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ORIGINAL ARTICLE

H12-(ADP)-liposomes for hemorrhagic shock in thrombocytopenia: Mesenteric artery injury model in rabbits

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Abstract

Background: Damage control resuscitation improves patient outcomes after severe hemorrhage and coagulopathy. However, effective hemostasis methods for these critical situations are lacking.

Objective: We evaluated the hemostatic efficacy of fibrinogen γ-chain (HHLGGAKQAGDV, H12)-coated, adenosine-diphosphate (ADP)-encapsulated liposomes (H12-[ADP]-liposomes) in thrombocytopenic rabbits with hemorrhagic shock.

Methods: Acute thrombocytopenia (80%) was induced in rabbits that also received mesenteric vessel injury, leading to hemorrhagic shock. Five minutes after injury, subjects received intravenous bolus injection with H12-(ADP)-liposomes (20 mg/kg), followed by isovolemic transfusion with stored red blood cells (RBCs)/platelet poor plasma (PPP) (RBC:PPP = 1:1 [vol/vol]), or lactated Ringer solution every 5 min to compensate blood loss. One group received H12-(phosphate buffered saline [PBS]) liposomes followed by RBC/PPP. Additional groups were received isovolemic transfusion with RBC/platelet rich plasma (PRP) (RBC:PRP = 1:1 [vol/vol]), RBC/PPP, PPP alone, or lactated Ringer solution.

Results: Treatment with H12-(ADP)-liposomes followed by RBC/PPP transfusion and RBC/PRP transfusion effectively stopped bleeding in all thrombocytopenic rabbits. In contrast, three of 10 rabbits treated with RBC/PPP failed hemostasis, and no rabbits receiving lactated Ringer solution stopped bleeding or survived. Twenty-four hours after hemorrhage, 80% of rabbits receiving H12-(ADP)-liposome followed by RBC/PPP transfusion survived and 70% of rabbits receiving RBC/PRP transfusion also survived, although RBC/PPP-transfused rabbits showed 40% survival. Rabbits receiving H12-(ADP)-liposomes followed by lactated Ringer solution showed a transient hemostatic potential but failed to survive. H12-(PBS)-liposomes showed no beneficial effect on hemostasis. Neither the PPP group nor the lactated Ringer group survived.

Conclusion: H12-(ADP)-liposome treatment followed by RBC/PPP may be effective in lethal hemorrhage after mesenteric vessel injury in coagulopathic rabbits.

KEYWORDS
hemorrhagic shock, mesenteric artery, platelet transfusion, resuscitation, thrombocytopenia

Essentials

- An initial hemostatic agent is necessary for acute care surgery.
- A new agent was developed for helping platelet aggregation.
- This agent stopped bleeding after severe vessel injury in animals with altered coagulation.
- This new treatment may be helpful for acute abdominal bleeding.

1 | INTRODUCTION

Abdominal vascular injuries are one of the most lethal injuries in trauma patients, which mortality ranges from 20% to 60%.[1,2] Patients with abdominal vascular injuries often exhibit the lethal vicious cycle of shock, consisting of acidosis, hypothermia, coagulopathy, and cardiac arrhythmias.[1] In such critical conditions, hemorrhage control and restoration of mesenteric tissue perfusion are crucial for life saving.[3] Mesenteric injury is found in approximately 5% of blunt trauma victims during laparotomy.[4] Prompt resuscitation is clinically important because it may cause significant blood loss or lead to bowel ischemia and necrosis, with eventually delayed rupture or ischemic strictures.[5,6] Mesenteric arterial injury frequently complicates coagulopathy, increasing mortality to approximately 40%.[7,8] Control of coagulopathy has been attempted by induction of damage control resuscitation (DCR) and massive transfusion protocol (MTP). Sorrentino et al. reported that refractory coagulopathy resulting from major abdominal vascular trauma decreased from 46% to 19% (during 1975–1980 vs 2004–2009) by adapting damage control surgery.[9] Moreover, institutional MTP may improve outcomes of patients with abdominal aortic injuries. Maciel et al. reported that introduction of MTP markedly increased patient overall survival from 14% to 47%.[10]

