Intraosseous transfusion of hemoglobin vesicles in the treatment of hemorrhagic shock with collapsed vessels in a rabbit model

Kohsuke Hagisawa,[1] Manabu Kinoshita,[2] Daizoh Saitoh,[3] Yuji Morimoto,[1] and Hiromi Sakai[4]

BACKGROUNDS: Intravenous transfusion sometimes encounters difficulty under prehospital conditions when peripheral vessels are collapsed and inaccessible. We investigated whether the cellular type hemoglobin-based oxygen carriers (Hemoglobin Vesicles: HbVs) allow intraosseous administration into blood circulation for the resuscitation of rabbits with severe hemorrhagic shock.

STUDY DESIGN AND METHODS: New Zealand white rabbits (2.5 kg average) were set in severe hemorrhagic shock [mean arterial pressure (MAP): 21 ± 2 mm Hg, Hb 5.1 ± 0.8 g/dL]. Immediately thereafter, 12 mL/kg of HbVs, 5% human serum albumin (HSA), autologous whole blood (WB), stored red blood cells (RBCs) or 36 mL/kg of Lactated Ringer's (LR) were intraosseously transfused, followed by an additional intraosseous transfusion with 8 mL/kg of HSA (following HbV, HSA or stored RBC transfusion), or WB or 24 mL/kg of LR (following LR transfusion), respectively.

RESULTS: Intraosseous transfusion of HbVs increased MAP (48 ± 9 mm Hg) and improved hypohemoglobinemia (7.1 ± 0.6 g/dL) as well as WB or RBC transfusion. In contrast, neither HSA nor LR improved hemodynamics or Hb levels. Seven out of 10 rabbits receiving HbVs survived for 24 hours, while only one out of 10 rabbits receiving LR survived (WB and RBC; 100% survivals, HSA; 30% survival).

CONCLUSIONS: Intraosseous infusion of HbVs might be an effective initial treatment to maintain hemodynamics during acute hemorrhagic shock. This approach could be used in emergency situations in which access to peripheral vessels is difficult.

Massive bleeding frequently leads to the collapse of peripheral vessels, which can make it difficult to achieve venous access prior to hospitalization. Several guidelines have recommended intraosseous infusion instead of intravenous infusion for urgent patients in the theater.[1,2] In addition, some papers have demonstrated the potential of intraosseous transfusion of blood product rather than infusion of colloid or crystalloid.[3,4] Colloidal solutions such as human serum albumin (HSA) retain blood pressure but completely lack oxygen carrying ability and exacerbate hemodilution.

Originally, intraosseous access was recommended for use in the resuscitation of critically ill children with difficult intravenous access.[5] Over time, the clinical use of intraosseous access increased, and its status of use has been elevated by influential organizations such as the American Heart Association Committee on Advanced Cardiac Life Support.[1] Intraosseous access is now recommended as a rescue technique to treat all critically ill patients regardless of age, when peripheral intravenous access is difficult or impossible.[6] A recent paper showed that advanced emergency medical technicians (EMTs) and paramedics achieved the same high rates of successful intraosseous access (95.2% and 95.6%, respectively; p = 0.84).[7]

From the [1]Department of Physiology and [2]Department of Immunology and Microbiology, National Defense Medical College; the [3]Division of Traumatology, National Defense Medical College Research Institute, Tokorozawa, Saitama; and the [4]Department of Chemistry, Nara Medical University, Kashihara, Nara, Japan.

Address reprint requests to: Manabu Kinoshita, MD, PhD, Department of Immunology and Microbiology, National Defense Medical College, 3-2 Namiki, Tokorozawa, Saitama 359-8513, Japan; e-mail: manabu@ndmc.ac.jp

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We have reported the efficacy of resuscitative transfusion with hemoglobin vesicles (HbVs) based on a rabbit model of massive hemorrhage under prehospital conditions. HbVs, in place of red blood cells (RBCs), can carry oxygen and can assist hemostasis with platelet transfusion even in thrombocytopenic coagulopathy.[8] The HbVs should be suitable for intraosseous access for the following reasons. First, HbVs do not require blood typing or cross-matching, a significant advantage in prehospital resuscitation. Second, the diameters of HbVs are quite small (φ = 250 nm), promoting smooth injection via the intraosseous space connected to the systemic circulation by a series of longitudinal Haversian canals. We previously examined the effect of intraosseous infusion of liposome-encapsulated hemoglobin that was a cellular type of hemoglobin-based oxygen carrier using a murine model.[9] However, the mice were too small to evaluate hemodynamic changes and hematological parameters in detail. Therefore, in the current study we used rabbits to more preciously examine these parameters including coagulation factors. HbVs is also composed of liposomes and limits the flow of cell-free plasma Hb, thereby inhibiting endothelial NO signaling and bioavailability causing cardiovascular dysfunction.[10,11] The aim of this study was to compare intraosseous HbV transfusion with the use of colloid (HSA), crystalloid fluid therapy, whole blood (WB) or RBC transfusion in rabbits in the setting of lethal hemorrhagic shock, where the mean arterial pressure (MAP) was approximately 20 mm Hg under conditions of vessel collapse.

