechanisms of IPF are unknown, a growing body of evidence suggests that both chronic inflammation and ROS (among other issues) appear to play a role in the onset or progression of IPF. Previous studies using human subjects with IPF have demonstrated that the generation of ROS from alveolar inflammatory cells, such as neutrophils and macrophages is enhanced and that this may promote alveolar epithelial cell injury and induce chronic inflammation, thus initiating or contributing to the development of pulmonary fibrosis [42,43]. In the present study, CO-HbV suppressed the cells count in BALF including neutrophils and alveolar macrophages (Fig. 4), and reduced the production of oxidation products (8-OH-dG and NO_2-Tyr), derived from nucleic acids and proteins, in the lung (Fig. 6A). In addition, the levels of cytokines (TNF-α, IL-6 and IL-1β) in lung tissue were significantly decreased as the result of the CO-HbV treatment (Fig. 5). It is well-known that inflammatory cells, including neutrophils and alveolar macrophages, are able to produce ROS *via* Nox2, which are essential to the development of pulmonary fibrosis in BLM-induced IPF model mice [44]. Nakahira et al. reported that ROS production was inhibited in LPS-treated macrophages when the cells were exposed to CO [45]. In addition, they also concluded that CO can form a complex with Nox2, indicating that CO is likely to modulate Nox2 activity [45]. These data suggest that CO-HbV would exert an inhibitory effect on the production of Nox2 in inflammatory cells, resulting in ameliorating the initiation and progression of BLM-induced pulmonary fibrosis.

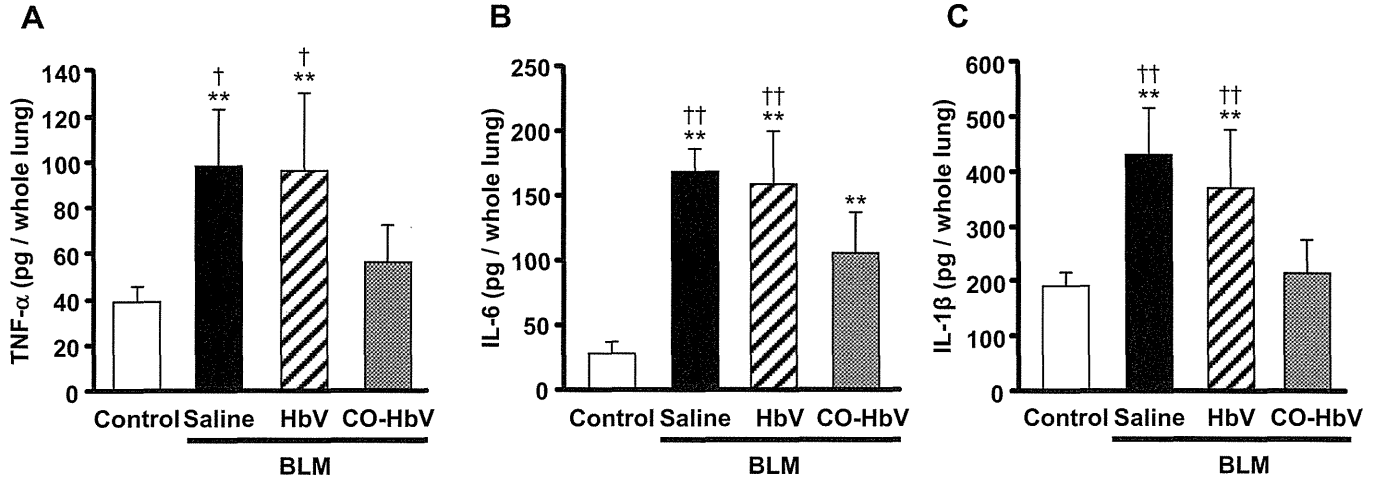


Fig. 5. Effect of CO-HbV on pulmonary inflammatory cytokines and chemokines in bleomycin-induced pulmonary fibrosis mice. The levels of cytokines and chemokine including (A) TNF-α, (B) IL-6 and (C) IL-1β in lung tissue on day 7. The amount of inflammatory cytokines and chemokine in whole lung tissue was measured by ELISA kit as described in the "Materials and methods" section. Each value represents the mean ± s.d. ($n = 5$). $^{**}P < 0.01$ versus control. $\dagger\dagger P < 0.01$ versus CO-HbV. $\dagger P < 0.05$ versus CO-HbV.

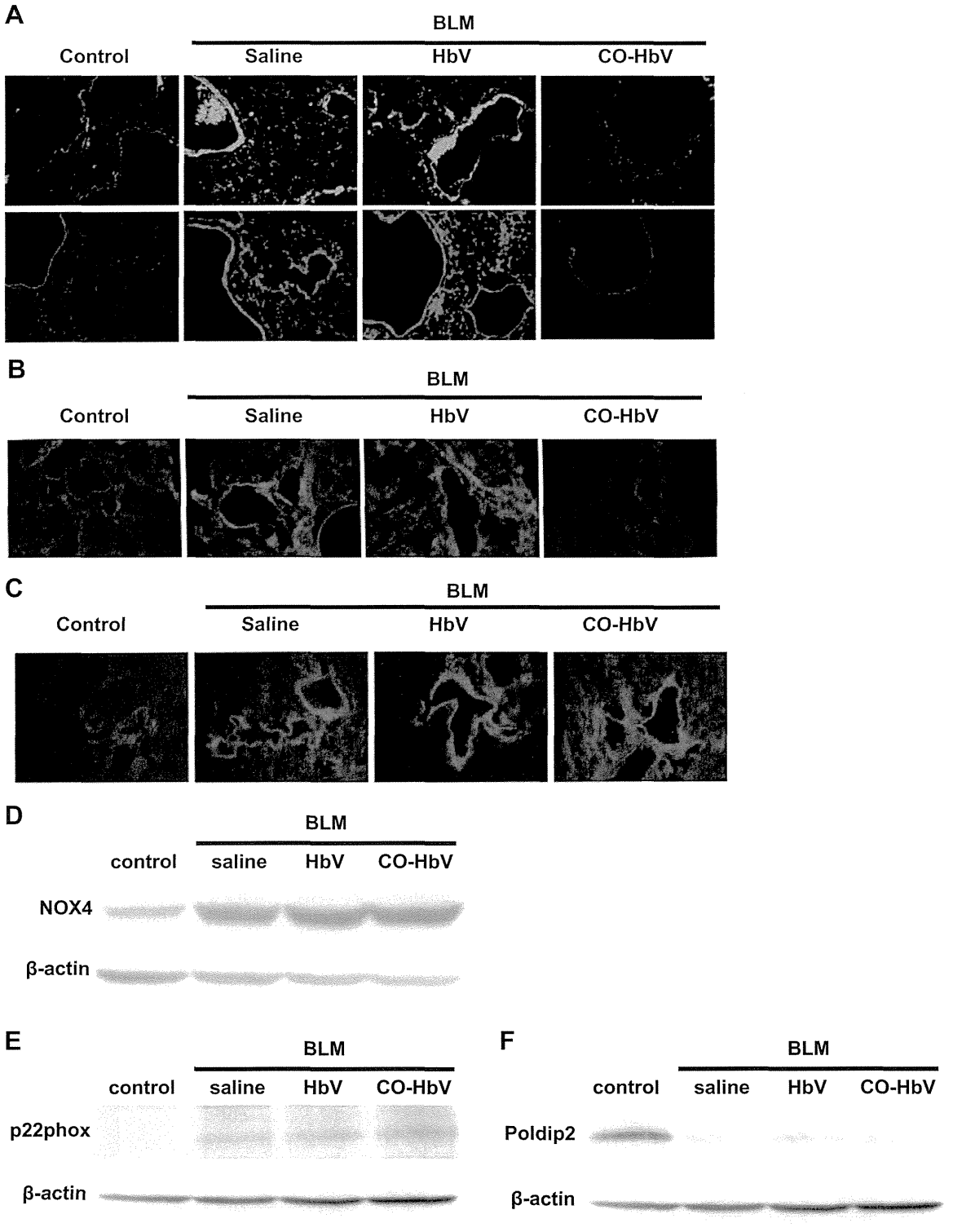


Fig. 6. Effect of CO-HbV on the generation of reactive oxygen species in lung tissue in bleomycin-induced pulmonary fibrosis mice. (A) The immunostaining of the lungs slice for the oxidative stress markers of nucleic acid (8-OH-dG; upper) and amino acid (NO_2-Tyr; lower). Mice were treated with bleomycin (BLM, 5 mg/kg) once on day 0. They were also administered saline, HbV or CO-HbV *via* the tail vein at 30 min before BLM treatment and 24 h after BLM treatment. Subsequently, the each immunostaining was performed on day 3 after BLM administration. (B) Production of pulmonary superoxide in bleomycin-induced pulmonary fibrosis mice on day 7 after BLM administration. Dihydroethidium was used to evaluate lung superoxide concentrations. (C-D)The protein expression of nicotinamide adenine dinucleotide phosphate oxidase 4 (Nox4) in lung tissue. Protein expression levels of Nox4 were determined by (C) immunostaining and (D) western blotting as described in the "Materials and methods" section. The protein expression of (E) $p22^{phox}$ and (F) polymerase delta interacting protein 2 (Poldip2)in lung tissue. Protein expression levels of both $p22^{phox}$ and Poldip2 were determined by western blotting as described in the "Materials and methods" section.

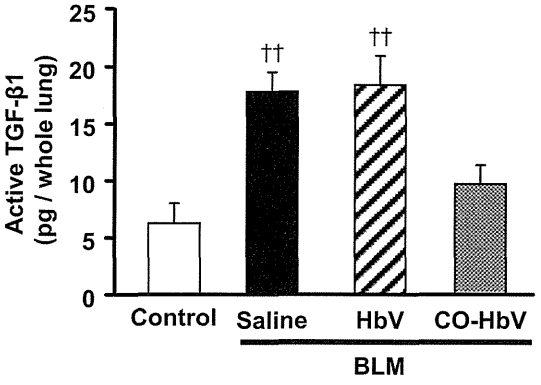


Fig. 7. Effect of CO-HbV on active TGF-β1 levels in bleomycin-induced pulmonary fibrosis mice. Active TGF-β1 levels in lung were determined on day 7 as described in the "Materials and methods" section. Each value represents the mean ± s.d. ($n = 6$). ††$P < 0.01$ versus CO-HbV.

In addition to Nox2, ROS are also generated by Nox4 and play a crucial role in the induction of alveolar epithelial cell death and the subsequent development of pulmonary fibrosis. In fact, it has been reported that Nox4 expression was increased in pulmonary fibrosis from patients with IPF [36,37], and the genetic or pharmacologic targeting of Nox4 abrogated fibrogenesis in murine models of lung injuries [37,46]. In our study, the Nox4 activity was suppressed by the administration of CO-HbV (Fig. 6B). Previously, the activity of Nox4, as determined by ROS generation, was thought to be exclusively dependent on the protein levels of Nox4 *in vitro* [47]. Interestingly, contrary with a previous *in vitro* study [47], our *in vivo* data using BLM-induced IPF model mice showed that the protein expression of Nox4 remained unchanged after the saline, HbV and CO-HbV treatment in BLM-induced IPF model mice (Fig. 6C and D). Although the activation mechanisms of Nox4 are largely unknown, at least part of the $p22^{phox}$ and Poldip2 would be related to Nox4 activity [38]. Therefore, we hypothesized that CO affected Nox4 activity *via* the membrane translocation of these two regulators, namely, $p22^{phox}$ and Poldip2. However, the protein expression of $p22^{phox}$ and Poldip2 in plasma membranes was not changed among the saline, HbV and CO-HbV treatment in BLM-induced IPF model mice (Fig. 6E and F). It therefore appears that CO inhibits a currently unknown pathway of Nox4 activation. A possible explanation for this issue is that CO interacts directly with the heme contained in Nox4. In fact, an interaction of Nox2, a heme protein, with CO was recently confirmed by a spectroscopic analysis [45]. Further investigation regarding this mechanism will be necessary to develop a comprehensive understanding of the effect of CO on tissue fibrosis.

There is an increasing body of evidence to suggest that epithelial–mesenchymal transition (EMT), a process whereby fully differentiated epithelial cells undergo a transition to a mesenchymal phenotype, thus giving rise to fibroblasts and myofibroblasts, may play a substantial role in a variety of pathogenic processes during pulmonary fibrogenesis. TGF-β1 has been implicated as functioning as a master switch in the induction of fibrosis in the lung, and is a major mediator of EMT in a number of physiological contexts, including pulmonary fibrosis [48]. In this regard, TGF-β1 is upregulated in the lungs of patients with IPF, and the expression of active TGF-β1 in the lungs of rats induces a dramatic fibrotic response, whereas an inability to respond to TGF-β1 affords protection from BLM-induced fibrosis [49]. The findings reported herein indicate that CO-HbV significantly reduced the active TGF-β1 content in lung tissue induced by the BLM treatment (Fig. 7). Several *in vitro* studies showed that ROS and inflammatory cytokines promoted TGF-β1 production in pulmonary epithelial cells and its subsequent activation [50,51]. Hence, these findings suggest that the suppression of ROS production and inflammatory cell infiltration at an early-stage of BLM treatment by CO-HbV eventually led to the suppression of active TGF-β1 production. In addition, it was reported that CO also suppressed TGF-β1-induced fibronectin and collagen production by fibroblasts and that this process was dependent, in part, on the transcriptional regulator Id1 [52]. It thus appears that CO inhibits both TGF-β1 production and some of TGF-β1 mediated signal pathways in the lung, and subsequently decreases the deposition of fibronectin and collagen, resulting in the suppression of pulmonary fibrosis.

Since the benefit of CO as a therapeutic agent has already been revealed pre-clinically in animal models of various human diseases [8], CO-HbV may not only be an effective therapeutic agent for the treatment of IPF, but also against other diseases in which the Nox family of proteins play an important role in disease progression, such as heart disease, rheumatoid arthritis, sepsis and cancer [53]. However, for clinical application of CO-HbV, there are a number of concern, particularly in relation to HbV as a carrier. Fortunately, it has already been demonstrated that HbV can be used safely as a carrier in animals, that HbV possesses a high biocompatibility, a low toxicity and does not accumulate in the body [54,55], indicating that HbV has the potential to function as a carrier of CO to diseased tissues in need of treatment without any detectable adverse effects. In addition, HbV has a good retention in the blood circulation in cynomolgus monkeys [23], and the half-life of HbV in humans was estimated to be approximately 3–4 days [56], which is long enough to function as a CO carrier. Furthermore, it is known that PEGylated liposomes show some unexpected pharmacokinetic properties, the so-called accelerated blood clearance phenomenon in which the long-circulation half-life is lost after being administered twice to the same animals [57]. Therefore, it is also concerned the pharmacokinetic properties after repeated infusion of CO-HbV, because it is expected that multiple injection of CO-HbV must be given for chronic and progressive diseases. In previous study, we demonstrated that the pharmacokinetics of HbV were negligibly affected by repeated injections at a massive dose [55].

5. Conclusions

The findings reported herein demonstrate that CO-HbV can inhibit the progression of pulmonary fibrosis. Furthermore, it can also be concluded that CO-derived anti-inflammatory and antioxidant effects are involved in its suppressive effect against pulmonary fibrosis progression and loss of lung mechanics. CO-HbV could be a new type of pharmaceutical therapeutic agent for using CO as a medical gas that would arrest ROS and inflammation-related disorders.

Acknowledgments

This work was supported in part by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare of Japan (201208035) and by a Grant-in-Aid for Young Scientist (B) from the Japan Society for the Promotion of Science (JSPS) (KAKENHI 24790159).