Moore et al. proposed staged laparotomy for treating coagulopathy.[11] Although emergency laparotomy followed by gauze packing may be effective for intraabdominal bleeding, it cannot be usually performed in prehospital resuscitation. Hemostasis by tourniquet is quite effective for extremity bleeding in prehospital resuscitation but there are no effective tools for trunk hemorrhage. Resuscitative Endovascular Balloon Occlusion of the Aorta and junctional tourniquet may be potential tools for trunk bleeding but require skillful techniques. Although hemostasis by platelet transfusion may be effective in prehospital settings, it is logistically difficult because of the short half-life of platelet concentrates. The recent pandemics of the COVID-19 further limits the available blood supply for transfusions worldwide.[12]

We have developed H12-(adenosine-diphosphate [ADP]) liposomes, which are effective for hemostasis against liver injury/hemorrhage with acute thrombocytopenic coagulopathy and acute pulmonary hemorrhage caused by blunt injury.[13,14] H12-(ADP)-liposomes accumulate at bleeding sites through interaction with activated platelets via glycoprotein IIb/IIIa and augment platelet aggregation by releasing ADP at the bleeding site. H12-(ADP)-liposomes remained intact in the blood circulation for up to 24 h after injection and accumulated at injured (bleeding) sites.[15] Thereafter, H12-(ADP)-liposomes released ADP and reinforced platelet aggregation with activated platelets within a few minutes.[16] The amount of ADP released from the liposome increased with decreasing lamellarity and with increasing membrane flexibility, owing to the conditions around the liposomes such as their compressed deformation by thrombus formation.[17] Released ADP was fully metabolized to allantoin and discharged to urine within 6 h.[15] H12-(ADP)-liposomes can be stored for at least 6 months at 4°C without shaking.[18] H12-(ADP)-liposomes may help hemostasis as an alternative to platelets in prehospital or urgent situations.[14,19]

Despite several trials,[20-22] an appropriate animal model of abdominal vascular injury complicated with severe acidosis and coagulopathy has not been provided. Therefore, we established a model of hemorrhagic shock with coagulopathy by vessel injury, based on our previous study.[23] Using this model, we evaluated the efficacy of H12-(ADP)-liposomes during initial DCR instead of platelet transfusion in thrombocytopenic rabbits with hemorrhagic shock caused by mesenteric vascular injury. In the current thrombocytopenic rabbits, platelet counts decreased to 50 ± 9 from 241 ± 35 ($\times 10^3/\mu l$) of normal rabbits (approximately 80% thrombocytopenia).

2 | MATERIALS AND METHODS

2.1 | 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 gained approval by the institutional review board (#16026). New Zealand White rabbits (2.5 ± 0.2 kg, male; Japan SLC, Hamamatsu, Japan) were used. The

rabbits had free access to standard feed and water during a 7-day adaptation period before the experiment. A crossmatch test was applied between a donor and a transfused animal to eliminate transfusion of incompatible blood types.

2.2 | Preparation of H12-(ADP)-liposomes

H12-(ADP)-liposomes were prepared as described previously.[8-11] Briefly, 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (1 g, 1.36 mmol), cholesterol (527 mg, 1.36 mmol), 1,5-dihexadecyl-N-succinyl-L-glutamate (189 mg, 272 μmol), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)] (52 mg, 9 μmol), and H12-polyethylene glycol-Glu2C18 (47 mg, 9 μmol) (Nippon Fine Chemical Co Ltd, Osaka, Japan) were dissolved in t-BuOH and freeze-dried. The resulting mixed lipids were hydrated with phosphate buffered saline (PBS) containing 1 mM ADP using Durapore (pore size, 0.45, 0.20 μm; Millipore, Tokyo) to prepare H12-(ADP)-liposomes. After washing the liposomes with PBS followed by centrifugation (100,000*g*, 30 min, 4°C), the remaining ADP was removed using Sephadex G25 (GE Healthcare Japan, Tokyo). The diameter of H12-ADP-liposomes were 179 ± 53 nm. When the total lipid concentration was 20 mg/ml, the concentrations of ADP inside and outside of liposomes were 0.0732 and 0.0016 mg/ml, respectively. The encapsulation efficiency of ADP was estimated as 8.76% (Table S1). We also prepared H12-(PBS)-liposomes (without ADP), which skipped the process of containing ADP.