MATERIALS AND METHODS

Rabbits

This study was conducted according to the guidelines of the Institutional Review Board for the Care of Animal Subjects of the National Defense Medical College and the ARRIVE guidelines. The Institutional Review Board approved this study (ethics approval number: #16026). New Zealand White rabbits (2.5 ± 0.2 kg, male; Japan SLC, Hamamatsu, Japan) were used.

Preparation of HbVs

HbVs were prepared as described previously.[8,10] Briefly, hemoglobin (Hb) was purified from outdated donated blood provided by the Japanese Red Cross Society (Tokyo, Japan). The encapsulated Hb (38 g/dL) contained 6.0 mM pyridoxal 5′-phosphate (Sigma Chemical Co) as an allosteric effector. The lipid bilayer was a mixture of 1,2-dipalmitoyl-sn-glycero-3-phosphatidyl choline, cholesterol, and 1,5-bis-O-hexadecyl-N-succinyl-L-glutamate at molar ratios of 5:4:0.9 (Nippon Fine Chemical Co Ltd) and 1,2-distearoyl-snglycero-3- phosphatidylethanolamine-N-PEG (0.3 mol %; NOF Corp). The HbVs were suspended in a physiologic salt solution as follows [Hb]: 10 g/dL; [lipids]: 8-10 g/dL and were deoxygenated in vials for storage at 4°C.

Acute hemorrhagic shock model

Twenty-eight rabbits were anesthetized using intramuscular injections of ketamine (25 mg/kg) and xylazine (10 mg/kg). Local anesthesia was achieved by subcutaneous injection of 1% lidocaine into the left inguinal area and below the left distal femur. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anesthetized rabbits were placed on a warming plate to maintain body temperature at 37°C. Aseptic techniques were adopted for all surgical procedures. Surgical catheters (polyethylene indwelling needle 20G; Terumo Co.) were inserted into the femoral artery and vein in each rabbit (at 0 min). Thereafter, 40 mL/kg of blood was drawn from the femoral artery (70% of total blood volume), and half compensative infusion of normal saline (20 mL/kg, 35% of total blood volume) was performed via the femoral vein to create animals that were hypotensive (MAP at 20 mm Hg) under conditions of hypohemoglobinemia (Hb below 6 g/dL). Such severe hypotension at approximately 20 mm Hg is achieved by reducing 35% of total blood volume and is essential for vessel collapse,[12] and a Hb level below 6 g/dL is critical for blood transfusion.[13] Rapid 35% saline infusion immediately after 70% hemorrhage could achieve these two conditions. We spent approximately 10 minutes for this blood withdrawal and saline infusion.

Preparation of WB and stored RBCs

Blood samples withdrawn as mentioned above were mixed with a 10% volume of 3.8% (w/v) sodium citrate for autologous WB transfusion. For preparing the stored RBCs, blood samples were centrifuged at 100 × *g* for 15 minutes, and the supernatant was used as platelet-rich plasma. The remaining sample was further centrifuged at 500 × *g* for 10 minutes and the supernatant was used as platelet-poor plasma. After removing the platelet-rich plasma and platelet-poor plasma, the remaining RBCs were washed with acid citrate dextrose solution, finally adding the same volume of mannitol adenine phosphate solution (D-mannitol 1.457, Adenine 0.014, Sodium Dihydrogen phosphate 0.094, w/v%, Terumo Co). The allogeneic RBC concentrates were stored at 4°C in a refrigerator and transfused within a week from the preparation. Table 1 shows their hematologic parameters.

Intraosseous transfusion of resuscitation fluid including HbVs

An intraosseous needle was placed in the left distal femur bone (EZ-IO PD 15 mm Intraosseous Infusion System, Teleflex Medical) within 2 minutes of the blood withdrawal. After successful drilling was confirmed by aspirating bone marrow, 12 mL/kg of HbVs, 5% HSA (albumin 5%, JB, Japan Blood Products Organization), stored RBCs, autologous WB,

TABLE 1. Hematological parameters of transfused bloods

	Hb concentrations (g/dL)	Ht (%)	PLT counts ($\times 10^3/\mu L$)
Whole blood (WB)	11 ± 1	35 ± 2	193 ± 42
Stored RBCs	12 ± 3	38 ± 10	52 ± 24

Data are means ± SD.