References

[1] Bjoraker JA, Ryu JH, Edwin MK, Myers JL, Tazelaar HD, Schroeder DR, et al. Prognostic significance of histopathologic subsets in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 1998;157:199–203.
[2] King Jr TE, Tooze JA, Schwarz MI, Brown KR, Cherniack RM. Predicting survival in idiopathic pulmonary fibrosis: scoring system and survival model. Am J Respir Crit Care Med 2001;164:1171–81.

[3] Schwartz DA, Helmers RA, Galvin JR, Van Fossen DS, Frees KL, Dayton CS, et al. Determinants of survival in idiopathic pulmonary fibrosis. Am J Respir Crit Care Med 1994;149:450–4.
[4] Noble PW, Albera C, Bradford WZ, Costabel U, Glassberg MK, Kardatzke D, et al. Pirfenidone in patients with idiopathic pulmonary fibrosis (CAPACITY): two randomised trials. Lancet 2011;377:1760–9.
[5] Rafii R, Juarez MM, Albertson TE, Chan AL. A review of current and novel therapies for idiopathic pulmonary fibrosis. J Thorac Dis 2013;5:48–73.
[6] Felton VM, Borok Z, Willis BC. N-acetylcysteine inhibits alveolar epithelial-mesenchymal transition. Am J Physiol Lung Cell Mol Physiol 2009;297: L805–12.
[7] Gurrieri C, Piazza F, Gnoato M, Montini B, Biasutto L, Gattazzo C, et al. 3-(2,4-dichlorophenyl)-4-(1-methyl-1H-indol-3-yl)-1H-pyrrole-2,5-dione (SB216763), a glycogen synthase kinase-3 inhibitor, displays therapeutic properties in a mouse model of pulmonary inflammation and fibrosis. J Pharmacol Exp Ther 2010;332:785–94.
[8] Motterlini R, Otterbein LE. The therapeutic potential of carbon monoxide. Nat Rev Drug Discov 2010;9:728–43.
[9] Otterbein LE, Zuckerbraun BS, Haga M, Liu F, Song R, Usheva A, et al. Carbon monoxide suppresses arteriosclerotic lesions associated with chronic graft rejection and with balloon injury. Nat Med 2003;9:183–90.
[10] Gentile MA. Inhaled medical gases: more to breathe than oxygen. Respir Care 2011;56:1341–57. discussion 57–9.
[11] Motterlini R, Mann BE, Foresti R. Therapeutic applications of carbon monoxide-releasing molecules. Expert Opin Investig Drugs 2005;14:1305–18.
[12] Chora AA, Fontoura P, Cunha A, Pais TF, Cardoso S, Ho PP, et al. Heme oxygenase-1 and carbon monoxide suppress autoimmune neuroinflammation. J Clin Invest 2007;117:438–47.
[13] Ferrandiz ML, Maicas N, Garcia-Arnandis I, Terencio MC, Motterlini R, Devesa I, et al. Treatment with a CO-releasing molecule (CORM-3) reduces joint inflammation and erosion in murine collagen-induced arthritis. Ann Rheum Dis 2008;67:1211–7.
[14] Hegazi RA, Rao KN, Mayle A, Sepulveda AR, Otterbein LE, Plevy SE. Carbon monoxide ameliorates chronic murine colitis through a heme oxygenase 1-dependent pathway. J Exp Med 2005;202:1703–13.
[15] Ameredes BT, Otterbein LE, Kohut LK, Gligonic AL, Calhoun WJ, Choi AM. Low-dose carbon monoxide reduces airway hyperresponsiveness in mice. Am J Physiol Lung Cell Mol Physiol 2003;285:L1270–6.
[16] Dubuis E, Potier M, Wang R, Vandier C. Continuous inhalation of carbon monoxide attenuates hypoxic pulmonary hypertension development presumably through activation of BKCa channels. Cardiovasc Res 2005;65:751–61.
[17] Fujita T, Toda K, Karimova A, Yan SF, Naka Y, Yet SF, et al. Paradoxical rescue from ischemic lung injury by inhaled carbon monoxide driven by derepression of fibrinolysis. Nat Med 2001;7:598–604.
[18] Foresti R, Bani-Hani MG, Motterlini R. Use of carbon monoxide as a therapeutic agent: promises and challenges. Intensive Care Med 2008;34:649–58.
[19] Omaye ST. Metabolic modulation of carbon monoxide toxicity. Toxicology 2002;180:139–50.
[20] Abe H, Azuma H, Yamaguchi M, Fujihara M, Ikeda H, Sakai H, et al. Effects of hemoglobin vesicles, a liposomal artificial oxygen carrier, on hematological responses, complement and anaphylactic reactions in rats. Artif Cells Blood Substit Immobil Biotechnol 2007;35:157–72.
[21] Sakai H, Suzuki Y, Sou K, Kano M. Cardiopulmonary hemodynamic responses to the small injection of hemoglobin vesicles (artificial oxygen carriers) in miniature pigs. J Biomed Mater Res A 2012;100:2668–77.
[22] Sakai H, Masada Y, Horinouchi H, Ikeda E, Sou K, Takeoka S, et al. Physiological capacity of the reticuloendothelial system for the degradation of hemoglobin vesicles (artificial oxygen carriers) after massive intravenous doses by daily repeated infusions for 14 days. J Pharmacol Exp Ther 2004;311:874–84.
[23] Taguchi K, Watanabe H, Sakai H, Horinouchi H, Kobayashi K, Maruyama T, et al. A fourteen-day observation and pharmacokinetic evaluation after a massive intravenous infusion of hemoglobin-vesicles (artificial oxygen carriers) in cynomolgus monkeys. J Drug Metab Toxicol 2012;3:128.
[24] Sakai H, Horinouchi H, Masada Y, Takeoka S, Ikeda E, Takaori M, et al. Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. Biomaterials 2004;25:4317–25.
[25] Sakai H, Horinouchi H, Tsuchida E, Kobayashi K. Hemoglobin vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. Shock 2009;31:507–14.
[26] Sakai H, Takeoka S, Park SI, Kose T, Nishide H, Izumi Y, et al. Surface modification of hemoglobin vesicles with poly(ethylene glycol) and effects on aggregation, viscosity, and blood flow during 90% exchange transfusion in anesthetized rats. Bioconjug Chem 1997;8:23–30.
[27] Tanaka R, Watanabe H, Kodama A, Chuang VT, Ishima Y, Hamasaki K, et al. Long-acting human serum albumin-thioredoxin fusion protein suppresses bleomycin-induced pulmonary fibrosis progression. J Pharmacol Exp Ther 2013;345:271–83.
[28] Sakai H, Tomiyama K, Masada Y, Takeoka S, Horinouchi H, Kobayashi K, et al. Pretreatment of serum containing hemoglobin vesicles (oxygen carriers) to prevent their interference in laboratory tests. Clin Chem Lab Med 2003;41:222–31.
[29] Furukawa M, Tanaka R, Chuang VT, Ishima Y, Taguchi K, Watanabe H, et al. Human serum albumin-thioredoxin fusion protein with long blood retention property is effective in suppressing lung injury. J Control Release 2011;154: 189–95.
[30] Woessner Jr JF. The determination of hydroxyproline in tissue and protein samples containing small proportions of this imino acid. Arch Biochem Biophys 1961;93:440–7.
[31] Tanaka K, Azuma A, Miyazaki Y, Sato K, Mizushima T. Effects of lecithinized superoxide dismutase and/or pirfenidone against bleomycin-induced pulmonary fibrosis. Chest 2012;142:1011–9.
[32] Kim-Mitsuyama S, Yamamoto E, Tanaka T, Zhan Y, Izumi Y, Izumiya Y, et al. Critical role of angiotensin II in excess salt-induced brain oxidative stress of stroke-prone spontaneously hypertensive rats. Stroke 2005;36:1083–8.
[33] Keane MP, Strieter RM. The importance of balanced pro-inflammatory and anti-inflammatory mechanisms in diffuse lung disease. Respir Res 2002;3:5.
[34] Piguet PF, Ribaux C, Karpuz V, Grau GE, Kapanci Y. Expression and localization of tumor necrosis factor-alpha and its mRNA in idiopathic pulmonary fibrosis. Am J Pathol 1993;143:651–5.
[35] Hunninghake GW. Antioxidant therapy for idiopathic pulmonary fibrosis. N Engl J Med 2005;353:2285–7.
[36] Amara N, Goven D, Prost F, Muloway R, Crestani B, Boczkowski J. NOX4/NADPH oxidase expression is increased in pulmonary fibroblasts from patients with idiopathic pulmonary fibrosis and mediates TGFbeta1-induced fibroblast differentiation into myofibroblasts. Thorax 2010;65:733–8.
[37] Hecker L, Vittal R, Jones T, Jagirdar R, Luckhardt TR, Horowitz JC, et al. NADPH oxidase-4 mediates myofibroblast activation and fibrogenic responses to lung injury. Nat Med 2009;15:1077–81.
[38] Lyle AN, Deshpande NN, Taniyama Y, Seidel-Rogol B, Pounkova L, Du P, et al. Poldip2, a novel regulator of Nox4 and cytoskeletal integrity in vascular smooth muscle cells. Circ Res 2009;105:249–59.
[39] Mauviel A. Transforming growth factor-beta: a key mediator of fibrosis. Methods Mol Med 2005;117:69–80.
[40] Raghu G, Collard HR, Egan JJ, Martinez FJ, Behr J, Brown KK, et al. An official ATS/ERS/JRS/ALAT statement: idiopathic pulmonary fibrosis: evidence-based guidelines for diagnosis and management. Am J Respir Crit Care Med 2011;183:788–824.
[41] Taguchi K, Urata Y, Anraku M, Maruyama T, Watanabe H, Sakai H, et al. Pharmacokinetic study of enclosed hemoglobin and outer lipid component after the administration of hemoglobin vesicles as an artificial oxygen carrier. Drug Metab Dispos 2009;37:1456–63.
[42] Cantin AM, North SL, Fells GA, Hubbard RC, Crystal RG. Oxidant-mediated epithelial cell injury in idiopathic pulmonary fibrosis. J Clin Invest 1987;79: 1665–73.
[43] Strausz J, Muller-Quernheim J, Steppling H, Ferlinz R. Oxygen radical production by alveolar inflammatory cells in idiopathic pulmonary fibrosis. Am Rev Respir Dis 1990;141:124–8.
[44] Manoury B, Nenan S, Leclerc O, Guenon I, Boichot E, Planquois JM, et al. The absence of reactive oxygen species production protects mice against bleomycin-induced pulmonary fibrosis. Respir Res 2005;6:11.
[45] Nakahira K, Kim HP, Geng XH, Nakao A, Wang X, Murase N, et al. Carbon monoxide differentially inhibits TLR signaling pathways by regulating ROS-induced trafficking of TLRs to lipid rafts. J Exp Med 2006;203:2377–89.
[46] Carnesecchi S, Deffert C, Donati Y, Basset O, Hinz B, Preynat-Seauve O, et al. A key role for NOX4 in epithelial cell death during development of lung fibrosis. Antioxid Redox Signal 2011;15:607–19.
[47] Serrander L, Cartier L, Bedard K, Banfi B, Lardy B, Plastre O, et al. NOX4 activity is determined by mRNA levels and reveals a unique pattern of ROS generation. Biochem J 2007;406:105–14.
[48] Willis BC, Borok Z. TGF-beta-induced EMT: mechanisms and implications for fibrotic lung disease. Am J Physiol Lung Cell Mol Physiol 2007;293:L525–34.
[49] Zhao J, Shi W, Wang YL, Chen H, Bringas Jr P, Datto MB, et al. Smad3 deficiency attenuates bleomycin-induced pulmonary fibrosis in mice. Am J Physiol Lung Cell Mol Physiol 2002;282:L585–93.
[50] Barcellos-Hoff MH, Dix TA. Redox-mediated activation of latent transforming growth factor-beta 1. Mol Endocrinol 1996;10:1077–83.
[51] Bellocq A, Azoulay E, Marullo S, Flahault A, Fouqueray B, Philippe C, et al. Reactive oxygen and nitrogen intermediates increase transforming growth factor-beta1 release from human epithelial alveolar cells through two different mechanisms. Am J Respir Cell Mol Biol 1999;21:128–36.
[52] Zhou Z, Song R, Fattman CL, Greenhill S, Alber S, Oury TD, et al. Carbon monoxide suppresses bleomycin-induced lung fibrosis. Am J Pathol 2005;166: 27–37.
[53] Al Ghouleh I, Khoo NK, Knaus UG, Griendling KK, Touyz RM, Thannickal VJ, et al. Oxidases and peroxidases in cardiovascular and lung disease: new concepts in reactive oxygen species signaling. Free Radic Biol Med 2011;51: 1271–88.
[54] Sakai H. Present situation of the development of cellular-type hemoglobin-based oxygen carrier (hemoglobin-vesicles). Curr Drug Discov Technol 2012;9:188–93.
[55] Taguchi K, Maruyama T, Otagiri M. Pharmacokinetic properties of hemoglobin vesicles as a substitute for red blood cells. Drug Metab Rev 2011;43:362–73.
[56] Taguchi K, Maruyama T, Iwao Y, Sakai H, Kobayashi K, Horinouchi H, et al. Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. J Control Release 2009;136:232–9.
[57] Abu Lila AS, Kiwada H, Ishida T. The accelerated blood clearance (ABC) phenomenon: clinical challenge and approaches to manage. J Control Release 2013;172:38–47.

Bioconjugate Chemistry

Article

pubs.acs.org/bc

Red Blood Cells Donate Electrons to Methylene Blue Mediated Chemical Reduction of Methemoglobin Compartmentalized in Liposomes in Blood

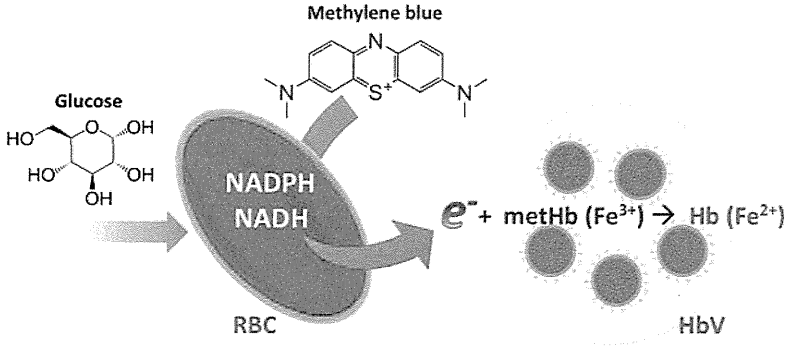
Hiromi Sakai,*[,†,‡,§] Bing Li,[‡] Wei Lee Lim,[‡,¶] and Yumika Iga[†]

[†]Department of Chemistry, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan

[‡]Waseda Bioscience Research Institute in Singapore, 11 Biopolis Way, #05-01/02, Helios, Singapore 138667

[§]Organization for University Research Initiatives, Waseda University, 513 Wasedatsurumaki-cho, Shinjuku, Tokyo 162-0041, Japan

[¶]School of Applied Science, Temasek Polytechnic, 21 Tampines Avenue 1, Singapore 529757



ABSTRACT: Electron-energy-rich coenzymes in cells, NADH and NADPH, are re-energized repeatedly through the Embden–Meyerhof and pentose–phosphate glycolytic pathways, respectively. This study demonstrates extraction of their electron energies in red blood cells (RBCs) for in vivo extracellular chemical reactions using an electron mediator shuttling across the biomembrane. Hemoglobin-vesicles (HbVs) are an artificial oxygen carrier encapsulating purified and concentrated Hb solution in liposomes. Because of the absence of a metHb-reducing enzymatic system in HbV, HbO_2 gradually autoxidizes to form metHb. Wistar rats received HbV suspension (10 mL/kg body weight) intravenously. At the metHb level of around 50%, methylene blue [MB^+; 3,7-bis(dimethylamino)phenothiazinium chloride] was injected. The level of metHb quickly decreased to around 16% in 40 min, remaining for more than 5 h. In vitro mixing of HbV/MB^+ with RBCs recreated the in vivo metHb reduction, but not with plasma. NAD(P)H levels in RBCs decreased after metHb reduction. The addition of glucose facilitated metHb reduction. Liposome-encapsulated NAD(P)H, a model of RBC, reduced metHb in HbV in the presence of MB^+. These results indicate that (i) NAD(P)H in RBCs reacts with MB^+ to convert it to leukomethylene blue (MBH); (ii) MB^+ and MBH shuttle freely between RBC and HbV across the hydrophobic lipid membranes; and (iii) MBH is transferred into HbV and reduces metHb in HbV. Four other electron mediators with appropriate redox potentials appeared to be as effective as MB^+ was, indicating the possibility for further optimization of electron mediators. We established an indirect enzymatic metHb reducing system for HbV using unlimited endogenous electrons created in RBCs in combination with an effective electron mediator that prolongs the functional lifespan of HbV in blood circulation.