2.3 | Acute thrombocytopenic rabbit model

Acute thrombocytopenia was achieved in rabbits essentially as described in our previous studies.[7,9] Rabbits were anesthetized using intramuscular injections of ketamine (25 mg/kg) and xylazine (10 mg/kg). Anesthesia was maintained with intravenous injections of pentobarbital (15 mg/kg) every 30 min during the experiment. The adequacy of anesthesia was monitored by the loss of the ear pinch reflex. Anaesthetized rabbits were placed on a warming plate to maintain the body temperature at 37°C. Aseptic techniques were adopted for all surgical procedures. Surgical catheters (polyethylene indwelling needle 20 G; Terumo Co., Tokyo, Japan) were inserted into the femoral artery and vein in each rabbit. Thereafter, 25 ml/kg of blood (sample 1) was withdrawn from the femoral artery, and the same volume of dextran 40 (Otsuka, Tokushima, Japan) was simultaneously transfused via the femoral vein. Forty minutes later, the next blood sample (25 ml/kg, sample 2) was withdrawn and the same volume of washed red blood cells (RBCs) prepared from sample 1 was transfused. This isovolemic blood exchange was repeated six times. The last transfusion of washed RBCs was performed without simultaneous blood withdrawal (Figure S1A). This blood exchange reduced platelet count to 50 ± 9 from 241 ± 35 ($\times 10^3$/μl) of normal rabbits (approximately 80% thrombocytopenia). Using other thrombocytopenic rabbits, we examined an aggregating function of the residual platelets using the collagen test and ADP test. In addition, we performed the H12-(ADP)-liposome test (instead of ADP test) with and without ADP agonist (Multiplate Analyzer, Roche Diagnostics International AG, Rotkreuz, Switzerland) before and after thrombocytopenia and at 5 min after mesenteric bleeding in this model. At that test, we added 1.05 mg of H12-(ADP)-liposome for 3 ml of whole blood, according to the bolus injection dose of H12-(ADP)-liposomes (20 mg/ml/kg) *in vivo* study.

2.4 | Preparation of washed RBCs, platelet rich plasma, and platelet poor plasma

As described previously,[7,9] blood samples withdrawn with a 10% volume of 3.8% (w/v) sodium citrate were centrifuged at 100 *g* for 15 min, and the supernatant was used as platelet rich plasma (PRP). The remaining sample was further centrifuged at 500 *g* for 10 min and the supernatant was used as platelet poor plasma (PPP). Thereafter, the remaining cells were washed with saline, diluted in 25 ml/kg of normal saline containing 5% human serum albumin, and transfused into the rabbit as washed RBCs. These PRP and PPP samples showed similar coagulation activity (fibrinogen, approximately 150 mg/dl; antithrombin [AT] III activity, 99%; prothrombin time [PT], 12 s; activated partial thromboplastin time [aPTT], 32 s) (Figure S1B).

2.5 | Preparation of allogeneic RBC concentrates from donor rabbits

Donor rabbits were anesthetized as described previously. Thereafter, 50 ml/kg of blood was drawn from the femoral artery, and the same volume of normal saline was simultaneously transfused via the femoral vein, as described previously.[7] Until their euthanasia (performed using intravenous injections of pentobarbital 100 mg/kg), the animals donated approximately 30 ml of RBC concentrate. In brief, after removing PRP and PPP, 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, Tokyo, Japan). The allogeneic RBC concentrates were stored at 4°C in a refrigerator until use.

2.6 | Mesenteric vessel injury and damage control resuscitation using H12-(ADP)-liposomes, RBCs, PRP, or PPP

After blood withdrawal and autologous RBC transfusion to make thrombocytopenia, rabbits underwent laparotomy and received mesenteric vessel injury at the jejunal mesentery approximately 10 cm anal side from the Treitz ligament (Figure S2). Bleeding volume from the mesenteric injury was measured every 5 min. At initial

5 min after bleeding, rabbits received intravenous bolus injection of H12-(ADP)-liposomes (20 mg/ml/kg). Immediately after H12-(ADP)-liposome injection, they received isovolemic transfusion with allogenic stored-RBC/PPP (RBC:PPP = 1:1 [vol/vol]) every 5 min to compensate the mesenteric hemorrhage (n = 10). In addition, we examined the effect of giving the H12-(ADP)-liposomes followed by infusion of lactated Ringer solution (n = 4) or PPP (n = 5), and examined the effect of H12-(PBS)-liposomes (without ADP) followed by RBC/PPP (n = 5) in this model. As a positive control group, isovolemic allogenic stored-RBC/PRP (RBC:PRP = 1:1 [vol/vol]) was transfused into the rabbits every 5 min (n = 10). As negative control groups, isovolemic allogenic stored-RBC/PPP (RBC:PPP = 1:1 [vol/vol]) was transfused into the rabbits every 5 min (n = 10) and isovolemic lactated Ringer solution (n = 5), or PPP alone (n = 4) was infused every 5 min (Figure 1). The bleeding from the injured vessel was quantitatively evaluated by passing the injured ileum through a hole cut in a surgical glove. The exsanguinating blood was then collected in the glove and its volume precisely measured (Figure S2A). After abdominal closure, rabbit survivals were monitored for 24 h under *ad libitum* feeding with laboratory diet and water. Postoperative analgesia was performed with intramuscular injections of buprenorphine (0.02 mg/kg) twice immediately after wound closure and 12 h later.