or 36 mL/kg (triplication of blood loss) of Lactated Ringer's (Lactec Injection, Otsuka) was intraosseously transfused into the rabbits approximately for 5 minutes (initial transfusion). Subsequently, an additional 8 mL/kg of 5% HSA was intraosseously transfused for 5 minutes (second transfusion) following HbV transfusion (HbV + 5% HSA group) (Fig. 1). An additional 8 mL/kg of 5% HSA or autologous WB was also intraosseously transfused following the initial 5% HSA or autologous WB (5% HSA group or WB group, respectively), and 24 mL/kg (triplication of blood loss) of Lactated Ringer's was similarly transfused following the initial Lactated Ringer's transfusion (Lactated Ringer's group) (Fig. 1). Thereafter, the rabbits were monitored for survival for 24 hours under ad libitum feeding with laboratory diet and water. Initial intraosseous transfusion volumes of 12 mL/kg of HbVs (Hb concentrate, 10 g/dL) included 1.2 g/kg of Hb, which is estimated at 90 g of Hb for a 75 kg person. Transfusion of 12 mL/kg stored RBCs (Hb concentrate,12 g/dL on average, Table 1) included 1.4 g/kg Hb, which is estimated at 105 g Hb for a human, and 12 mL/kg WB (11 g/dL on average, Table 1) included 1.3 g/kg Hb, the equivalent of 98 g Hb for a person. Shackleford et al. described prehospital blood transfusion during medical evacuation of combat casualties in Afghanistan.[14] In that report, 1-2 units of RBCs were transfused during evacuation. Because 1 unit of packed RBCs contains 50-80 g of Hb, transfusion of Hb in 12 mL/kg of HbVs, stored RBCs or WB corresponds to 1-2 units of packed RBCs for a human patient. We think that such transfusion volumes of Hb are appropriate for initial prehospital resuscitation.

Measurement of blood cell counts and blood gas analysis

Arterial blood samples were withdrawn for the measurement of blood cell counts, and for blood gas analyses at the following four points: before and after the blood withdrawal, after the initial intraosseous transfusion, and second transfusion (Fig. 1). Blood cell counts were measured using an Erma PCE 170 hematology analyzer (Erma). However, the Hb concentration in blood containing HbVs could not be accurately determined because the liposome capsules interfered with the spectrophotometric measurement of Hb absorbance. The "actual Hb concentrations" (Fig. S1, available as supporting information in the online version of this paper) were estimated by a method reported previously.[8] Analysis of blood gas, including plasma lactate levels, was performed by an ABL 80 blood gas analyzer (Radiometer).

Analyses of WB coagulation activity

The coagulation activity of WB was examined using a Sonoclot® Coagulation and Platelet Function Analyzer (Sienco) as previously described.[8] The Sonoclot signal typically describes coagulation parameters including clotting time (CT), which indicates the period up to the beginning of fibrin formation, and clot rate (CR), which indicates the slope of fibrin gel formation that is affected by both the rate of the fibrinogen to fibrin conversion and the amount of fibrinogen.

Analyses of plasma nitric oxide [NO_2^-/NO_3^- (NOx)]

Plasma NO_2^-/NO_3^- (NOx) levels were measured at the following four points: before and after blood withdrawal, after the first intraosseous transfusion and the second transfusion. Blood samples were collected in heparin and centrifuged at 50,000 × *g* at 4°C for 20 minutes to remove the HbV particles. The supernatants were stored at −80°C until measurement. Plasma NO_2^-/NO_3^- (NOx) levels were measured at Eicom Laboratory (Eicom corporation), using a high-performance liquid chromatography-Griess system, as previously described.[8]

Histopathologic examinations

For histopathologic examinations, the rabbits treated with HbV, WB, HSA, or LR euthanized 1 hour after the hemorrhage (n = 2 in each group). Their lung and kidney were removed from the subject rabbits. Excised organs were fixed by 20% formalin for 2 days and processed to paraffin embedding blocks to stain with hypoxia-induced factor-1a.

Immunohistochemistry of Hypoxia-Inducible factor-1alpha (HIF-1alpha) protein

After deparaffinization, specimen slides were placed in 0.6% methanol in phosphate buffered saline (PBS) for 40 minutes. The slides were incubated with purified mouse anti-HIF-1alpha antibody (NB100-105, Novus Biologicals) diluted 1:50 in 1.5% normal goat serum in PBS for 16 hours at room temperature. The slides were washed and then incubated with goat anti-mouse IgG (ab205719, Abcam) diluted 1:2000 in PBS for 1 hour at room temperature. Reactions were visualized with 3, 3-diaminobenzidine (DAB), and counterstained with hematoxylin.

STATISTICAL ANALYSES

Statistical analyses were performed using the Stat View 4.02 J software package (Abacus Concepts). Survival rates were compared by the Wilcoxon signed rank test. Continuous variables were compared using the unpaired student's t-test or the one-