INTRODUCTION

NADH, NADPH, and their oxidized forms, NAD^+ and $NADP^+$, are electron mediators of various biological processes including energy metabolism, mitochondrial functions, calcium homeostasis, antioxidation/generation of oxidative stress, gene expression, immunological functions, aging, and cell death.[1,2] Within circulating red blood cells (RBCs) in the bloodstream, these mediators are key elements for glycolysis and antioxidative functions.[3] During a long history of evolution, mammalian RBCs have lost the nuclei to focus their functions to oxygen transport and glycolysis, which are important to maintain the metabolism of the host body and protect it from oxidative damage. During the glycolysis, high electron energies are produced and charged repeatedly as NADPH and NADH. $NADP^+$ is reduced to NADPH by glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose–phosphate pathway. NADPH is used mainly for glutathione reductase and NADPH-flavin metHb reductase,[4] both for protection from oxidative damage. NAD^+ is reduced to NADH by glyceraldehyde 3-phosphate

Received: April 8, 2014
Revised: May 26, 2014
Published: May 30, 2014

   dx.doi.org/10.1021/bc500153x | *Bioconjugate Chem.* 2014, 25, 1301–1310

dehydrogenase (GAPDH) in the Embden–Meyerhof pathway. NADH is used mainly for NADH-Cytochrome b_5 reductase to reduce metHb,[5] and for lactate dehydrogenase to convert pyruvate to lactate.[6] Consequently, the electron energies of NADH and NADPH are consumed within RBCs. We have developed the idea of extracting these intracellular electron energies across the biomembrane and of using them for extracellular chemical reactions in blood circulation. This is a method of reduction of metHb in artificial oxygen carriers.

Hemoglobin-based oxygen carriers (HBOCs) have been studied worldwide for clinical use because they are expected to alleviate transfusion-related problems such as (i) the possibility of infection (HIV, hepatitis, etc.) because of a "window period" in which the amount of virus is too low to detect, (ii) the possibility of blood-type mismatching by human error, and (iii) the possibility of blood shortages in an emergency situation because of the short shelf life of donated blood (3–6 weeks refrigerated).[7−10] Hemoglobin-vesicle (HbV) is an HBOC encapsulating purified human Hb solution in a phospholipid vesicle (liposome) to shield toxic effects of cell-free Hbs.[10−12] Earlier studies clarified the safety and efficacy of HbVs as a transfusion alternative and for oxygen therapeutics using animal models.[13−20] Actually, HbVs do not contain the metHb-reducing enzymatic system that is present in the original RBCs. Hb is purified from outdated human blood by pasteurization (60 °C, 12 h) to achieve the utmost safety from any possibility of infection.[21] During this procedure, all the unstable enzymes, including the metHb-reducing system, are denatured and eliminated. As a result, Hb in HbV gradually increases the metHb level after intravenous injection. MetHb in HbV cannot be reduced by plasma ascorbic acid (AsA) because of the high barrier functionality of the lipid bilayer membrane of HbV with no ionophore. We attempted to create some nonenzymatic systems within HbV to reduce metHb.[22−27] However, these methods are not practical because of the systemic complexity and limited efficacy for metHb reduction.

In fact, our pilot in vivo study (see Figure 1 in this paper) of intravenous single injection of methylene blue [MB^+; 3,7-bis(dimethylamino)phenothiazinium chloride] following HbV injection demonstrated the effective reduction of metHb in HbV. MB^+ is a water-soluble phenothiazine dye that is commonly used as one component of the Giemsa stain solution in pathological studies, an electron mediator in electrochemical reactions, and therapeutic drugs for neurological disease, methemoglobinemia, and so forth.[28−30] Because HbV contains no potential electron donor, this phenomenon implies that the injected MB^+ in plasma was somehow reduced by biological electron donors, possibly those in RBCs. The reduced leukomethylene blue (MBH) penetrated across the lipid bilayer membrane of HbV to reduce metHb. This study was undertaken to identify the source of electrons to reduce metHb in HbV and to clarify all electron pathways. We also studied its long-term effectiveness for in vivo studies using MB^+ and the possibility of other potential electron mediators.

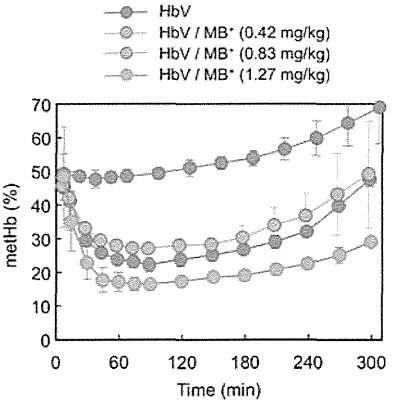


Figure 1. Time course of the level of metHb in HbV after intravenous administration into Wistar rats (n = 3, mean ± SD). HbV with the metHb level of 50% was injected. Without MB^+, the level increased gradually. Subsequent MB^+ injection showed dramatic reduction of the level of metHb, depending on the amount of MB^+.

RESULTS

In Vivo Reduction of metHb in HbV by Injection of MB^+. HbV with the metHb level of nearly 50% was injected into the rat femoral vein at a dose rate of 10 mL/kg, which corresponded to about 18% of whole blood volume (56 mL/kg). Subsequent injection of MB^+ (0.42 mg/kg) rapidly decreased the level of metHb to about 30%, but it returned to a gradual increase (Figure 1). With increasing the amount of MB^+ to 0.83 and 1.27 mg/kg, the level of metHb decreased. It was 16% at 60 min after injection of 1.27 mg/kg MB^+. Accordingly, the reduction of metHb is dependent on the dose amount of MB^+. The low level of metHb was sustained for over 6 h by injecting 1.27 mg MB^+/kg body weight.

In Vitro Reduction of metHb in HbV by MB^+ and RBCs. Both fresh rat whole blood and washed RBCs in the presence of MB^+ showed an effective metHb reduction in HbV from 50% to 10–15% in 5 min, and the low metHb level was sustained for 60 min (Figure 2). With decreasing amount of washed RBCs, the metHb reduction is minimized. Rat plasma alone showed no metHb reduction at all. These results indicate that electron donors are present in RBCs, but not in plasma,

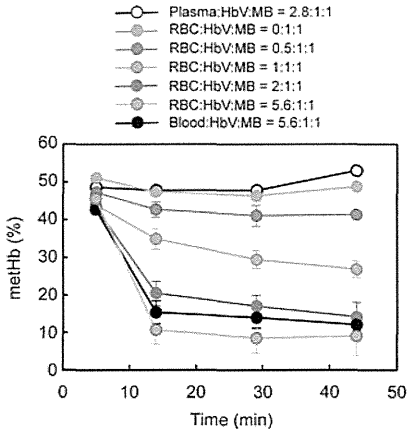


Figure 2. In vitro reduction of metHb in HbV by the addition of blood components and MB^+ (n = 3, mean ± SD). "Blood" was fresh whole blood collected from Wistar rats and heparinized. "Plasma" was obtained by centrifugation of whole blood. The whole blood [Hb] was around 12 g/dL. "RBC" was washed RBCs. The mixing ratio, blood:HbV:MB = 5.6:1:1 by vol corresponds to the in vivo experiment of injection of 10 mL/kg HbV and 10 mL/kg MB^+ to a Wistar rat that possesses blood volume of 56 mL/kg. Because the hematocrit of whole blood was around 50%, the mixing ratio of plasma:HbV:MB was adjusted to 2.8:1:1 by vol. The presence of RBCs contributes to the metHb reduction in HbV.

dx.doi.org/10.1021/bc500153x | Bioconjugate Chem. 2014, 25, 1301−1310

and that the MB^+-MBH redox system works as a mediator to transport electrons to metHb in HbVs. Both levels of the reduced forms, NADH and NADPH, significantly decreased in RBCs after metHb reduction in HbV (Figure 3), indicating that NADH and NADPH would be the electron donors.

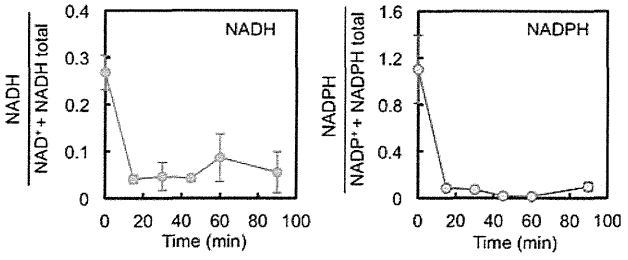


Figure 3. Decreased levels of NADH and NADPH in rat RBCs after the reduction of metHb in HbV in the presence of MB^+ (n = 3, mean ± SE).

External addition of α-D-glucose to washed RBCs at the beginning showed effective metHb reduction of HbV for over 4 h (Figure 4). However, without α-D-glucose, the level of metHb

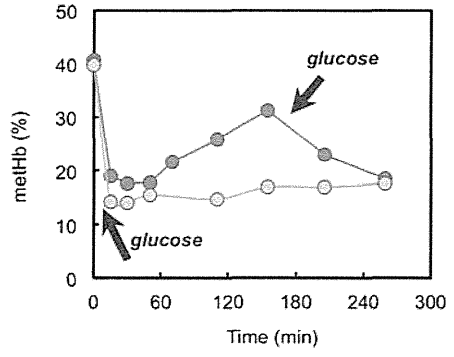


Figure 4. Addition of α-D-glucose to washed RBCs affects the metHb reduction in HbV in the presence of MB^+. The mixing ratio RBC:HbV:MB:glucose = 2:1:1:1 by vol. The pink plots show that the addition of α-D-glucose from the beginning exhibits more effective and durable metHb reduction in HbV. Blue plots show that a late addition of α-D-glucose improves metHb reduction in HbV.

increased gradually. Addition of α-D-glucose after 3 h dramatically facilitated the metHb reduction. These results demonstrate that the concentration of electron donors in RBCs was increased by the facilitated glycolysis in RBCs.

Partition Experiment of MB^+ in HbV and RBC. This experiment was performed to determine the percentage of MB^+ binding to the surface or into the membrane of the HbV or rat RBC. The results of MB^+ partition into HbV showed no obvious dependence on the mixing volume ratio of MB^+/HbV (0.1–2.0), and the percentage of MB^+ incorporated into HbV was 37.7 ± 2.9% (mean ± SD). The same tendency was confirmed with rat RBCs, and the percentage of MB^+ incorporated into rat RBCs was 35.5 ± 6.3%.

In Vitro Reduction of metHb in HbV by MB^+ and Chemical Reagents. MB^+ has characteristic absorption with the λ_{max} at 650 nm. It decreases with the addition of an electron donor (Figure 5). Addition of NADPH and NADH showed rapid reduction of MB^+ to MBH. The reaction completed within 20 min. Cys completed the reaction within 40 min. GSH

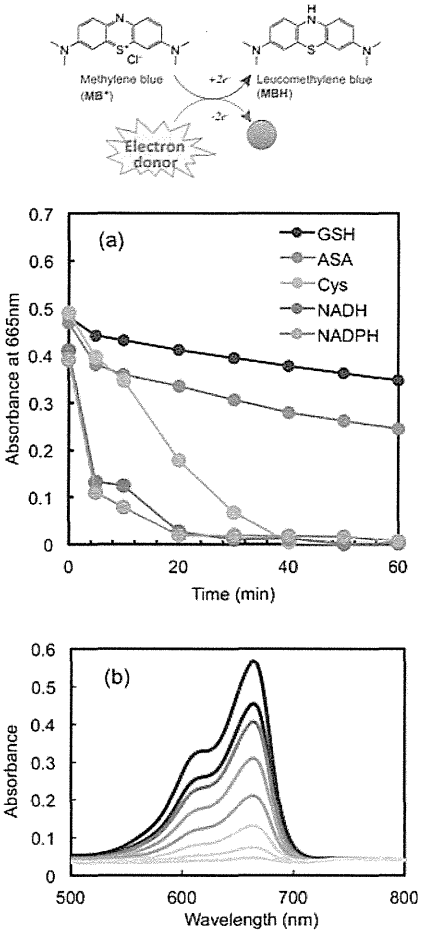


Figure 5. (a) Time course of MB^+ reduction by a reductant (AsA, GSH, Cys, NADH, or NADPH) in anaerobic conditions in pH 7.4 phosphate buffered saline. The reaction mixture in a total of 5 mL contained 6.8 μM of MB and 1000 μM of one electron donor. (b) Change of absorption spectra of MB^+ during reduction by Cys (0 → 60 min).

and ASA showed extremely slow reduction. The reduction was not completed even after 60 min.

Liposome Encapsulated NADPH or NADH as a Model of RBCs Can Reduce metHb in HbV. All the liposome encapsulated NADPH or NADH showed rapid reduction of metHb in HbV by the addition of MB^+ (Figure 6), which indicates that MB^+ permeates across the lipid membrane into the NADPH-liposome or NADH-liposome and that MB^+ is reduced to form MBH, which diffuses out from the NADPH-liposome or NADH-liposome and then enters into HbV to reduce metHb in HbV. The phase transition temperature of DPPC is 41 °C; that of DMPC is 23 °C. Therefore, the lipid bilayer membrane containing DPPC is expected to possess a generally lower molecular fluidity and a higher barrier functionality than those of DMPC. However, no marked difference was observed between DPPC and DMPC liposomes for the reduction of metHb in HbV, indicating that MB^+ and MBH freely shuttle across the lipid bilayer membranes of both liposomes.

Practical in Vivo Study of HbV and MB^+. Injection of HbV alone (metHb less than 5%) into Wistar rats showed a gradual metHb increase, as shown in Figure 7. Co-injection of

dx.doi.org/10.1021/bc500153x | Bioconjugate Chem. 2014, 25, 1301–1310

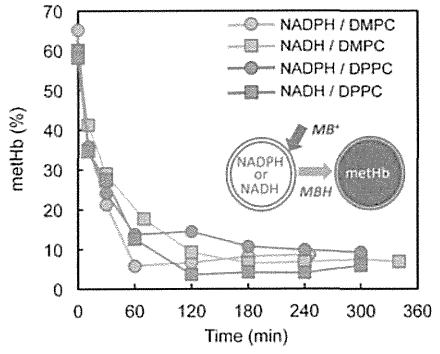


Figure 6. Reduction of metHb in HbV by the addition of MB^+ and liposome-encapsulated NADPH or NADH in an anaerobic condition. The liposome membranes consist of DPPC/cholesterol/DHSG/PEG_{5000}-DSPE or DMPC/cholesterol/DHSG/PEG_{5000}-DSPE = 5/4/0.9/0.03 by mol. All the liposomes showed reduction of metHb in HbV by the addition of MB^+.