2.7 | Measurement of blood cell counts and coagulation factors

Blood cell counts were measured using a hematology analyzer (Erma PCE 170, Erma, Tokyo, Japan) at five time points: before the experiment, after completing the blood exchange, and 5, 15, and 30 min after mesenteric injury. To measure the plasma fibrinogen levels, AT III activity, PT, and aPTT, blood samples were collected in heparinized syringes and centrifuged at 500 × g at 4°C for 10 min, at four time points: before experiment, after completing the blood

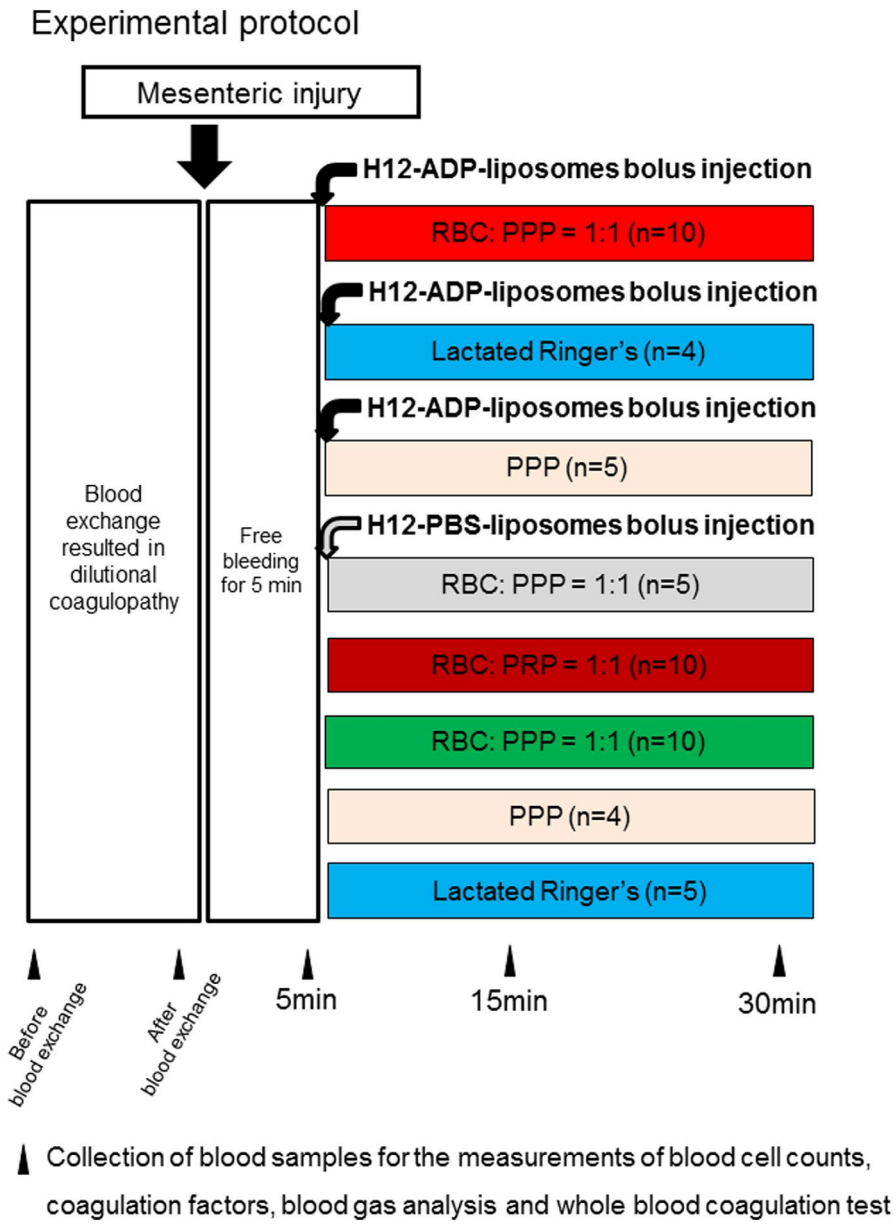

FIGURE 1 Experimental design of resuscitation following mesenteric hemorrhage in rabbits with thrombocytopenic coagulopathy. Thrombocytopenia was induced in rabbits by repeated blood withdrawals and isovolemic transfusion of autologous washed RBCs. Thereafter, mesenteric injury was made in the jejunum, resulting in hemorrhagic shock due to mesenteric bleeding. At initial 5 min after bleeding, rabbits received H12-(ADP)-liposomes or H12-(PBS)-liposomes intravenous bolus injection (20 mg/ml/kg), immediately followed by an isovolemic transfusion with allogenic stored-RBC/PPP (RBC:PPP = 1:1 [vol/vol]) every 5 min to compensate the mesenteric hemorrhage. Isovolemic RBC/PRP (RBC:PRP = 1:1 [vol/vol]) group, RBC/PPP (RBC:PPP = 1:1 [vol/vol]) group, PPP group, and lactated Ringer solution group, were compared for outcomes and hemodynamic/hematologic parameters. ADP, adenosine-diphosphate; PBS, phosphate buffered saline; PPP, platelet poor plasma; RBC, red blood cell

exchange, and 15 and 30 min after injury. These parameters were measured at the Sanritsu Zelcova Laboratory (Tokyo, Japan).

2.8 | Analyses of whole blood coagulation activity

The coagulation activity of whole blood was examined as previously described (Sonoclot Coagulation & Platelet Function Analyzer, Sienco, Morrison, CO).[9,10] 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. We also measured the clot amplitude. Because Sonoclot measurements took at least 20 min for each sample, whole blood samples were analyzed at four time points: before experiment, after completing the blood exchange, and 15 and 30 min after mesenteric injury.

2.9 | Measurement of blood gases and lactate levels