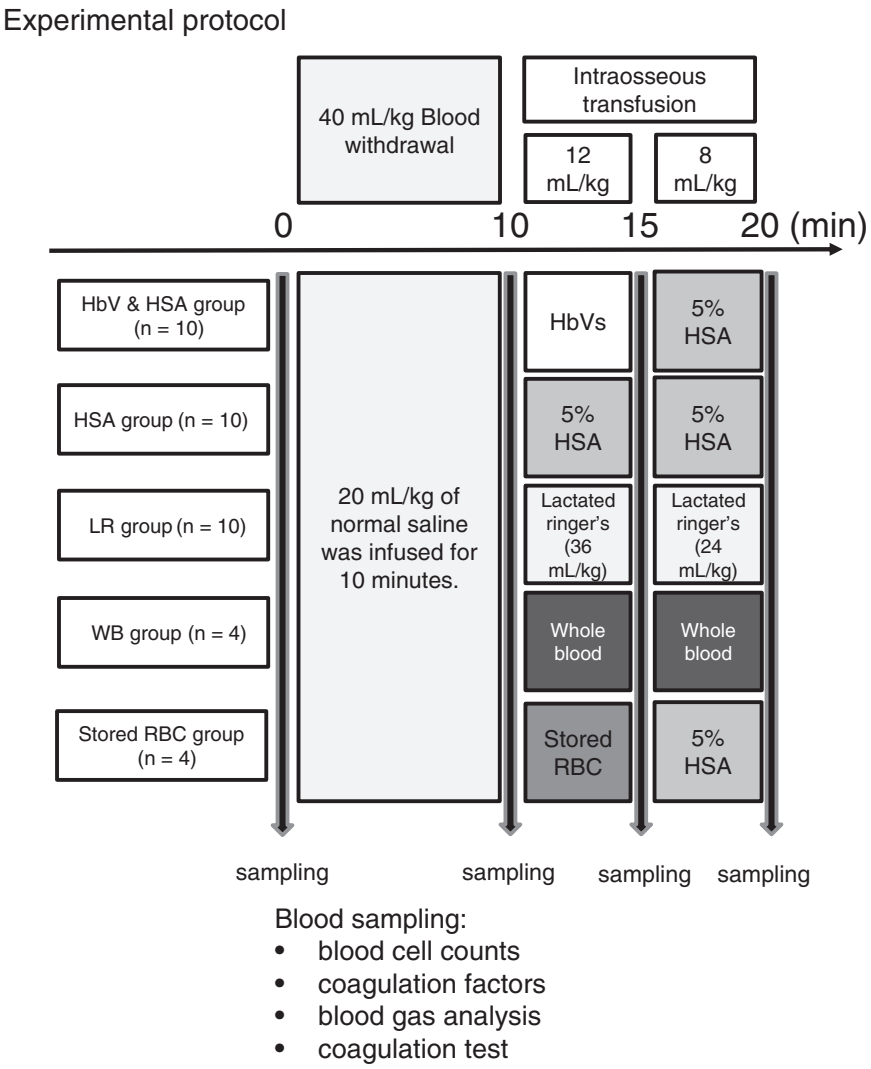


Fig. 1. Experimental design of intraosseous resuscitation following severe hemorrhagic shock. Within 2 minutes of blood withdrawal, 12 mL/kg of HbVs (n = 10), 5% HSA (n = 10), stored RBCs (n = 4), autologous WB (n = 4), or 36 mL/kg of lactated Ringer's (n = 10) was intraosseously transfused into the rabbits (initial transfusion). Subsequently, an additional 8 mL/kg of 5% HSA was intraosseously transfused (second transfusion after 20 min) following HbV transfusion (HbV + 5% HSA group) (Fig. 1). An additional 8 mL/kg of 5% HSA or autologous WB was also intraosseously transfused following the initial 5% HSA transfusion or autologous whole blood (WB) (5% HSA group or WB group), and 24 mL/kg of Lactated Ringer's was similarly transfused following the initial Lactated Ringer's transfusion (Lactated Ringer's group).

way analysis of variance followed by the Bonferroni post-hoc test. Data are presented as the means ± SD, with $p < 0.05$ considered to be statistically significant.

RESULTS

Acute hemorrhagic shock by blood withdrawal

After 40 mL/kg of blood was withdrawn from the femoral artery, normal saline was then infused, equivalent to a half volume of blood loss (20 mL/kg). This procedure resulted in severe hypotension and hypohemoglobinemia (MAP: 20 ± 2 mmHg, Hb: 5.5 ± 0.8 g/dL). Plasma lactate levels were increased, and moderate coagulopathy occurred, due to the decreases in platelet counts and CR, and showed the prolongation CT were observed (Fig. 3C, 5, Table 2). Nonetheless, systemic fluid balance was unchanged by hyperventilation (Table 2).

Mitigation of fatal anemia by intraosseous transfusion with HbVs

Intraosseous transfusion of HbVs immediately improved MAP and Hb concentration as well as those of WB or stored RBCs, whereas 5% HSA and Lactated Ringer's infusions increased MAP but did not improve Hb concentration (Fig. 2A, B). Intraosseous transfusion of HbVs did not affect