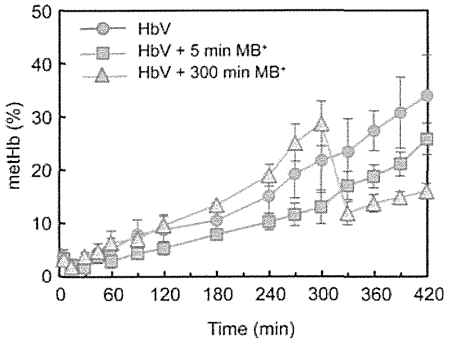


Figure 7. Time course of the level of metHb in HbV after intravenous administration into Wistar rats (n = 3, mean ± SD). HbV with the metHb level of <5% was injected. Without MB^+, the level increased gradually. Co-injection of MB^+ showed a lower rate of metHb increase. Late MB^+ injection at around 300 min showed significant metHb reduction.

MB^+ at the beginning showed a slowed increase in metHb. Injection of MB^+ at 5 h showed the rapid reduction of metHb. Accordingly, MB^+ injection can improve the functional life span of HbV.

Other Electron Mediators for metHb Reduction in HbV. Among the other electron mediators tested, AZA, BCB, TLB, and TDB showed similar metHb reduction to that of MB^+ (Figure 8). However, PM tended to return to a higher metHb level and TA showed insufficient metHb reduction.

DISCUSSION

Our primary finding is that the high electron energies present within RBCs are used effectively for extracellular chemical reaction, namely, reduction of metHb compartmentalized in HbV in circulating blood of Wistar rats. Here we propose the main electron pathway. The injected MB^+ in plasma diffuses across the biomembrane of RBCs, and NADPH and NADH in RBCs reduce MB^+ to MBH, which permeates across the hydrophobic lipid bilayer membranes of both RBC and HbV, and transfers an electron to metHb in HbV. Thus, NADPH and NADH provide electrons to metHb in HbV via the shuttling MB^+-MBH redox system (Scheme 1).

We have studied physicochemical and biochemical characterization,[11,12,43] safety,[15−17] and efficacy[13,14,18,19] of HbV for

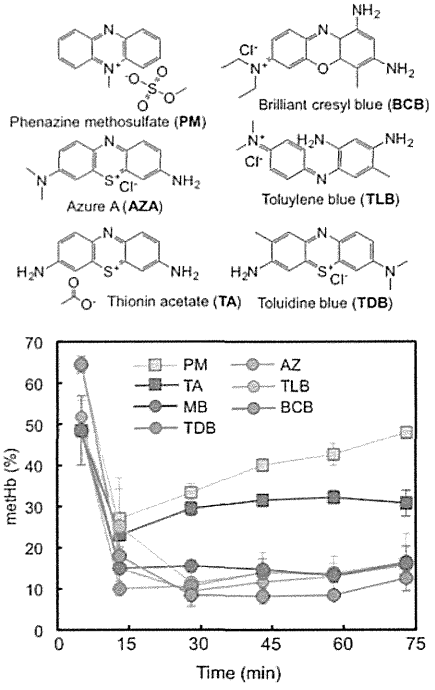


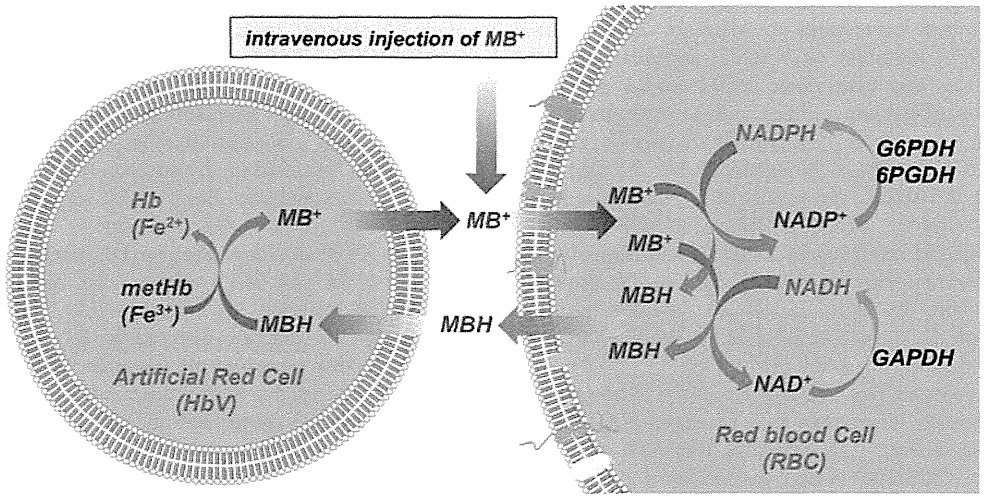
Figure 8. In vitro reduction of metHb in HbV by the addition of RBCs and a series of potential electron mediators. The mixing volume ratio of RBC:HbV:electron mediator = 5.6:1:1. The concentration of an electron mediator solution was adjusted to 0.35 mM. [Hb] of the RBC suspension was adjusted at 12 g/dL. HbV suspension contained about 50% metHb. Among six potential electron mediators, AZA, BCB, TLB, and TDB showed effective metHb reduction as did MB^+. However, PM and TA showed less effectiveness (n = 3, mean ± SD).

clinical usage as a transfusion alternative and oxygen therapeutic. HbVs contain no metHb reducing enzymatic system that exists in original RBCs. Hb is purified from outdated human blood by pasteurization to ensure the utmost safety from infection.[21] This strategy contrasts against others by maintaining the original metHb reducing system in red blood cells.[31,32] During our Hb purification procedure, all enzymes including the metHb-reducing system are denatured and eliminated. Therefore, Hb in HbV gradually increases the metHb level and loses its oxygen carrying capacity. One important point is that encapsulation of Hb in liposomes can shield all the toxic effects of the further oxidization to ferrylhemoglobin and iron release induced by the addition of excess amount of hydrogen peroxide.[33] On the other hand, to prolong the functional life span of HbV, establishment of a metHb reducing system has long been a challenging subject in our research group.[24,34] To reduce the level of metHb in HbV, we tested coencapsulation of a series of thiols[23] and catalase,[25] photoreduction using flavin,[27] construction of catalase-like activity using metHb and tyrosine,[26] and so forth. Unfortunately, these methods are not practical because of the low efficiency and the addition of other new components to encapsulate into HbVs, which complicates the whole preparation for clinical use.

In the case of cell-free chemically modified Hb solutions such as intramolecular cross-linked Hb and polymerized Hb, the rate of autoxidation is reduced in the bloodstream because metHb can react directly with reducing agents that are present in plasma, such as AsA.[35−38] Nearly 100 μM of AsA is present in

dx.doi.org/10.1021/bc500153x | Bioconjugate Chem. 2014, 25, 1301−1310

Scheme 1. Proposed Electron Transfer Pathway from RBCs to metHb in HbV[a]



[a]Intravenously injected MB^+ is first dispersed in plasma of blood. MB^+ permeates across the biomembrane of RBCs to the cytoplasmic space, where it reacts with NADPH and NADH to be reduced to MBH. The oxidized $NADP^+$ is energized repeatedly to NADPH using 6-phosphogluconate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH) in the pentose phosphate pathway. The oxidized NAD^+ is energized repeatedly to NADH by glyceraldehyde 3-phosphate dehydrogenase (GAPDH) in the Embden–Meyerhof pathway. The reduced MBH permeates across the biomembrane of RBCs to the extracellular phase and diffuses across the lipid bilayer membrane of HbV and transfers an electron to ferric metHb to form ferrous Hb. The oxidized MB^+ again diffuses out across the lipid bilayer membrane of HbV and across the biomembrane of RBCs repeatedly.

healthy rat blood plasma.[39] However, in the case of HbV, Hb is shielded by the lipid bilayer (liposome) so that AsA cannot permeate and thereby reduce metHb in HbV. In our rat pilot experiment, the injection of MB^+ alone reduced the metHb level in HbV effectively from 50% to around 16%, at doses of 10 mL/kg, which correspond to 18 vol % of circulating blood volume. To clarify the mechanism of metHb reduction, some in vitro experiments were conducted using fresh rat blood, plasma, and washed RBCs. In the rat blood and washed RBC suspension, the reduction of metHb in HbV was observed equally in the presence of MB^+. However, reduction was not observed in the rat plasma. The concentration of AsA in rat plasma, around 100 μM,[39] would be insufficient to reduce MB^+ rapidly. Our result, which showed that MB^+ is reduced directly by RBCs, clearly illustrates that the main electron donor is present in RBCs. We specify that the electron donors in RBCs are NADPH and NADH based on the following reasons: (1) The level of NADPH and NADH decreased after metHb reduction. (2) Increased glycolysis facilitated metHb reduction in HbV. (3) The reduction of MB^+ by NADPH and NADH are much faster than by AsA or GSH. (4) Liposomes encapsulated NADPH and NADH, a model of RBCs, showed effective metHb reduction. (5) Oxidation–reduction potentials (E'_0 at 20 °C, pH 7.0) of Hb (Fe^{3+}/Fe^{2+}), MB^+/MBH, and $NAD(P)^+$/NAD(P)H are +0.14, +0.01, and −0.32 V, respectively.[40] The direction of electron transfer from NAD(P)H to metHb via MB^+ is reasonable.

Results of a partition experiment revealed that nearly 35–40% of MB^+ are incorporated in both RBC and HbV lipid membranes. Ionic interaction between the positively charged MB^+ and the negatively charged lipid membranes of both HbV and RBC would facilitate the incorporation of MB^+ into the hydrophobic lipid membranes.[41] Judging from the chemical structure, the reduced form MBH is more easily incorporated into the lipid membranes. Actually, liposome-encapsulated NADPH and NADH mixed with MB^+ reduced metHb in HbV effectively. No marked difference was found between DPPC (T_c = 41 °C) and DMPC (T_c = 23 °C) liposomes, which should differ in terms of membrane fluidity.[42] This result would correspond to the fast MB^+ uptake into both liposomes. In fact, we tried to measure the rate of MB^+ incorporation into the DPPC- and DMPC-containing liposomes. Because of the technical difficulty in the ultracentrifuge, we could not track the entire incorporation profile, but it was clarified that MB^+ incorporation was quite fast and equilibrated within a few minutes. Both the NAD(P)H-encapsulated liposomes and HbV bear PEG chains on their surface, which would prevent direct contact with each other.[43] PEG-modified HbV would not directly contact RBCs bearing glycocalyx layer on the surface. Therefore, the experimental results suggest that both MB^+ and MBH freely move across the hydrophobic lipid membranes of both RBC and HbV and diffuse through aqueous phase between the membranes.

MB^+ [3,7-bis(dimethylamino)phenothiazinium chloride], a water-soluble phenothiazine dye, has been used clinically for the primary and standard therapy of methemoglobinemia.[28] MB^+ is also used for photochemical virus inactivation of biological products,[44] therapeutic drug for a rescue of destructive shock,[29] as an antimalarial agent,[45] for a treatment of a neurological disorder such as Alzheimer's disease,[46] and vasoplegia during cardiac surgery with cardiopulmonary bypass[30] as an elective inhibitor of nitric oxide cyclic guanosine monophosphate. The safety of MB^+ injection is studied extensively, especially in hemodynamic and vascular endothelial functions.[28,47,48] According to the instruction of commercially available "methylenblau VITIS i.v." (Neopharma GmbH & Ci. KG), the maximum dosage of MB^+ to human for single dosage is 2 mg/kg. The repeated injection should be less than 7 mg/kg because of various side effects.[34] In our experiment, the maximum dosage of MB^+ was 1.3 mg/kg, which was much lower than the limited dosage.

dx.doi.org/10.1021/bc500153x | Bioconjugate Chem. 2014, 25, 1301–1310

In the case of rescue from methemoglobinemia,[28,49] it has been believed that hydrophilic MB^+ in blood after intravenous administration would be reduced to MBH by RBC plasma membrane NADPH reductase or NADPH-dependent metHb reductase, and the less hydrophilic MBH would permeate across the hydrophobic biomembrane and be incorporated in RBCs. Then MBH would nonenzymatically transfer electron to intracellular metHb in RBCs. No extracellular reaction had been considered. In contrast, our results show clearly that MB^+ reacts easily and directly with NADPH and NADH aqueous solutions and liposome-encapsulated NADPH and NADH with no enzyme. Only re-energizing $NADP^+$ and NAD^+ requires glycolytic enzymatic reactions in RBCs. So-called plasma membrane oxidoreductases[50] would not be required in our system.

MB^+ can be reduced by AsA and GSH[51,52] although the reaction rate is much lower than that of either NADPH or NADH, as shown in Figure 5a, indicating that AsA and GSH within RBCs can contribute, in part, as electron donors to the reduction of MB^+. However, GSH in RBCs is used for reduction of oxidized AsA, dehydro ascorbic acid (DHA), and the oxidized GSH (GSSG) is reduced by NADPH-dependent glutathione reductase.[3] AsA is used as a reactive oxygen scavenger. The resulting mono dehydro ascorbic acid (MDA) is reduced to AsA by NADH-dependent MDA reductase. Consequently, all electrons originate from NADPH and NADH, the oxidized forms of which are re-energized repeatedly during glycolysis.

The defense mechanism to oxidative stress is different between animal species. Biosynthesis of ascorbic acid is present in rats but not in humans and guinea pigs and the superoxide dismutase activity is higher in humans and guinea pigs than in rats.[53] The plasma ascorbic acid level is higher in rats than in humans and guinea pigs.[39] Oxidized dehydroascorbate is reduced by cytochrome *b*561, one plasma membrane oxidoreductase, residing on human and guinea pig RBCs but not on rat RBCs.[54] In fact, we conducted some in vitro experiments using blood from humans and guinea pigs, and it was clarified that both human and guinea pig RBCs reduced metHb in HbV in the presence of MB^+ as did rat RBCs (data not shown here). Therefore, we believe that all mammalian RBCs with NAD(P)H are a potent electron donor which is created by the glycolytic mechanism, and the evolutional difference in ascorbic acid biosynthesis does not affect our results.

Intravenously injected MB^+ in plasma would directly contact not only RBCs, but also endothelial cells in the vasculature, where MB^+ might be reduced by NADPH and NADH in the endothelial cells. The reduced MBH would come out to the plasma phase and would be expected to contribute to metHb reduction in HbV. However, we infer that RBCs are the main source of the electron donor to metHb in HbV because the in vitro mixing experiment using blood (Figure 2) recreates in vivo results.

We tested other potential electron mediators:[55−57] phenothiazines (AZA, E'_0 = 0.013 V; TA, E'_0 = 0.063 V; TDB, E'_0 = 0.011 V), indamine (TLB, E'_0 = 0.115 V), phenazine (PM, E'_0 = 0.080 V), and phenazonium (BCB, E'_0 = 0.047 V). Their E'_0 values are similar to that of MB^+ (+0.01 V) and are intermediate between those of $Hb(Fe^{3+}/Fe^{2+})$ (+0.14 V) and $NAD(P)^+/NAD(P)H$ (−0.32 V). All of them in combination with RBCs initially showed reduction of metHb in HbV, as shown in Figure 8. Among the chemicals tested, AZA, TDB, TLB, and BCB showed similar metHb reduction to that of MB^+. However, PM tended to return to a higher metHb level and TA showed insufficient metHb reduction. A variety of candidate chemicals as electron mediators are known that might differ not only in redox potential, but also in hydrophobicity for biomembrane permeability and reactivity to metHb and molecular O_2. Optimizing metHb reduction is expected to be a challenging study in terms of not only efficacy but also safety. Actually, Kiese et al.[57] reported that TDB is less toxic than MB^+ for the treatment of methemoglobinemic patients. Further study must be conducted to overcome the limitations of this MB^+ system. The reduced MBH would transfer an electron to molecular O_2, and induce reactive oxygen species that are believed to be mostly scavenged by superoxide dismutase and catalase present in RBC. Even so, the generation of reactive oxygen species is expected to be a reason that the metHb level in HbV did not fall below 10%.