Blood gases and plasma lactate levels were measured at four time points: before experiment, after completing the blood exchange, and 15 and 30 min after mesenteric injury, using a blood gas analyzer (ABL 80, Radiometer, Copenhagen, Denmark). Numbers were reduced because we were unable to get blood samples from all rabbits.

2.10 | Observation with transmission electron microscope

Two additional rabbits were prepared for H12-ADP-liposome + RBC/PPP group. The mesenteric vessel specimens were obtained from the injury site at 1 h after injury. These specimens were prefixed with a fixative containing 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 3 h at 4°C, followed by postfixing in 1% osmium tetroxide in 0.1 mol/L phosphate buffer (pH 7.4) for 2 h at 4°C, dehydration, and embedding in epoxy resin. For selection of the bleeding site lesion, semithin sections were stained with toluidine blue. Ultrathin sections stained with uranyl acetate and lead citrate were then examined under an electron microscope (JEM 1400; JEOL, Tokyo, Japan) at an accelerating voltage of 80 kV.

2.11 | Statistical analyses

Statistical analyses were performed using a software package (Stat View 4.02J, Abacus Concepts, Berkeley, CA). Survival rates were compared by Wilcoxon signed-rank test. Statistical evaluations between two groups were made using Student *t* test, and other statistical evaluations were performed using a one-way analysis of variance, followed by a Bonferroni *post hoc* test. Data are presented as means ± SD, with $p < 0.05$ considered to be statistically significant.

3 | RESULTS

3.1 | Acute thrombocytopenia in rabbits after blood exchange and platelet plasma apheresis

After isovolemic blood exchange and platelet plasma apheresis, rabbits showed marked decreases in platelet counts ($50 \pm 8 \times 10^3$/μl) and plasma fibrinogen levels (59 ± 23 mg/dl), indicating severe coagulopathy (PT and aPTT were out of measurable range). They also showed significant prolongation of CT and reduction of CR (Table 1). RBC counts were $3.7 \pm 0.6 \times 10^6$/μl and hemoglobin (Hb) concentrations were 8 ± 1 g/dl in rabbits, which indicated mild anemia from loss of RBCs during the blood exchange. All rabbits showed a moderately reduced mean arterial pressure (MAP; 48 ± 7 mm Hg) at the end of blood exchange/platelet plasma apheresis (Table 1, Figure 2). They also showed lactic acidosis: pH declined to 7.15 ± 0.08, whereas plasma lactate levels were elevated to 7 ± 4 mmol/L (Table 1).