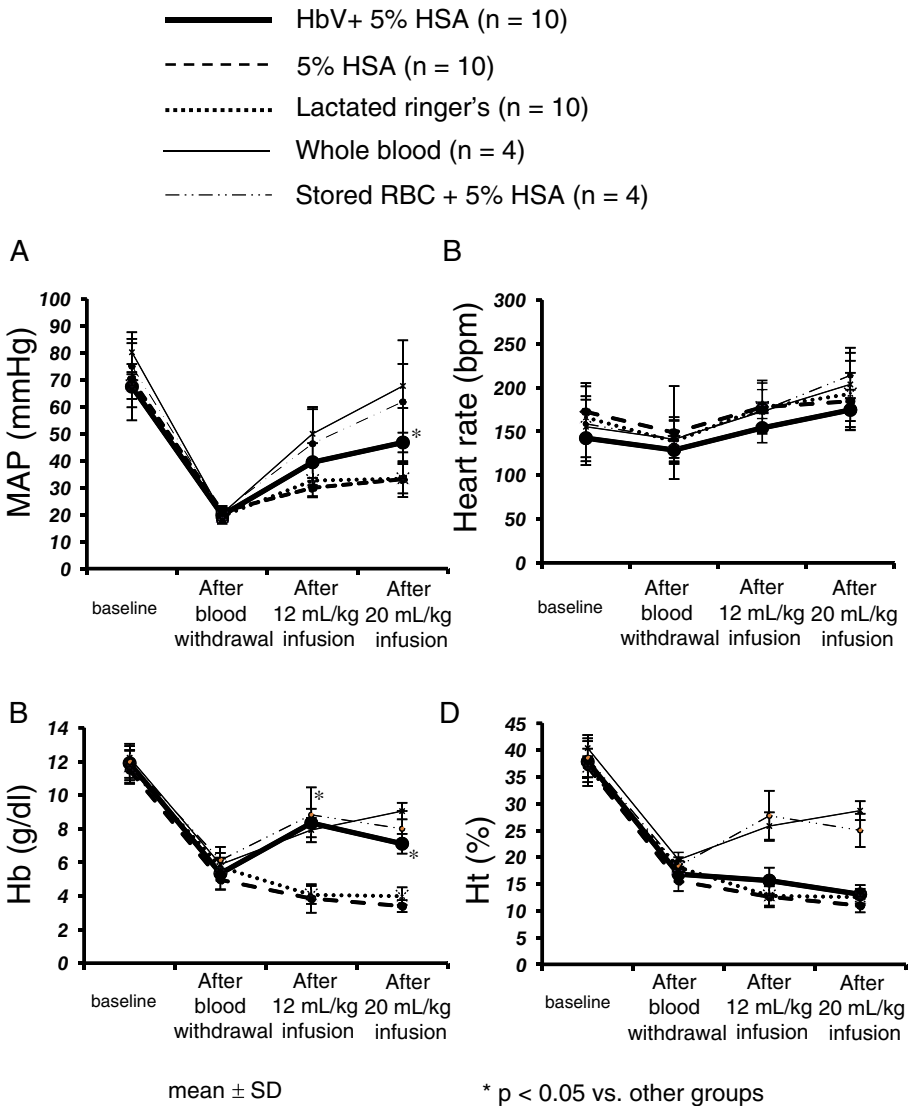


Fig. 2. Changes in mean arterial pressure and blood cell counts in rabbits. HbV administration transfusion kept MAP above 40 mm Hg (A) and Hb concentration above 6 g/dL (C), whereas heart rate change was not significant (B). Ht decreased to 15% in the same manner as other groups (D). $p < 0.05$ by ANOVA.

the hematocrit (Ht) because HbVs were quite small and did not contribute to the measured Ht (Fig. 2C).

Additional intraosseous transfusion of 5% HSA following the initial HbV or stored RBC transfusion further increased the MAP (Fig. 2A), though their Hb concentrations were slightly reduced (Fig. 2B). As expectedly, WB transfusion augmented MAP and Hb concentration after its additional transfusion. Neither additional intraosseous transfusion of 5% HSA following the initial 5% HSA transfusion (5% HSA group) nor additional transfusion of Lactated Ringer's following the initial Lactated Ringer's transfusion (Lactated Ringer's group), which was threefold-transfusion volume, improved these parameters (Fig. 2A, B).

At the end of intraosseous transfusions (initial and second), the HbV + 5% HSA group as well as WB and stored RBC groups showed moderate lactic acidosis (Fig. 3A, C). However, both 5% HSA and Lactate Ringer's groups tended to show further severe lactic acidosis (Fig. 3A, C). Serum HCO_3^- levels were similar among all the group, whereas CtO_2 levels were theoretically proportional to Hb concentration, which were divided into the following three groups at the end of the experiment: WB and RBC transfusion groups, HbV + 5% HSA group, and 5% HSA and Lactated Ringer's groups (Fig. 3D).

As results of those different hemodynamical responses, intraosseous transfusion of HbVs followed by 5% HSA, as well as stored RBC or WB transfusion, significantly increased rabbit survivals in comparison to intraosseous transfusion of 5% HSA alone or Lactated Ringer's (Fig. 4). It should be noted that WB transfusion took a significantly