In spite of such limitations, we confirmed through in vivo experimentation that coinjection of MB^+ and HbV from the beginning exhibited a lower rate of autoxidation. Late injection of MB^+ at 5 h after single-HbV injection exhibited a rapid reduction of metHb in HbV, as shown in Figure 7. Periodic repeated injection would decrease metHb repeatedly. Actually, our other study showed that three periodic injections of MB^+ at 7, 24, and 48 h after HbV administration maintained a low metHb level for over 50 h. They prolonged the oxygen carrying capacity of HbV. This will be reported elsewhere.

CONCLUSION

We established a system to extract electron energies of RBCs for extracellular chemical reaction using an electron mediator that shuttles across the bilayer membrane. Using this method, reduction of metHb, which is shielded by the liposomal membrane, was performed effectively. Other than HBOCs, our research results imply the possibility of utilizing intracellular abundant glycolytic energy for other electrochemical reactions for in vivo devices.

MATERIALS AND METHODS

Preparation of Hb-Vesicles (HbVs). HbVs were prepared using a method reported previously with only slight modifications.[20,21,43] Human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). First, Hb was stabilized using carbonylation (HbCO) and pasteurized (60 °C for 12 h) for virus inactivation. All unstable enzymes were eliminated during this procedure. The obtained Hb solution was concentrated by ultrafiltration to 42 g/dL. Subsequently, pyridoxal 5′-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 1. Here PLP was used instead of 2,3-diphosphoglyceric acid (2,3-DPG) because 2,3-DPG is chemically unstable. The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-*O*-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3-phosphatidylethanolamine-*N*-PEG_{5000} (NOF Corp., Tokyo, Japan) at the molar composition of 5/4/0.9/0.03. The encapsulated HbCO was converted to HbO_2 by exposing the liquid membranes of HbVs to visible light under an O_2 atmosphere. Finally the Hb concentration of the suspension

dx.doi.org/10.1021/bc500153x | *Bioconjugate Chem.* 2014, 25, 1301−1310

was adjusted to 10 g/dL. The particle size distribution, 280 ± 50 nm, was measured using a light-scattering method (Nanoparticle analyzer, Model SZ-100; Horiba, Ltd., Kyoto, Japan). The original HbV had metHb level of less than 5%. HbV was incubated at 37 °C for about 20 h to increase the metHb level to about 50%.

Injection of HbV and MB^+ into Wistar Rats. All experimental protocols were approved by the IACUC of Biological Resource Center, A*STAR, Singapore, and Nara Medical University, Japan. The protocols comply with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources Commission on Life Sciences, National Research Council – National Academy of Sciences (Washington, DC: National Academy Press, 1996). Experiments were conducted using 21 male Wistar rats (8–10 wks 300–350 g b.w.). The rats were anesthetized by inhalation of 1.5%-isoflurane (Halocarbon Products Corp., North Augusta, SC, USA) mixed with air using a vaporizer (TK-7; Biomachinery, Chiba, Japan) throughout the experiment (fraction of inspired O_2, FiO_2 = 21%; flow rate, 1.5 L/min) while spontaneous breathing was maintained. Polyethylene catheters (SP-31 tubing, OD 0.8 mm, ID 0.5 mm; Natsume Seisakusho Co., Ltd., Tokyo) filled with saline solution containing 10 IU/mL heparin (B. Braun Medical Industries S/B, Penang, Malaysia) were introduced through the left femoral artery and vein. The arterial catheter was connected to a transducer connected to a BP Amp (MLT0670; AD Instruments) and monitored with software (PowerLab; AD Instruments).

Pilot Study. The systemic blood volume was estimated as 56 mL/kg body weight.[13] HbV with the metHb level of about 50% was injected intravenously (10 mL/kg, 1 mL/min). Five minutes later, an MB^+ solution (Sigma-Aldrich; dissolved in PBS at 0.3 mM) was injected (3.3, 6.6, 10 mL/kg, respectively, corresponding to 0.42, 0.83, and 1.27 mg MB^+ /kg). The rat was kept anesthetized for over 5 h for periodical blood collection from the artery. After the experiment, the rat was euthanized by phlebotomy.

Arterial blood (<75 μL) was periodically collected directly into a heparinized glass capillary for hematocrit measurement (Hirschmann Laborgerate GmbH & Co. KG, Eberstadt, Germany). It was centrifuged for 1 min at 12,000 rpm using a centrifuge machine (3220; Kubota Corp., Tokyo, Japan). RBCs precipitated at the bottom, whereas HbV was dispersed in the upper plasma phase. About 20 μL of the plasma phase was diluted with 3 mL of PBS in a Thunberg cuvette that was sealed with a rubber cap. The solution was then deoxygenated using gentle N_2 gas bubbling for 5 min. Consequently, HbV contained only metHb and deoxyHb. Visible light absorption spectra were measured using a spectrophotometer (V-650; Jasco Corp., Tokyo, Japan) that was equipped with an integral sphere (ISN-470) to minimize the effect of light scattering. The spectra were measured between 300 and 500 nm, and the level of metHb was calculated from the absorption ratios at 405 nm (metHb) and 430 nm (deoxyHb).

Practical Injections of HbV and MB^+. HbV with the metHb level below 5% was injected (10 mL/kg, 1 mL/min). The first group received coinjection of MB^+ and HbV. MB^+ was injected 5 min after injection of HbV (10 mL/kg, corresponded to 1.27 mg MB^+/kg). The second group received MB^+ 300 min after HbV injection. The third group received HbV only. The level of metHb was monitored for 7 h using the same method as that described above.

In Vitro Reduction of metHb in HbV in the Presence of Blood Components and MB^+. Heparinized fresh blood was collected from the femoral artery or posterior vena cava of an anesthetized Wistar rat. The blood was centrifuged (2000 rpm, 10 min). Thereby a transparent plasma layer was obtained. After removing the buffy coat (white cells and platelets), the sedimented RBC layer was dispersed in the same volume of phosphate buffered saline (PBS, pH 7.4; Gibco, Paisley, UK). This procedure was repeated twice. The obtained washed RBC concentrate was finally suspended in PBS at [Hb] = 12 g/dL. Whole blood, washed RBC, or plasma was mixed with HbV of about 50% metHb content and incubated at 37 °C. After 5 min, MB^+ (0.3 mM) was added and mixed gently. The change of metHb level of HbV was measured spectrophotometrically as described above. To clarify the origin of electrons, α-D-glucose (100 mM in PBS; Aldrich) as a starting material of glycolysis in RBCs was added at the beginning or at 150 min to the concentration of 10 mM.

For the mixture of washed RBC with HbV and MB^+ at the volume ratio of 2:1:1, the levels of NAD^+, NADH, $NADP^+$, and NADPH in RBCs were monitored using NADP/NADPH and NAD/NADH Quantitation Kits (PromoKine; PromoCell GmbH, Heidelberg, Germany). The procedure followed the instruction manual, except that the RBC concentrate (20 μL) was diluted in an extraction buffer and filtered through 10 kDa centrifugal filter (Ultracel 10K; Amicon Inc.) to remove Hb for the colorimetric assay. The ratios of NAD(P)H to the sum of $NAD(P)^+$ and NAD(P)H were calculated.

Partition Experiment of MB^+ in HbV and RBC. Appropriate volumes of HbV ([Hb] = 3 g/dL) and MB^+ (0.34 mM) were mixed in a tube (MB^+/HbV volume ratio = 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, and 2.0; total 1.5 mL), incubated at 37 °C for 40 min, and ultracentrifuged at 40,000*g* for 1 h. The amount of MB^+ in the supernatant was calculated from the weight of the supernatant and the MB^+ concentration obtained from the absorbance at 665 nm. From the value, the percentage of MB^+ adhering to HbV was determined. The method was repeated for rat RBCs.

In Vitro MB^+ Reduction Using Electron Donors. Reaction profiles of MB^+ and a series of electron donors were tested in anaerobic condition: L-glutathione (GSH; Sigma-Aldrich), L-cysteine (Cys; Kanto Chemical Co. Inc., Tokyo), L-(+)-ascorbic acid (AsA; Wako Pure Chemical Industries Ltd., Osaka, Japan), NADH (Sigma-Aldrich), and NADPH (Sigma-Aldrich). The MB^+ solution dissolved in PBS was mixed with a solution of one electron donor at [MB^+] = 6.8 μM and [electron donor] = 1000 μM in a Thunberg cuvette. For attaining the anaerobic condition, the MB^+ solution in the cuvette was bubbled with N_2 gas beforehand for 10 min. The solution of an electron donor in another glass vial was bubbled with N_2, and collected carefully with a gastight syringe and inserted into the cuvette. Absorption spectra of MB^+ were measured using the spectrophotometer between 500–800 nm. The reduction was monitored at the peak wavelength of 665 nm that corresponds to MB^+.

Liposome Encapsulated NADPH or NADH as a Model of RBCs. Two different powdered mixed lipids were used for liposome formation. One is the same with the lipids of HbV (DPPC/cholesterol/DHSG/DSPE-PEG_{5000} = 5/4/0.9/0.03 by mol). The other includes 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC; Nippon Fine Chemical Co. Ltd.) instead of DPPC (DMPC/cholesterol/DHSG/DSPE-PEG_{5000} = 5/4/0.9/0.03 by mol). NADH or NADPH was dissolved in PBS at

dx.doi.org/10.1021/bc500153x | *Bioconjugate Chem.* 2014, 25, 1301–1310

the concentration of 25 mg/mL. The powdered mixed lipid (0.3 g) was dispersed in 5 mL of NADH or NADPH solution and stirred overnight for encapsulation. The particle diameter was regulated using the stepwise extrusion method with the final filter of 0.22 μm pore size (FM; Millipore). The unencapsulated outer NADPH or NADH was removed using a gel filtration using Sepharose CL-4B (Sigma Chemical Corp.). On the gel filtration column (i.d. 1.5 cm; length 18 cm), 1 mL of the liposome suspension was embedded. The filtrated liposome fraction was obtained. Then the whole lipid concentration was adjusted to about 0.6–0.7 g/dL. The liposome suspension was mixed with HbV with 50% metHb level and incubated at 37 °C in anaerobic condition. After 5 min MB^+ was injected to initiate electron transfer. The volume ratio of NAD(P)H-encapsulated liposome:HbV:MB^+ was 10:1:1. The surfaces of both the NAD(P)H-encapsulated liposomes and HbV are PEG-modified. The change of metHb level was monitored spectrophotometrically as described above. We confirmed that the liposomes did not release NAD(P)H during the procedure.

Other Electron Mediators for metHb Reduction in HbV. Instead of MB^+, other electron mediators were tested for metHb reduction, including Azure A (AZA; MP Biomedicals), phenazine methosulfate (PM; MP Biomedicals, Santa Ana, USA), thionin acetate (TA; Wako Pure Chemical Industries Ltd.), toluidine blue O (TDB; Waldeck GmbH & Co., Munster, Germany), Brilliant cresyl blue (BCB; MP Biomedicals), and toluylene blue (TLB; MP Biomedicals). They were dissolved at 0.35 mM in PBS without further purification. The washed rat RBC suspension ([Hb] = 12 g/dL), HbV with 50% metHb level, and a solution of an electron mediator were mixed at the volume ratio of 5.6:1:1. The change of metHb level in HbV was monitored using the method described above.

AUTHOR INFORMATION

Corresponding Author

*Tel and Fax: +81-(0)744-29-8810. E-mail: hirosakai@naramed-u.ac.jp.

Present Address

Wei Lee Lim, Bioprocessing Technology Institute, A*star, Singapore.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): Of the authors, H.S. is an inventor holding patents related to the production and utilization of Hb-vesicles.

ACKNOWLEDGMENTS

This research was supported by a Grant-in-Aid for Scientific Research (Kiban B, No. 25282136) from the Japan Society for the Promotion of Sciences (JSPS), Health and Labour Sciences Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan, and Strategic Research Platforms for Private University: a Matching Fund Subsidy from Ministry of Education, Culture, Sports, Science and Technology, Japan. The authors acknowledge Dr. Hirohisa Horinouchi and Mr. Yasuhisa Seishi (School of Medicine, Keio University, Tokyo, Japan) for meaningful discussion related to animal experiments, and Ms. Tomoko Kure and Ms. Karin Kettisen (Nara Medical University) for discussion related to metHb reduction mechanism.

REFERENCES

(1) Braun, R. D., Santhanam, K. S. V., and Elving, P. J. (1975) Electrochemical oxidation in aqueous and nonaqueous media of dihydropyridine nucreotides NMNH, NADH, and NADPH. *J. Am. Chem. Soc. 97*, 2591–2598.

(2) Ying, W. (2008) NAD^+/NADH and $NADP^+$/NADPH in cellular functions and cell death: regulation and biological consequences. *Antioxid. Redox. Signal. 10*, 179–206.

(3) May, J. M., Qu, Z. C., and Morrow, J. D. (2001) Mechanism of ascorbic acid recycling in human erythrocytes. *Biochim. Biophys. Acta 1528*, 159–166.

(4) Nagai, M., Yubisui, T., and Yoneyama, Y. (1980) Enzymatic reduction of hemoglobins M Milwaukee-1 and M Saskatoon by NADH-cytochrome b_5 reductase and NADPH-flavin reductase purified from human erythrocytes. *J. Biol. Chem. 255*, 4599–4602.

(5) Tomoda, A., Yubisui, T., Tsuji, A., and Yoneyama, Y. (1979) Kinetic studies of methemoglobin reduction by human red cell NADH cytochrome b_5 reductase. *J. Biol. Chem. 254*, 3119–3123.

(6) Nakayama, Y., Kinoshita, A., and Tomita, M. (2005) Dynamic simulation of red blood cells metabolism and its application to the analysis of a pathological condition. *Theor. Biol. Model. 2*, 18.

(7) Chang, T. M. (2005) Therapeutic applications of polymeric artificial cells. *Nat. Rev. Drug Discovery 4*, 221–235.

(8) Xiong, Y., Liu, Z. Z., Georgieva, R., Smuda, K., Steffen, A., Sendeski, M., Voigt, A., Patzak, A., and Bäumler, H. (2013) Nonvasoconstrictive hemoglobin particles as oxygen carriers. *ACS Nano 7*, 7454–7461.

(9) Alagic, A., Koprianiuk, A., and Kluger, R. (2005) Hemoglobin-superoxide dismutase-chemical linkages that create a dual-function protein. *J. Am. Chem. Soc. 127*, 8036–8043.

(10) Sakai, H. (2012) Cellular-type hemoglobin-based oxygen carrier (hemoglobin-vesicles) as a transfusion alternative and for oxygen therapeutics. *Curr. Drug Discovery Technol. 9*, 188–193.

(11) Sakai, H., Sato, A., Masuda, K., Takeoka, S., and Tsuchida, E. (2008) Encapsulation of concentrated hemoglobin solution in phospholipid vesicles retards the reaction with NO, but not CO, by intracellular diffusion barrier. *J. Biol. Chem. 283*, 1508–1517.

(12) Sakai, H., Sato, A., Sobolewski, P., Takeoka, S., Frangos, J. A., Kobayashi, K., Intaglietta, M., and Tsuchida, E. (2008) NO and CO binding profiles of hemoglobin vesicles as artificial oxygen carriers. *Biochim. Biophys. Acta 1784*, 1441–1447.

(13) Sakai, H., Horinouchi, H., Tsuchida, E., and Kobayashi, K. (2009) Hemoglobin vesicles and red blood cells as carriers of carbon monoxide prior to oxygen for resuscitation after hemorrhagic shock in a rat model. *Shock 31*, 507–514.

(14) Kobayashi, M., Mori, T., Kiyono, Y., Tiwari, V. N., Maruyama, R., Kawai, K., and Okazawa, H. (2012) Automatic labeling method for injectable ^{15}O-oxygen using hemoglobin-containing liposome vesicles and its application for measurement of brain oxygen consumption by PET. *J. Cereb. Blood Flow Metab. 32*, 33–40.

(15) Taguchi, K., Maruyama, T., Iwao, Y., Sakai, H., Kobayashi, K., Horinouchi, H., Tsuchida, E., Kai, T., and Otagiri, M. (2009) Pharmacokinetics of single and repeated injection of hemoglobin-vesicles in hemorrhagic shock rat model. *J. Controlled Release 136*, 232–239.

(16) Kaga, M., Li, H., Ohta, H., Taguchi, K., Ogaki, S., Izumi, H., Inagaki, M., Tsuchiya, S., Okamura, K., Otagiri, M., Sakai, H., and Yaegashi, N. (2012) Liposome-encapsulated hemoglobin (hemoglobin-vesicle) is not transferred from mother to fetus at the late stage of pregnancy in the rat model. *Life Sci. 91*, 420–428.

(17) Takahashi, D., Azuma, H., Sakai, H., Sou, K., Wakita, D., Abe, H., Fujihara, M., Horinouchi, H., Nishimura, T., Kobayashi, K., and Ikeda, H. (2011) Phagocytosis of liposome particles by rat splenic immature monocytes makes them transiently and highly immunosuppressive in ex vivo culture conditions. *J. Pharmacol. Exp. Ther. 337*, 42–49.

(18) Seishi, Y., Horinouchi, H., Sakai, H., and Kobayashi, K. (2012) Effect of the cellular-type artificial oxygen carrier hemoglobin vesicle as a resuscitative fluid for prehospital treatment: experiments in a rat uncontrolled hemorrhagic shock model. *Shock 38*, 153−158.
(19) Sakai, H., Masada, Y., Horinouchi, H., Yamamoto, M., Ikeda, E., Takeoka, S., Kobayashi, K., and Tsuchida, E. (2004) Hemoglobin-vesicles suspended in recombinant human serum albumin for resuscitation from hemorrhagic shock in anesthetized rats. *Crit. Care Med. 32*, 539−545.
(20) Sakai, H., Horinouchi, H., Masada, Y., Takeoka, S., Ikeda, E., Takaori, M., Kobayashi, K., and Tsuchida, E. (2004) Metabolism of hemoglobin-vesicles (artificial oxygen carriers) and their influence on organ functions in a rat model. *Biomaterials 25*, 4317−4325.
(21) Sakai, H., Takeoka, S., Yokohama, H., Seino, Y., Nishide, H., and Tsuchida, E. (1993) Purification of concentrated hemoglobin using organic solvent and heat treatment. *Protein Expr. Purif. 4*, 563−569.
(22) Sakai, H., Onuma, H., Umeyama, M., Takeoka, S., and Tsuchida, E. (2000) Photoreduction of methemoglobin by irradiation in the near-ultraviolet region. *Biochemistry 39*, 14595−14602.
(23) Takeoka, S., Sakai, H., Kose, T., Mano, Y., Seino, Y., Nishide, H., and Tsuchida, E. (1997) Methemoglobin formation in hemoglobin vesicles and reduction by encapsulated thiols. *Bioconjugate Chem. 8*, 539−544.
(24) Takeoka, S., Ohgushi, T., Nishide, H., and Tsuchida, E. (1997) Reduction of methemoglobin via electron transfer across the bilayer membrane of Hb-vesicles. *Bull. Chem. Soc. Jpn. 70*, 1171−1178.
(25) Teramura, Y., Kanazawa, H., Sakai, H., Takeoka, S., and Tsuchida, E. (2003) Prolonged oxygen-carrying ability of hemoglobin vesicles by coencapsulation of catalase *in vivo*. *Bioconjugate Chem. 14*, 1171−1176.
(26) Atoji, T., Aihara, M., Sakai, H., Tsuchida, E., and Takeoka, S. (2006) Hemoglobin vesicles containing methemoglobin and L-tyrosine to suppress methemoglobin formation *in vitro* and *in vivo*. *Bioconjugate Chem. 17*, 1241−1245.
(27) Sakai, H., Masada, Y., Onuma, H., Takeoka, S., and Tsuchida, E. (2004) Reduction of methemoglobin via electron transfer from photoreduced flavin: restoration of O_2-binding of concentrated hemoglobin solution coencapsulated in phospholipid vesicles. *Bioconjugate Chem. 15*, 1037−1045.
(28) Bradberry, S. M. (2003) Occupational methaemoglobinaemia. Mechanisms of production, features, diagnosis and management including the use of methylene blue. *Toxicol. Rev. 22*, 13−27.
(29) Jang, D. H., Nelson, L. S., and Hoffman, R. S. (2013) Methylene blue for distributive shock: a potential new use of an old antidote. *J. Med. Toxicol. 9*, 242−249.
(30) Ozal, E., Kuralay, E., Yildirim, V., Kilic, S., Bolcal, C., Kücükarslan, N., Günay, C., Demirkilic, U., and Tatar, H. (2005) Preoperative methylene blue administration in patients at high risk for vasoplegic syndrome during cardiac surgery. *Ann. Thorac. Surg. 79*, 1615−1619.
(31) Ogata, Y., Goto, H., Kimura, T., and Fukui, H. (1997) Development of neo red cells (NRC) with the enzymatic reduction system of methemoglobin. *Artif. Cells Blood Substit. Immobil. Biotechnol. 25*, 417−427.
(32) Liu, Z. C., and Chang, T. M. (2008) Long-term effects on the histology and function of livers and spleens in rats after 33% toploading of PEG-PLA-nano artificial red blood cells. *Artif. Cells Blood Substit. Immobil. Biotechnol. 36*, 513−524.
(33) Takeoka, S., Teramura, Y., Atoji, T., and Tsuchida, E. (2002) Effect of Hb-encapsulation with vesicles on H_2O_2 reaction and lipid peroxidation. *Bioconjugate Chem. 13*, 1302−1308.
(34) Tsuchida, E., Sou, K., Nakagawa, A., Sakai, H., Komatsu, T., and Kobayashi, K. (2009) Artificial oxygen carriers, hemoglobin vesicles and albumin-hemes, based on bioconjugate chemistry. *Bioconjugate Chem. 20*, 1419−1440.
(35) McGown, E. L., Lyons, M. F., Marini, M. A., and Zegna, A. (1990) Reduction of extracellular methemoglobin by erythrocytes. *Biochim. Biophys. Acta 1036*, 202−206.
(36) den Boer, P. J., Bleeker, W. K., Rigter, G., Agterberg, J., Stekkinger, P., Kannegieter, L. M., de Nijs, I. M., and Bakker, J. C. (1992) Intravascular reduction of methemoglobin in plasma of the rat *in vivo*. *Biomater. Artif. Cells Immob. Biotechnol. 20*, 647−650.
(37) Harrington, J. P., Gonzalez, Y., and Hirsch, R. E. (2000) Redox concerns in the use of acellular hemoglobin-based therapeutic oxygen carriers: The role of plasma components. *Artif. Cells Blood Substitutes Immob. Biotechnol. 28*, 477−492.
(38) Vandegriff, K. D., Malavalli, A., Minn, C., Jiang, E., Lohman, J., Young, M. A., Samaja, M., and Winslow, R. M. (2006) Oxidation and haem loss kinetics of poly(ethylene glycol)-conjugated haemoglobin (MP4): dissociation between in vitro and in vivo oxidation rates. *Biochem. J. 399*, 463−471.
(39) Buehler, P. W., D'Agnillo, F., Hoffman, V., and Alayash, A. I. (2007) Effect of endogenous ascorbate on oxidation, oxygenation and toxicokinetics of cell-free modified hemoglobin after exchange transfusion in rat and guinea pig. *J. Pharmacol. Exp. Therapeut. 323*, 49−60.
(40) Bunn, H. F., and Forget, B. G. (1986) *Hemoglobin: molecular, genetic, and clinical aspects*, W.B. Saunders Company, Philadelphia.
(41) Nakagaki, M., Katoh, I., and Handa, T. (1981) Surface potential of lipid membrane estimated from the partitioning of methylene blue into liposomes. *Biochemistry. 20*, 2208−2212.
(42) Antunes-Madeira, M. C., Almeida, L. M., and Madeira, V. M. (1990) Effects of lindane on membrane fluidity: intramolecular excimerization of a pyrene derivative and polarization of diphenylhexatriene. *Biochim. Biophys. Acta 1022*, 110−114.
(43) Sakai, H., Sato, A., Takeoka, S., and Tsuchida, E. (2007) Rheological properties of hemoglobin vesicles (artificial oxygen carriers) suspended in a series of plasma-substitute solutions. *Langmuir 23*, 8121−8.
(44) Lozano, M., Cid, J., and Muler, T. H. (2013) Plasma treated with methylene blue and light: clinical efficacy and safety profile. *Transfus. Med. Rev. 47*, 235−240.
(45) Wainwright, M., and Amaral, L. (2005) Review: The phenothiazinium chromophore and the evolution of antimalarial drugs. *Tropical Med. Int. Health 10*, 501−511.
(46) Schirmer, R. H., Adler, H., Pickhardt, M., and Mandelkow, E. (2011) Review "Lest we forget you − methylene blue···". *Microbiol. Aging 2325*, e7−e16.
(47) Menardi, A. C., Viaro, F., Vicente, W. V., Rodrigues, A. J., and Evora, P. R. (2006) Hemodynamic and vascular endothelium function studies in healthy pigs after intravenous bolus infusion of methylene blue. *Arq. Bras. Cardiol. 87*, 525−532.
(48) DiSanto, A. R., and Wagner, J. G. (1972) Pharmacokinetics of highly ionized drugs III: Methylene blue-blood levels in the dog and tissue levels in the rat following intravenous administration. *J. Pharmaceut. Sci. 61*, 1090−1094.
(49) May, J. M., Qu, Z. C., and Cobb, C. E. (2004) Reduction and uptake of methylene blue by human erythrocytes. *Am. J. Physiol. Cell Physiol. 286*, C1390−8.
(50) Kennett, E. C., and Kuchel, P. W. (2006) Plasma membrane oxidoreductases: Effects on erythrocyte metabolism and redox homeostasis. *Antioxid. Redox Signal. 8*, 1241−1247.
(51) Kelner, M. J., and Alexander, N. M. (1985) Methylene blue directly oxidizes glutathione without the intermediate formation of hydrogen peroxide. *J. Biol. Chem. 260*, 15168−15171.
(52) Mowry, S., and Ogren, P. J. (1999) Kinetics of methylene blue reduction by ascorbic acid. *J. Chem. Educ. 76*, 970−973.
(53) Nandi, A., Mukhopadhyay, C. K., Ghosh, M. K., Chattopadhyay, D. J., and Chatterjee, I. B. (1972) Evolutionary significance of vitamin C biosynthesis in terrestrial vertebrates. *Free Radic. Biol. Med. 22*, 1047−1054.
(54) Su, D., May, J. M., Koury, M. J., and Asard, H. (2006) Human erythrocyte membranes contain a cytochrome *b*561 that may be involved in extracellular ascorbate recycling. *J. Biol. Chem. 281*, 39852−39859.

(55) Fultz, M. L., and Durst, R. A. (1982) Mediator compounds for the electrochemical study of biological redox systems: a compilation. *Anal. Chim. Acta 140*, 1−18.
(56) Williamson, J. (1959) Drug resistance in trypanosomes, effects of metabolic inhibitors, pH and oxidation-reduction potential on normal and resistant typanosoma rhodesiense. *Br. J. Pharmacol. 14*, 443−455.
(57) Kiese, M., Lorcher, W., Weger, N., and Zierer, A. (1972) Comparative studies of the effects of toluidine blue and methylene blue on the reduction of ferrihaemoglobin in man and dog. *Eur. J. Clin. Pharmacol. 4*, 115−118.

Normothermic Preservation of the Rat Hind Limb With Artificial Oxygen-carrying Hemoglobin Vesicles

Jun Araki,[1] Hiromi Sakai,[2,3] Dai Takeuchi,[1,4] Yu Kagaya,[1,5] Kensuke Tashiro,[1] Munekazu Naito,[6] Makoto Mihara,[1] Mitsunaga Narushima,[1] Takuya Iida,[1] and Isao Koshima[1]

Background. For managing major limb amputation, it is important to consider ischemic time and reperfusion injury by free radicals after the blood supply is reestablished. State of preservation during transplant surgery is crucial for the survival and function of the tissue, graft, or organ. In this study, we confirmed the effect of intermittent blood flow in rat ischemic hind limb and developed a new oxygenic preservation method using artificial oxygen carrying hemoglobin vesicles (HbVs). **Methods.** We first compared a continuous ischemic model and an intermittent reflow model on rat hind limb. At postoperative day 7, hind limbs were evaluated. Next, we performed total amputation, normothermic preservation by perfusion with extracellular-trehalose-Kyoto (ETK) solution or HbV, and microsurgical replantation of the left hind limb. Venous efflux was analyzed, the amputated limb evaluated after 6 hr perfusion, and the replantation outcome of each model was compared. **Results.** In our early study, 24 hr continuous ischemic model necrotized, but intermittent reflow model almost survived except for partial necrosis at postoperative day 7. Scar tissue on the right limb showed myonecrosis and infiltration of inflammatory cells. Skeletal muscle on the right limb was structurally well maintained. Hemoglobin vesicle–treated limbs appeared to have much better oxygenation than ETK-treated limbs. Aerobic respiration remained in the amputated limb, gastrocnemius muscle was well maintained, and the overall replantation was successful in the limb preserved using HbV. **Conclusion.** These studies demonstrated that oxygenic preservation is effective for rat ischemic limb, suggesting that this method may be useful for other replantation and transplantation surgeries.

(*Transplantation* 2015;99: 687–692)

Of late, allotransplantations involving vascularized composite tissue, such as face,[1] hand,[2] larynx,[3] and trachea,[4] have been successful, wherein ischemic time affects functional success to a greater degree than other organ transplants. In limb replantation or transplantation, the harvested extremity must be revascularized within 4 to 6 hr[5-7] to avoid extensive reperfusion injury and complete loss of the limb because of vascular leakage, edema, and subsequent tissue necrosis.[8] The outcome of extremity transplantation may be optimized by the choice of donor[9,10] and planning of the surgical intervention,[11,12] but the ischemic time before successful revascularization remains crucial and so far is a limiting factor.[8] Bone, tendon, fat, and skin tolerate long-term ischemia relatively well; however, muscular, vascular, and neural tissues are easily mortally injured, which is related to tissue necrosis and loss of function or compartment syndrome as a consequence of reperfusion injury.[5,6,8]

Ex vivo organ perfusion has been performed to preserve kidney[13] and lung[14] however, there are few reports of optimization of limb preservation.[8] Tissue-preserving potential and the feasibility of extremity perfusion using autologous blood have been investigated. Artificial oxygen carriers are considered to have higher potential than autologous blood for

Received 4 September 2014. Revision requested 26 September 2014.

Accepted 26 September 2014.