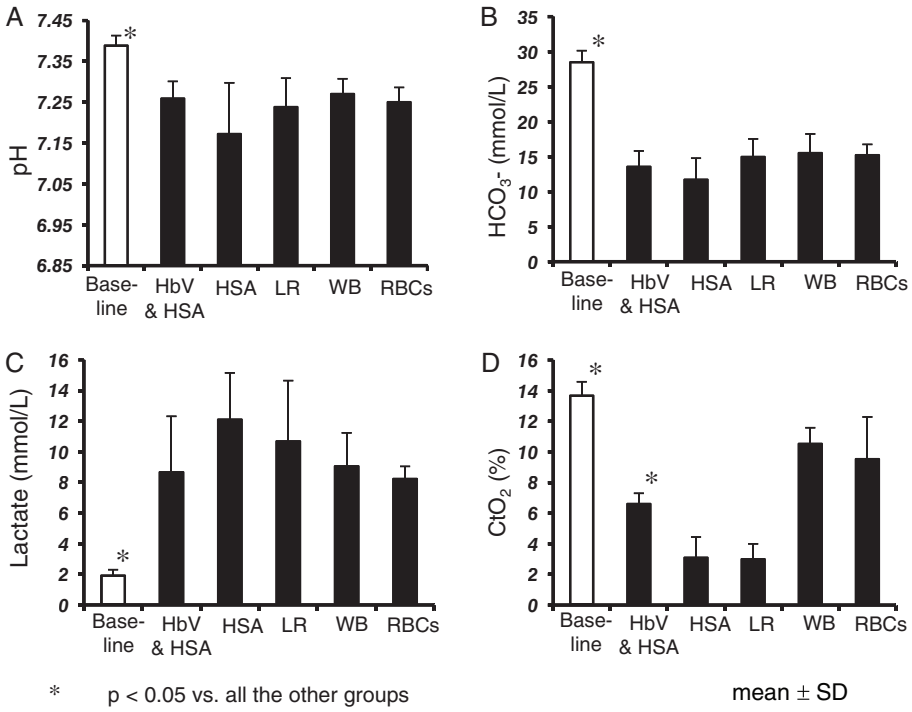


Fig. 3. Parameters of metabolic acidosis in rabbits 5 minutes after intraosseous resuscitation. HbV + 5% HSA treatment mitigated acidosis (A, B, E) and elevation of plasma lactate levels at the end of the experiment (C). The other two groups failed in lethal metabolic acidosis. In particular, 5% HSA significantly attenuated the ratio of arterial oxygen content as low as one-sixth of normal range (A, D) and shifted the severe acidemia (pH declined less than 7.2). $p < 0.05$ by ANOVA.

longer infusion time than did HbV + 5% HSA (24 ± 3 vs. 18 ± 3 min, $p < 0.05$), because WB transfusion needed high infusion pressure (>300 mmHg) to administer into the bone marrow space.

Coagulopathy due to hemodilution

After the second transfusion, 5% HSA group markedly prolonged CT but reduced CR (Fig. 5A, B), suggesting a severe coagulopathy. Lactated Ringer's group also showed a reduced CR (Fig. 5B). In contrast, HbVs +5% HSA group showed almost similar CT and CR to those of WB and stored RBC groups (Fig. 5A, B), suggesting a prevention of exacerbating coagulopathy. Intraosseous transfusion of 5% HSA most severely affected hemodilution among all the groups, as Ht levels fell to levels as low as 25%. Unlike the 5% HSA group, the HbVs +5% HSA group prevented the exacerbation of coagulopathy, maintaining the CT and CR values as well as WB and RBC group (Fig. 5).

Analysis of plasma nitric oxide [NO_2^-/NO_3^- (NOx)]

All rabbits, including HbV-transfused rabbits, showed significantly decreased plasma NO_2^-/NO_3^- (NOx) levels due to hemodilution after the hemorrhage followed by partial saline replacement. However, no differences in the plasma NO_2^-/NO_3^- (NOx) levels were observed among the three

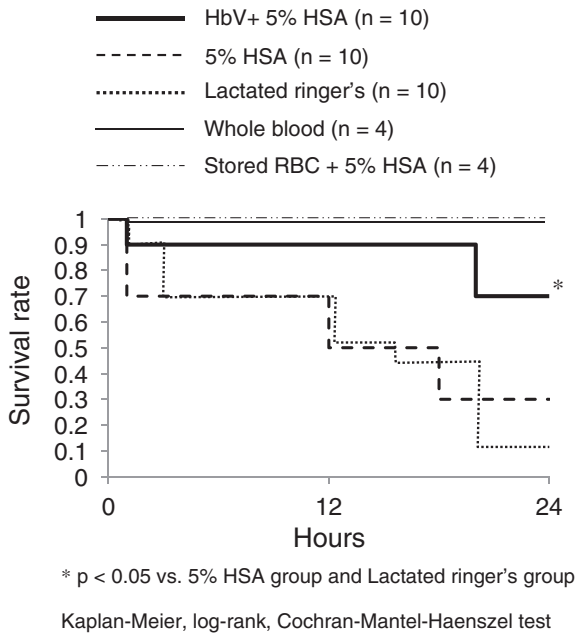
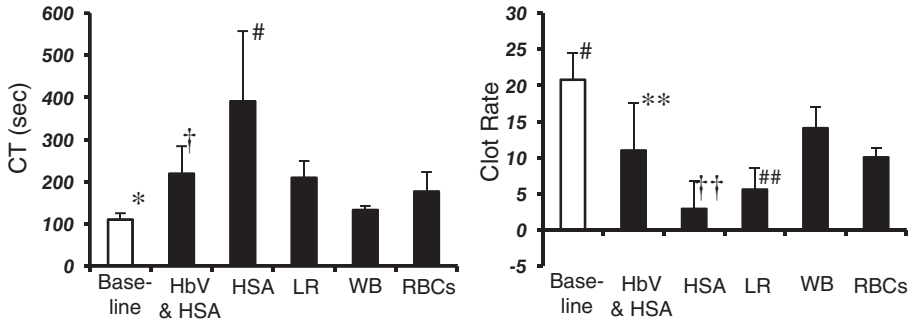


Fig. 4. Survival frequencies of rabbits. Seven out of 10 rabbits receiving HbVs +5% HSA survived for 24 hours, whereas 7 out of 10 rabbits receiving 5% HSA died within 20 hours and 9 out of 10 rabbits receiving Lactated Ringer's died. $p < 0.05$ by Kaplan–Meier, log-rank, Cochran–Mantel–Haenszel test.