[1] *Department of Plastic Surgery, University of Tokyo, Tokyo, Japan.*

[2] *Artificial Red Cells Group, Waseda Bioscience Research Institute in Singapore (WABIOS), Biopolis, Singapore.*

[3] *Department of Chemistry, School of Medicine, Nara Medical University, Nara, Japan.*

[4] *Department of Plastic Surgery, Saitama Medical Center, Saitama Medical University, Saitama, Japan.*

[5] *Department of Plastic Surgery, University of Yamanashi Hospital, Yamanashi, Japan.*

[6] *Department of Anatomy, Aichi Medical University, Aichi, Japan.*

Research funding from the Nakatomi Foundation, JSPS KAKENHI (B) 22300161, and Health and Labour Sciences Research Grants (Health Science Research Including Drug Innovation) from the Ministry of Health, Labour and Welfare, Japan.

The authors declare no conflicts of interest.

Of the authors, H.S. is an inventor in the patents related to the production and utilization of Hb vesicles.

J.A. designed the study, performed the surgeries, performed the statistical analyses, and co-wrote the article. H.S. designed and prepared the hemoglobin vesicles and co-wrote the article. D.T., Y.K., and K.T. assisted in the surgeries. M.N. designed and performed functional examination. M.M., M.N., T.I., and I.K. supervised the study and revised the article.

Correspondence: Jun Araki, M.D., Department of Plastic Surgery, University of Tokyo Graduate School of Medicine, 7-3-1, Hongo, Bunkyo-Ku, Tokyo 113-8655, Japan. (arakij-pla@h.u-tokyo.ac.jp).

Supplemental digital content (SDC) is available for this article. Direct URL citations appear in the printed text, and links to the digital files are provided in the HTML text of this article on the journal's Web site (www.transplantjournal.com).

ISSN: 0041-1337/15/9904-687

DOI: 10.1097/TP.0000000000000528

clinical application because the recipient may undergo invasive or emergency surgery. Hemoglobin vesicles (HbVs) are artificial oxygen carriers that encapsulate purified human Hb solution in phospholipid vesicles (liposomes).[15] The safety and efficacy of HbVs as a transfusion alternative and as an oxygen therapeutic agent have been studied extensively using animal models.

In this study, we demonstrated that intermittent blood supply was effective for the preservation of composite tissue using a rat ischemic hind limb model. In addition, we developed new perfusate for composite tissue using a combination of conventional extracellular-trehalose-Kyoto (ETK) solution and HbVs.

RESULTS

Comparative Study of a 24-Hr Continuous Ischemic Model and an Intermittent Reflow Model on Hind Limb

During the 24-hr experiments, macroscopically, no significant differences were observed between the right and left limbs (see **Figure S1A, SDC,** http://links.lww.com/TP/B89). At postoperative day 7, the right limbs (24-hr continuous ischemic model) necrotized, whereas the left limbs (intermittent reflow model) survived except for partial necrosis (see **Figure S1B, SDC,** http://links.lww.com/TP/B89). Scar tissue on the right limbs showed myonecrosis and infiltration of inflammatory cells (see **Figure S1C, D, SDC,** http://links.lww.com/TP/B89). Skeletal muscle on the light limbs was structurally well maintained (see **Figure S1E, F, SDC,** http://links.lww.com/TP/B89). Black (see **Figure S1C, E, SDC,** http://links.lww.com/TP/B89) and white (see **Figure S1D, F, SDC,** http://links.lww.com/TP/B89) scale bars indicate 200 and 25 μm, respectively.

Normothermic Perfusion and Blood Gas Analysis

The ETK and ETK + HbV perfusates were analyzed by a blood gas analyzer before perfusion. Limbs perfused by ETK only presented a white ischemic color (Figure 1A), whereas limbs perfused by ETK + HbV presented a red color (Figure 1B). Three important parameters indicating oxygen metabolism, PO_2, PCO_2, and lactic acid, were compared between ETK and ETK + HbV perfused limbs. PO_2 levels were maintained at 80 mm Hg in the ETK efflux but dropped to 30 mm Hg in the ETK + HbV efflux, and they were significantly different (Figure 1C). PCO_2 levels were 8.2 ± 2.1 mm Hg in the ETK efflux and 19.6 ± 10.1 mm Hg in the ETK + HbV efflux 1 hr after perfusion, and then both were gradually dropped. They were not significantly different during 6 hr perfusion (Figure 1D). Lactic acid levels were 2.7 ± 0.3 mm Hg in the ETK efflux and 1.8 ± 0.6 mm Hg in the ETK + HbV efflux 1 hr after perfusion, and then both were gradually dropped. They were not significantly different until 3 hr after perfusion but the ETK + HbV efflux showed significantly higher levels than ETK efflux from 4 hr to 6 hr after perfusion (Figure 1E).

Macroscopic and Microscopic Observations of Amputated Rat Limb After Normothermic Preservation for 6 Hr

Amputated rat limbs storage by wet and cold (4°C) showed no macroscopic changes or weight increase rate (Figure 2A). Limbs perfused by ETK only developed severe edema and a weight increase rate of 130% (Figure 2B). Limbs perfused by ETK + HbV experienced moderate edema, with a corresponding weight increase rate of 110% (Figure 2C). On histologic examination of gastrocnemius muscle, compared to limbs in wet and cold storage (Figure 2D), the muscle fibers were shrunk, and interstitial edema was severe in the muscle tissues perfused by ETK only (Figure 2E). Muscle tissue perfused with ETK + HbV, however, was preserved with only minor signs of shrinking of muscle fibers and interstitial edema (Figure 2F).

Replantation, Limb Survival, and Function Outcome

Replantation was successfully performed for all 12 limbs. Limbs preserved by wet and cold appeared to have insufficient blood circulation, causing limb necrosis within 3 days of surgery (Figure 3A). Limbs perfused by ETK only

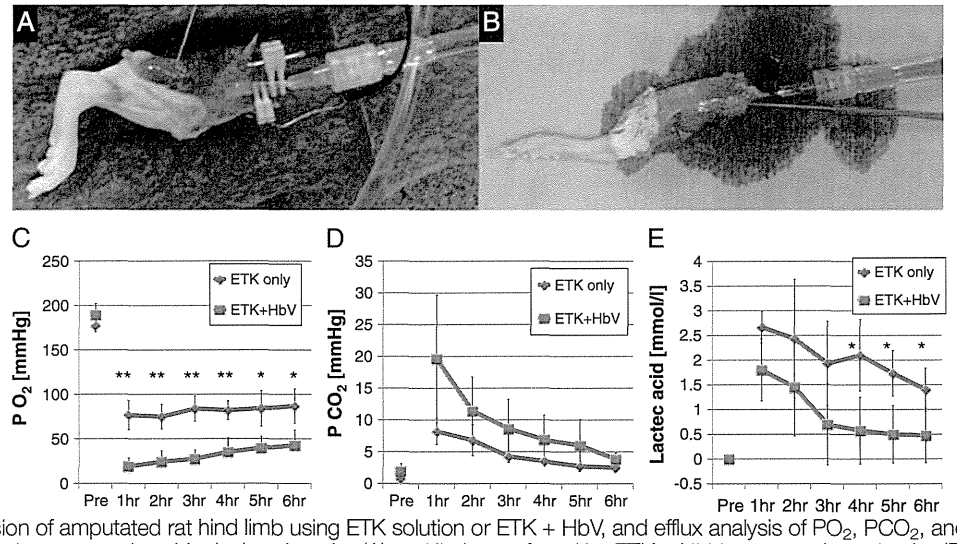


FIGURE 1. Perfusion of amputated rat hind limb using ETK solution or ETK + HbV, and efflux analysis of PO_2, PCO_2, and lactic acid. Limbs perfused by ETK only presented a white ischemic color (A) and limbs perfused by ETK + HbV presented a red color (B). Three important parameters describing oxygen metabolism, PO_2 (C), PCO_2 (D), and lactic acid (E), suggested that aerobic respiration remained in the amputated limb perfused by ETK + HbV. (C–E) Statistical significance was defined as $^*P<0.05$ and $^{**}P<0.01$. ETK, extracellular-trehalose-Kyoto; HbV, hemoglobin vesicles.

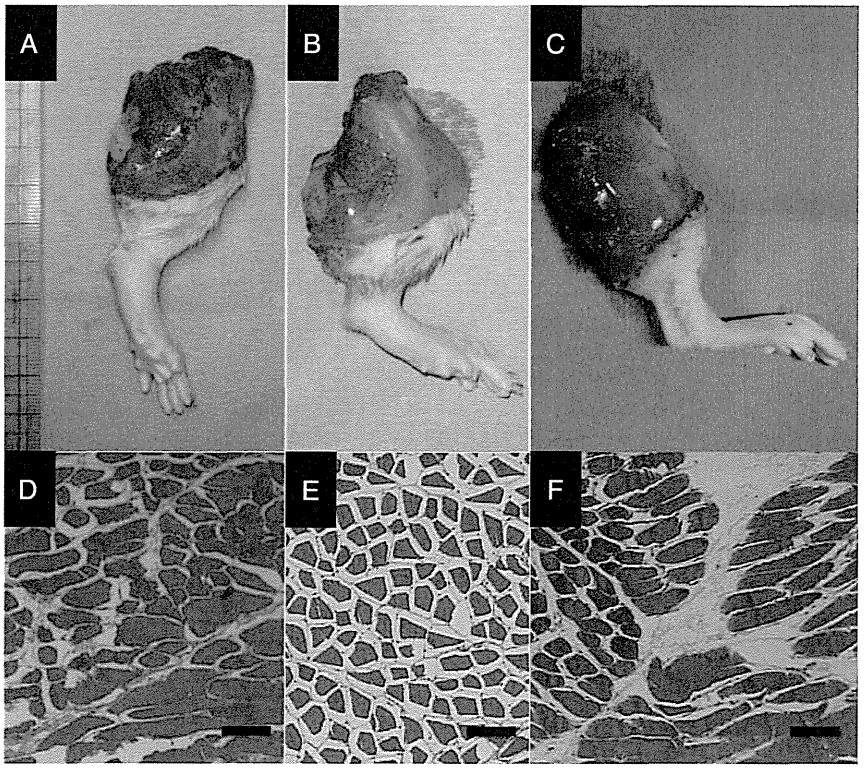


FIGURE 2. Macroscopic and microscopic findings of amputated rat limb after normothermic preservation for 6 hr. Amputated rat limbs preserved by wet and 4°C cold condition had no change in macroscopic appearance or weight increase rate (A). Limbs perfused by ETK only (B) or ETK + HbV (C) developed edema and weight gain. On histologic examination of gastrocnemius muscle, compared to limbs in cold storage (D), the muscle fibers were shrunk and interstitial edema was severe in the limb perfused by ETK only (E), whereas the limb perfused by ETK + HbV was preserved with only minor signs of muscle fiber shrinking and interstitial edema (F). Black scale bars indicate 100 μm. ETK, extracellular-trehalose-Kyoto; HbV, hemoglobin vesicles.

appeared to have blood circulation the day after surgery (Figure 3B), but animals were dead within 3 days of surgery because of body strength loss from lack of feeding, likely the result of the ischemia-reperfusion injury. However, limbs perfused by ETK + HbV showed excellent blood circulation and survived for 2 weeks after operation (Figure 3C). In addition, functional examination showed the rats could walk using the replanted limb at postoperative 3 months (Figure 3D, E; also see **Videos, S1** and **S2, SDC,** http://links.lww.com/TP/B90 and http://links.lww.com/TP/B154).

DISCUSSION

In this early experimental trial, intermittent blood flow provided a much better outcome than continuous ischemia for rat limb preservation at room temperature. Only a 10-min perfusion every 4 hr appeared to result in insufficient blood supply, but was nonetheless effective in preserving the limb (see **Figure. S1, SDC,** http://links.lww.com/TP/B89). It is said that as in health under normal physiologic conditions, there is a safety margin of oxygen delivered to the tissues.[16]

With this in mind, we performed a series of limb preservation studies with oxygen carriers. Autologous blood is often used in such experiments,[17] but the clinical utility of this approach is limited. There are few studies on organ preservation using ETK and hemoglobin.[18] We therefore used HbVs for perfusion and replantation studies. The suspension of Hb within vesicles has advantages, such as sufficient stability, both in storage and in blood circulation, absence of blood type complications and pathogens, and prompts degradation by the reticuloendothelial system. Initial animal studies demonstrated the potential of HbV as a transfusion alternative. The smaller average particle size of HbVs (0.25 μm), compared to RBCs (8 μm), allows better perfusion through narrow capillaries. Recent advances in HbV research suggest this approach may offer unique advantages in the management of major limb amputation.[15]

We used ETK as a control study and as a base of an oxygenic preservation fluid. For normothermic preservation, ETK, as an organ preservation fluid, is known to be superior to the University of Wisconsin and intracellular-trehalose-Kyoto solutions.[19] Whereas ETK has a high-sodium/low-potassium composition similar to that of extracellular fluid, University of Wisconsin has a low-sodium/high-potassium composition similar to that of intracellular fluid.[20] In this study, we succeeded in replantation of the rat hind limb after approximately 8 hr warm ischemia using HbVs mixed with ETK in a ratio of 1:1 (ETK + HbV). Total 8 hr warm ischemia included 2-hr interval for cannulation of the femoral vessels and the replantation surgery before and after 6 hr of normothermic perfusion. Interestingly, this method had much better survival and functional results than 6 hr cold storage with 2-hr interval, which was the gold standard to preserve organs. A previous study showed that only two of 10 replantations of the rat hind limb survived after 4 hr warm ischemia.[21] This achievement roughly doubled the ischemic time. However, further studies need to be conducted to optimize solution composition for prolonging the ischemic time.

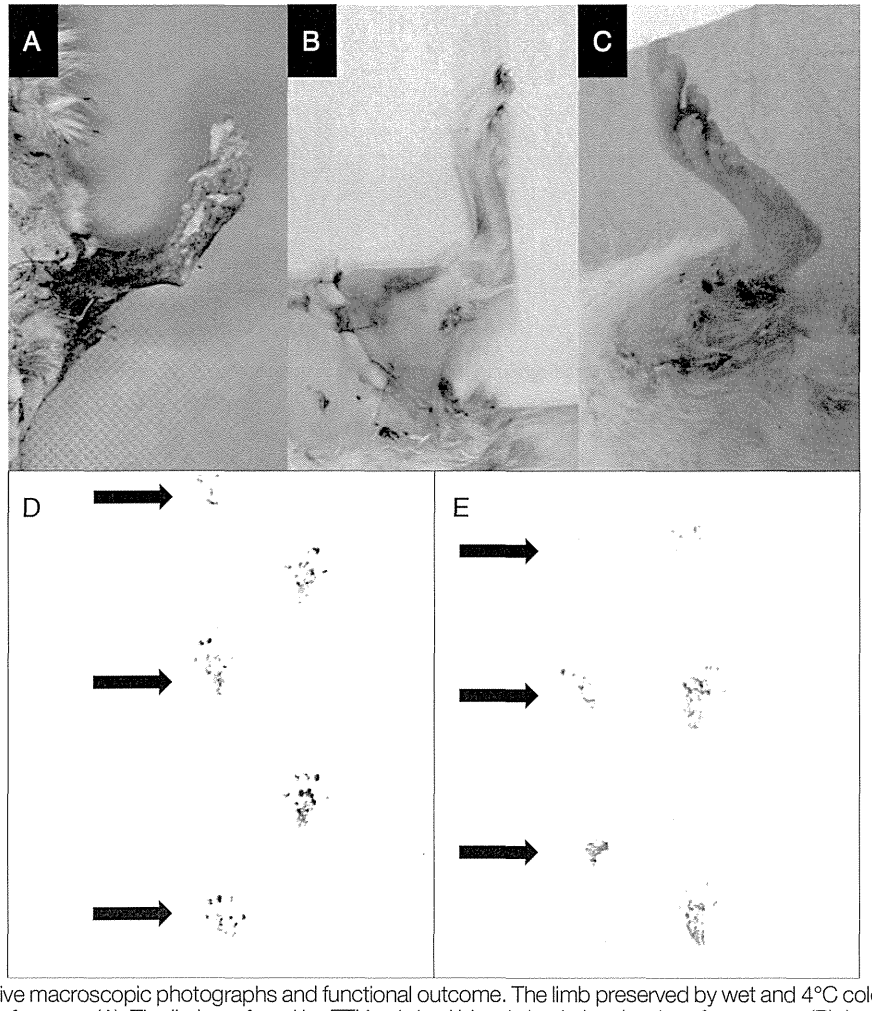


FIGURE 3. Postoperative macroscopic photographs and functional outcome. The limb preserved by wet and 4°C cold condition showed necrosis within three days of surgery (A). The limb perfused by ETK only had blood circulation the day after surgery (B), but the animals were dead three days later because of body strength loss and lack of feeding, likely derived from ischemia-reperfusion injury. Limbs perfused by ETK + HbV showed excellent blood circulation and survived two weeks post operation (C). In addition, functional examination showed the rats could walk using the replanted limb at postoperative 3 months (E), although it was not perfect compared to normal rat (D). ETK, extracellular-trehalose-Kyoto; HbV, hemoglobin vesicles.

Many reports describe oxygen delivery to tissues and organs. The oxygen content in a fluid (blood) of 1 dL [$C(O_2)$] may be expressed by the following equation[22]:

$C(O_2)$ [(O_2)mL/(fluid)dL] = $P(O_2)$ × 0.0225 + Hb × $S(O_2)$ × 1.306,

where $P(O_2)$ is the mean oxygen partial pressure (15.4 kPa in arterial blood under 20% FiO_2), Hb is the hemoglobin concentration (g/dL), and $S(O_2)$ is the oxygen saturation (generally close to 1).

As indicated in this equation, hemoglobin in red blood cells usually contains most (~99%) of the oxygen in the blood, whereas the remaining plasma portion contains only approximately 1%.[23] The final Hb concentration was adjusted to 5 g/dL during ETK + HbV administration. This level is not sufficient but appears to provide much better oxygenation than ETK only for the amputated limb. In fact, blood gas analysis clearly showed that aerobic respiration was maintained in the amputated limb (Figure 1) and gastrocnemius muscle (Figure 2), and that replantation was successful (Figure 3) in the ETK + HbV treatment group.

In this reperfusion study, using artificial oxygen carriers, we have not observed macrophage phagocytosis of HbV in the limb (Figure 2E), which is in contrast to that observed in the liver and spleen (reticuloendothelial system) in systemic perfusion studies. This suggests that HbV does not remain in the composite tissue graft after heparinized saline irrigation. Organ-perfusing storage solution, including ETK, does not correspond to a category of pharmaceutical products in Japan. As such, HbV holds significant potential for clinical application.

Amputated limbs are currently preserved by keeping them cool and moist. New preservation methods using artificial oxygen-carrying HbVs may be useful for replantation surgery of not only limbs but also the other parts of body, such as scalp,[24] ear,[25] nose,[26] lip,[27] and genitalia,[28] in clinical practice. Moreover, when safety and efficacy of oxygenic preservation methods have been established in these composite grafts, they could be applied to transplantation of other vital organs including heart, lung, liver, kidney, and intestine.

MATERIALS AND METHODS

Animals

Fifteen healthy adult male Wistar rats weighing 400 to 470 g (average 430 g) were included in this study. Two rats

were used for an experimental comparative study of a 24-hr continuous ischemic model and an intermittent reflow model on hind limb. Other 12 rats were used for amputation of the left limb, preservation by perfusion, and replantation. The other one was used for normal control of functional examination. Animals were allowed access to food and water before and after surgery. All animal experiments were approved by the University of Tokyo Animal Care and use Committee with institutional guidelines (M-P13-044) and by the IACUC of the Biological Resource Center of A*STAR (110700).

Comparative Study of a 24-Hr Continuous Ischemic Model and an Intermittent Reflow Model on Hind Limb

Animals were anesthetized by intraperitoneal injection of pentobarbital and placed supine. Through a bilateral semicircular inguinal incision, the femoral arteries and veins were dissected and clamped by microvessel clips (BEAR, Tokyo, Japan). Skin incisions were then closed, and the animals were allowed to recover from anesthesia. After 4 hr of clamping, only the left side femoral vessels were declamped, and the left limb reperfused for 10 min under anesthesia. Animals were administered heparin (50 U; Ajinomoto, Tokyo, Japan) intraperitoneally in every intervention, and the administration was repeated five times. Finally, bilateral femoral vessels were declamped 24 hr after initial clamping, and both limbs reperfused. At postoperative day 7, hind limbs were macroscopically and microscopically evaluated.

Preparation of Perfusate With Artificial Oxygen-Carrying HbVs

The ETK, an organ preservation fluid, was purchased from Otsuka Pharmaceutical Company (Osaka, Japan). One bag (1000 mL) contained sodium gluconate (21.814 g), KH_2PO_4 (0.885 g), K_2HPO_4 (3.222 g), hydroxyethyl starch (30.0 g), and trehalose dehydrate (45.3 g) (pH 7.3–7.4). The HbVs were prepared using a previously reported method.[29-31] Briefly, human Hb solution was obtained through purification of outdated RBCs provided by the Japanese Red Cross Society (Tokyo, Japan). Hemoglobin was then stabilized by carbonylation (HbCO) and concentrated by ultrafiltration to 38 g/dL. Subsequently, pyridoxal 5′-phosphate (PLP; Sigma Chemical Co., St. Louis, MO) was added to the HbCO solution as an allosteric effector at a molar ratio of PLP/Hb tetramer = 1.0. The Hb solution with PLP was then mixed with lipids and encapsulated in vesicles. The lipid bilayer comprised 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylcholine, cholesterol, 1,5-O-dihexadecyl-*N*-succinyl-L-glutamate (Nippon Fine Chemical Co. Ltd., Osaka, Japan), and 1,2-distearoyl-*sn*-glycerol-3- phosphatidylethanolamine-*N*-PEG5000 (NOF Corp., Tokyo, Japan) at a molar ratio of 5, 4, 0.9, and 0.03, respectively. The particle diameter was regulated by extrusion. Encapsulated HbCO was converted to HbO_2 by exposing the liquid membranes of HbVs to visible light under an O_2 atmosphere. Finally, the Hb concentration of the suspension was adjusted to 10 g/dL. Hemoglobin vesicles were mixed with ETK in a ratio of 1:1.

Normothermic Preservation With Perfusion Fluids, Efflux Analysis, and Replantation of Rat Hind Limb

Animals were anesthetized by intraperitoneal injection of pentobarbital and inhalation of isoflurane in supine position. A circumferential incision was made at the midthigh region of the left hind limb and extended into the skin and the underlying musculature. The inferior epigastric vessels were isolated and cauterized. The femoral muscles were cut with an electric scalpel and the femur transected with a bone rongeur. The femoral nerve and femoral vessels were then clamped and cut. Finally, the left hind limb was amputated. The femoral artery of the amputated limb was cannulated with a 27-G blunt needle and injected with heparinized saline to wash out the blood. Four limbs were maintained under wet condition in cold storage chamber (4°C), four limbs were perfused by ETK only, and four limbs were perfused by a perfusate prepared with HbV and ETK (ETK + HbV) through the femoral artery. The perfusion speed was under 1 mL per min. The femoral vein was also cannulated with a polyethylene tube (SP-31 tubing, OD 0.8 mm, ID 0.5 mm; Natsume Seisakusho Co Ltd, Tokyo, Japan), and efflux fluid was analyzed with a blood gas analyzer (ABL80 FLEX, Radiometer, Copenhagen, Denmark) at hourly intervals. During perfusion, the amputated limb was wrapped in gauze moistened with normal saline and placed in a sterile at room temperature (23°C–27°C). Before and after 6 hr of perfusion, the amputated limb was weighed, and a biopsy of the gastrocnemius muscle was performed. The replantation procedure was performed approximately 6 hr after the initiation of ischemia time before 8 hr of the total warm ischemia time. Replantation was performed with the amputated femur fixated to the residual proximal femur in a retrograde fashion through the knee with a 23-gauge needle. The femoral artery and vein were then anastomosed under an operative microscope in an interrupted suture using 10-0 nylon. Vessel clamps were removed and anastomotic patency confirmed using the milk test for 20 min before skin closure. The total warm ischemic time was approximately 8 hr. The muscular layer was sutured circumferentially, and the skin closed. An effort was made to close the limb muscle and skin layer with the hip in an adducted and flexed position to minimize tension on the anastomosis. An Elizabethan collar was used to allow the animals to consume unlimited food and water while preventing autocannibalization. Animals were intraperitoneally injected antibiotic and anti-inflammatory drugs twice per day for 7 days and observed in separate cages after surgery.

Histologic Examination

As described above, 12 rat hind limbs were isolated and preserved by three ways: wet and cold storage (n = 4), perfusion by ETK (n = 4), and perfusion by ETK + HbV (n = 4). After 6 hr of perfusion, the gastrocnemius muscle biopsies were collected. All samples were fixed in 4% buffered formaldehyde solution and used for paraffin embedding and histologic evaluation (hematoxylin-eosin staining).

Functional Examination

Walking Track Analysis was performed to examine replanted hind limb after 3 months of replantation as described previously.[32] Briefly, rats were allowed conditioning trials in a walking track (8.2 × 42 cm) darkened at one end. White office paper cut to the appropriate dimensions was placed on the bottom of the track. The rat's hind limbs were dipped in Chinese ink, and the rat was permitted to walk down the track, leaving its hind footprints on the paper. In addition walking appearance was taken by a digital camera (COOLPIX S8200; Nikon Corporation, Tokyo, Japan).

Statistical Analysis

Results are described as means ± standard deviations. Comparisons between the two groups were performed using the unpaired Student *t* test. Comparisons of more than two groups were made by analysis of variance with the Bonferroni correction. Statistical significance was defined as P less than 0.05.

ACKNOWLEDGMENT

The authors thank Ms. Ayako Sato, Makiko Hieda, Yukari Yamashita, Kana Kizuki, and Yoshiko Watabe for their technical assistance.

REFERENCES

1. Siemionow M, Papay F, Alam D, et al. Near-total human face transplantation for a severely disfigured patient in the USA. *Lancet*. 2009;18:203.
2. Jones JW, Gruber SA, Barker JH, et al. Successful hand transplantation. One-year follow-up. Louisville Hand Transplant Team. *N Engl J Med*. 2000;343:468.
3. Strome M, Stein J, Esclamado R, et al. Laryngeal transplantation and 40 month follow up. *N Engl J Med* 2001;344:1676.
4. Delaere P, Vranckx J, Verleden G, et al. Tracheal allotransplantation after withdrawal of immunosuppressive therapy. *N Engl J Med* 2010;14:138.
5. Steinau H-U. *Limb Replantation and Postischemia Syndrome of Acute Ischemic-Induced Myopathy and Reperfusion Injury*. New York: Springer Verlag 1988;9:33.
6. Blaisdell FW. The pathophysiology of skeletal muscle ischemia and the reperfusion syndrome: a review. *Cardiovasc Surg* 2002;10:620.
7. Muramatsu I, Takahata N, Usui M, et al. Metabolic and histologic changes in the ischemic muscles of replanted doglegs. *Clin Orthop Relat Res* 1985;196:292.
8. Constantinescu MA, Knall E, Xu X, et al. Preservation of amputated extremities by extracorporeal blood perfusion; a feasibility study in a porcine model. *J Surg Res* 2011;171:291.
9. Petruzzo P, Lanzetta M, Dubernard JM, et al. The international registry on hand and composite tissue transplantation. *Transplantation* 2008;86:487.
10. Hettiaratchy S, Randolph MA, Andrew Lee WP. Long-term consideration of hand transplantation. *Transplantation* 2003;75:1605.
11. Atkins SE, Winterton RIS, Kay SP. Upper limb amputations: where, when and how to replant. *Curr Orthop* 2008;22:31.
12. Lloyd MS, Teo TC, Pickford MA, et al. Preoperative management of the amputated limb. *Emerg Med J* 2005;22:478.
13. Moers C, Smits JM, Maathuis MH, et al. Machine perfusion or cold storage in deceased-donor kidney transplantation. *N Engl J Med* 2009;360:7.
14. Cypel M, Yeung JC, Liu M, et al. Normothermic ex vivo lung perfusion in clinical lung transplantation. *N Engl J Med* 2011;364:1431.
15. Sakai H, Sou K, Horinouchi H, et al. Haemoglobin-vesicles as artificial oxygen carriers: present situation and future visions. *J Intern Med* 2008; 263:4.
16. Thomas D. The physiology of oxygen delivery. *Vox Sang*. 2004; 87(Suppl 1):70.
17. Müller S, Constantinescu MA, Kiermeir DM, et al. Ischemia/reperfusion injury of porcine limbs after extracorporeal perfusion. *J Surg Res* 2013; 181:170.
18. Yamaoka I, Kikuchi T, Arata T, et al. Organ preservation using a photosynthetic solution. *Transplant Res* 2012;1:2.
19. Iwai S, Kikuchi T, Kasahara N, et al. Impact of normothermic preservation with extracellular type solution containing trehalose on rat kidney grafting from a cardiac death donor. *PLoS One* 2012;7:e33157.
20. Moen J, Claesson K, Pienaar H, et al. Preservation of dog liver, kidney, and pancreas using the Belzer-UW solution with a high-sodium and low-potassium content. *Transplantation* 1989;47:940.
21. Salgado CJ, Jamali AA, Ortiz JA, et al. Effects of hyperbaric oxygen on the replanted extremity subjected to prolonged warm ischaemia. *J Plast Reconstr Aesthet Surg* 2010;63:532.
22. Lumb AB, Nair S. Effects of increased inspired oxygen concentration on tissue oxygenation: theoretical considerations. *Eur J Anaesthesiol* 2010;27:275.
23. Araki J, Kato H, Doi K, et al. Application of normobaric hyperoxygenation to an ischemic flap and a composite skin graft. *Plast Reconstr Surg Glob Open* 2014;2:e152.
24. Cheng K, Zhou S, Jiang K, et al. Microsurgical replantation of the avulsed scalp: report of 20 cases. *Plast Reconstr Surg* 1996;97:1099.
25. Kind GM, Buncke GM, Placik OJ, et al. Total ear replantation. *Plast Reconstr Surg*. 1997;99:1858.
26. Hammond DC, Bouwense CL, Hankins WT, et al. Microsurgical replantation of the amputated nose. *Plast Reconstr Surg* 2000;105:2133.
27. Walton RL, Beahm EK, Brown RE, et al. Microsurgical replantation of the lip: a multi-institutional experience. *Plast Reconstr Surg* 1998;102:358.
28. Griffiths M, Britto J, Frame J. 5-year follow-up of replantation of penis and testis in a child. *Lancet* 2003;361:263.
29. Sakai H, Takeoka S, Yokohama H, et al. Purification of concentrated hemoglobin using organic solvent and heat treatment. *Protein Expr Purif* 1993;4:563.
30. Sakai H, Masada Y, Takeoka S, et al. Characteristics of bovine hemoglobin as a potential source of hemoglobin-vesicles for an artificial oxygen carrier. *J Biochem* 2002;131:611.
31. Sakai H, Sou K, Tsuchida E. Hemoglobin-vesicles as an artificial oxygen carrier. *Methods Enzymol* 2009;465:363.
32. Wong KH, Naidu M, David P, et al. Peripheral nerve regeneration following crush injury to rat peroneal nerve by aqueous extract of medicinal mushroom *Hericium erinaceus* (Bull.: Fr) Pers. (Aphyllophoromycetideae). *Evid Based Complement Alternat Med* 2011;2011:580752.