* p < 0.05 vs. HbV group, 5% HSA group and Lactated ringer's group,
† p < 0.05 vs. 5% HSA group,
# p < 0.05 vs. all the other groups,
** p < 0.05 vs. 5% HSA group and Lactated ringer's group,
†† p < 0.05 vs. HbV group, Whole blood and RBC group,
p < 0.05 vs. HbV group and Whole blood group

mean ± SD

Fig. 5. Analyses of whole blood (WB) coagulation activity. HbVs +5% HSA treatment prevented exacerbation of coagulopathy, maintaining CT (A) and Clot rate (B) values. 5% HSA and Lactated Ringer's treatments worsened coagulopathy as designated by prolongation of CT and decrease of clot rate. p < 0.05 by ANOVA.

groups after intervention (Table S1, available as supporting information in the online version of this paper), suggesting that HbVs did not have a potent NO scavenging effect.

HbV transfusion attenuates HIF-1alpha expression in the kidney

Rabbits infused with 5% HSA showed strong focal HIF-1alpha staining in the proximal convoluted tubules of the kidneys 1 hour after the transfusion period (Fig. S2B, available as supporting information in the online version of this paper, indicated by arrows), whereas the HbV- or LR-infused rabbits showed only weak and diffuse HIF-1alpha staining (Fig. S2A, C, available as supporting information in the online version of this paper). There was no staining noted in the kidney of the WB-transfused rabbits (Fig. S2D, available as supporting information in the online version of this paper). The lungs and livers showed no staining for HIF-1alpha in any of the transfused rabbits' groups (Fig. S3A-D, S4A-D, available as supporting information in the online version of this paper).

DISCUSSION

In the prehospital setting, intraosseous transfusion of HbVs may be a promising approach for the provision of resuscitative fluid in the treatment of severe hemorrhagic shock accompanied by collapse of peripheral vessels. Our data suggest that HbVs may preserve the oxygen supply for critical organs and prevent coagulopathy. In recent papers using hemoglobin-based solutions, the lowest MAP during

TABLE 2. Changes in the hematologic variables and coagulation factors in rabbits before and after blood withdrawal

Variables	Before blood withdrawal (n = 38)	After blood withdrawal (n = 38)
MAP (mm Hg)	71 ± 11	20 ± 2*
Hb concentrations (g/dL)	11.8 ± 0.9	5.5 ± 0.8*
Ht (%)	38 ± 3	18 ± 3*
PLT counts ($\times 10^3/\mu L$)	229 ± 59	78 ± 38*
CT (sec)	113 ± 27	182 ± 50*
Clot rate	20.9 ± 7.3	9.7 ± 5.9*
pH	7.39 ± 0.05	7.39 ± 0.06
PaO_2 (mm Hg)	63 ± 11	158 ± 24*
HCO_3- (mmol/L)	29 ± 3	17 ± 4*
BE (mmol/L)	3.7 ± 3.6	−6.8 ± 3.8*
Lactate (mmol/L)	2.1 ± 1.3	5.5 ± 2.3*

Data are mean ± SD.
* p < 0.05 versus before blood exchange.

hemorrhagic shock was set at 30-40 mmHg in their animal studies.[15-18] In the current study, we demonstrated the treatment with hemoglobin-based oxygen carrier in a critical situation in which animals suffered severe hypotension (MAP, 20 mmHg) and anemia (Hb concentration, 6 g/dL) with the elevation of plasma lactate levels (5 mmol/L).

Severe hemorrhage often induces vasoconstriction and ultimately, the collapse of peripheral vessels, which makes it difficult to deliver a transfusion. Using a swine model, Gomez et al. reported that cardiovascular collapse was defined as a MAP <30 mmHg for 10 minutes or <20 mmHg for 10 seconds after the bleeding of 35% total blood volume. After cardiovascular collapse, lactate levels were elevated approximately 6 mmol/L.[19] In line with this report, our current study using a rabbit model showed severely decreased MAP approaching lethality when intraosseous access was delayed (data not shown). We found that rapid 35% saline infusion immediately after 70% hemorrhage achieved both hypotensive (MAP = 20 mmHg) and hypohemoglobinemia (Hb below 6 g/dL), conditions in which blood transfusion is strongly recommended.[12,13] Animals also showed marked decreases in Ht levels (15%), contributing to hemodynamic dysfunction.[19-21] The plasma lactate levels were elevated to 5.4 ± 2.3 mmol/L.

For the treatment of severe hemorrhagic shock, Lactated Ringer's was toxic in the resuscitation of rats showing MAP at 25-30 mmHg, whereas it was beneficial in the treatment of moderate hemorrhagic shock with respect to initial survival, acid–base parameters, and organ damage.[22] In line with this report, Lactated Ringer's infusion exacerbated lactemia in our current model.

In the current study, 5% HSA rescued the animals to some extent because it maintained the MAP. Das et al. reported that HSA was effective for the treatment of moderate hemorrhagic shock without any septic complication.[23] Nevertheless, 5% HSA infusion also retained the vascular space with fluid, resulting in serious hemodilution that exacerbated anemia, lactic acidosis, and coagulopathy in our current model.

Prehospital blood transfusion can improve the outcomes of hemorrhagic patients.[12] As an alternative to seeking peripheral venous access in the bleeding patient, Bjerkvig et al. suggest that fresh WB transfusion through the intraosseous route is safe, reliable, and provides sufficient flow for resuscitation.[24] In our current study, intraosseous WB transfusion failed in half of the cases (four out of eight). Huge hematomas occurred in the post-tibial space because of intolerance to high infusion pressure. Therefore, WB transfusion requires a gentle and long infusion time, resulting in failure to prevent elevation of serum lactate levels.

The use of HbVs facilitates this strategy, overcoming the lethal hypoxia of hemorrhagic shock. Moreover, HbV helped to improve acidosis because protons are captured by deoxy-Hb transforming from oxy-Hb. HbVs are nanosized particles that enable rapid infusion even via an intraosseous route, a crucial property for emergency care. In our current study, the speed of infusion was 1.2 mL/kg/min, which was approximately twice as fast as that of fresh WB transfusion reported by Bjerkvig et al (0.5 mL/kg/min).[24] HbVs may be suitable for use in emergency vehicles because they have long shelf lives (approximately 1 year), no need of blood typing or cross-match test, and no risk of blood contamination.[11] Moreover, they may not exacerbate coagulopathy; prompt HbV treatment via intraosseous access may preserve the oxygen supply for critical organs. In this critical situation, HbVs might directly contribute as a pressor regulating vascular tone as erythrocytes.[25] From the viewpoint of rheology, the presence of particles is essential for maintaining blood pressure and the coagulation system.[26] We confirmed that the content of free Hb in the HbV product is less than 2%. The stability of HbV in blood circulation is extremely high and it does not induce lysis. According to the results of the circulation persistence of radioisotope labeled HbV,[27] the outer lipid membrane and the inner Hb circulate together in blood. Once they are captured by macrophages, they are degraded and excreted through urine and feces.

In our previous study, HbVs were suspended in HSA for treatment of hemorrhagic shock because the HbVs particles, as well as erythrocytes, do not contribute to colloid osmotic pressure.[28] However, we modified that procedure to separate the effects of HbVs and 5% HSA. We postulated that responding to oxygen debt was of primary importance for this model of injury. Thus, 5% HSA was delivered to stabilize the blood pressure. Moreover, a simple procedure is preferable in a prehospital environment to avoid mixing two agents at the necessary ratio.

LIMITATIONS

Following traumatic injury, bleeding can be slowed by emergency intervention. However, patients face the risk of renewed bleeding following resuscitation and there are risks of inflammation, coagulation, and pathologic endothelial effects. In our current study, we focused on the hemodynamics, oxygen supply and blood coagulation as affected by intraosseous infusion of HbVs. Hence, we did not characterize organ injury, which influences systemic inflammatory responses and the coagulation system following traumatic injury. Infusion of normal saline proceeding the intraosseous treatment might be a confused scenario, although this procedure offered the reproducible severe hypotension and hypohemoglobinemia. However, we felt it more important to focus on severe hypotension and anemia.

CONCLUSIONS

Intraosseous infusion of HbVs might be effective initial treatment to maintain hemodynamics during acute hemorrhagic

shock. This approach could be used in emergency situations in which access to peripheral vessels is difficult.

CONFLICTS OF INTEREST

The authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Fig. S1. Relationship between measured Hb concentrations and actual Hb concentrations. We made various concentrations of pure HbV solution (1, 2, 3, and 4 g/dL of actual Hb) and measured their Hb concentrations using an Erma PEC 170 hematology analyzer. "Measured Hb concentrations" were exactly 4.36-fold higher than the "actual Hb concentrations" ($y = 4.36x$, $R^2 = 1$).

Fig. S2. Histopathological findings in the kidney. HE staining shows no abnormal structural changes.

Fig. S3. Histopathological findings in the kidney. The 5% HSA-infused rabbits showed strong focal HIF-1alpha staining in the proximal convoluted tubules of the kidneys 1 hour after the transfusion period (B, indicated by arrows), whereas the HbV- or LR-infused rabbits showed a weak and diffuse HIF-1alpha staining (A, C). There was no staining noted for the kidney of the WB-transfused rabbits (D).

Fig. S4. Histopathological findings in the lungs. The lungs showed negative staining for HIF-1alpha in all of transfused rabbits' groups.

Fig. S5. Histopathological findings in the livers. The livers showed negative staining for HIF-1alpha in all of the transfused rabbits' groups.

Table S1. Changes in plasma NO_2^-/NO_3^- (NOx) levels in rabbits.