3.2 | Hemorrhagic shock with coagulopathy by mesenteric injury

Rabbits then received the mesenteric vessel injury. During the initial 5 min, their blood loss reached 16 ± 6 ml, which was approximately 11% of total blood volume (estimated as 54 ml/kg[24]) At 5 min after the bleeding, MAP, Hb concentrations, and platelet counts further reduced to 28 ± 5 mmHg, 6 ± 1 g/dl, and $45 \pm 11 \times 10^3$/μl, respectively (Table 1, Figure 2). These parameters characterized coagulopathy as defined by Moore et al.[11] (MAP < 30 mmHg, Hb < 6 g/dl, platelet $< 50 \times 10^3$/μl, pH < 7.2).

The area under the aggregation curve assessed by the collagen test or ADP test was not significantly changed between before experiment and after mesenteric vessel bleeding, which indicated that aggregating function of the residual platelets was well preserved in this model. H12-(ADP)-liposome test with ADP agonist showed similar results to the ADP test and H12-(ADP)-liposome test without ADP agonist showed no response *ex vivo* (Table S2).

3.3 | Hemostasis, bleeding volumes, and survivals after infusion/transfusion treatments

Administration of H12-(ADP)-liposomes followed by transfusion with RBC/PPP as well as transfusion with RBC/PRP effectively stopped bleeding in all thrombocytopenic rabbits. Their bleeding times were similar (24 ± 20 vs 25 ± 20 min) (Figure 3A) and their total blood loss were 39 ± 41 and 37 ± 28 ml, respectively. In contrast, RBC/PPP transfusion alone as well as administration

TABLE 1 Changes in hematologic variables and coagulation factors of rabbits before and after blood withdrawal

Variables	Before blood withdrawal ($n = 53$)	After blood withdrawal ($n = 53$)	5 min after injury ($n = 53$)
MAP (mmHg)	70 ± 11	49 ± 7*	26 ± 5**
Hb concentration (g/dl)	11.7 ± 0.8	7.9 ± 1.2*	6.7 ± 1.0**
HCT (%)	38 ± 4	26 ± 4*	22 ± 4**
PLT count ($\times 10^3/\mu l$)	241 ± 35	50 ± 9*	45 ± 11**
Fibrinogen concentration (mg/dl) ($n = 43$)	176 ± 23	57 ± 22*	Not measured
AT3 activity (%) ($n = 27$)	79 ± 8	26 ± 5*	Not measured
PT (s) ($n = 27$)	5.0 ± 0.2	>15	Not measured
aPTT (s) ($n = 27$)	26.0 ± 5.4	>75	Not measured
CT (s)	109 ± 24	398 ± 213*	Not measured
CR	19.4 ± 6.8	2.7 ± 1.6*	Not measured
Clot amplitude	97 ± 13	38 ± 11*	Not measured
pH	7.37 ± 0.07	7.18 ± 0.10*	Not measured
CtO_2 (vol%)	13 ± 2	9 ± 2*	Not measured
PaO_2 (mm Hg)	67 ± 15	165 ± 28*	Not measured
HCO_3- (mmol/L)	29 ± 4	10 ± 3*	Not measured
BE (mmol/L)	4 ± 4	−17 ± 4*	Not measured
Lactate (mmol/L)	3 ± 1	7 ± 4*	Not measured

Note: Data are mean ± SD.

Abbreviations: aPTT, activated partial thromboplastin time; CR, clot rate; CT, clotting time; CtO_2, arterial oxygen content; Hb, hemoglobin; HCT, hematocrit; MAP, mean arterial pressure; PLT, platelet.

*$p < 0.05$ vs before blood withdrawal.

**$p < 0.05$ vs before and after blood withdrawal.

of H12-(PBS)-liposomes followed by transfusion with RBC/PPP delayed hemostasis; bleeding time was 44 ± 35 and 57 ± 28 min and total blood loss: 69 ± 35 and 86 ± 16 ml, respectively. These blood loss volumes were significantly larger than that of the H12-(ADP)-liposomes with RBC/PPP group (Figure 3B). Eventually, three of 10 (30%) rabbits transfused with RBC/PPP, one of five (20%) rabbits administered with H12-(PBS)-liposomes followed by RBC/PPP, one of five (20%) rabbits with H12-(ADP)-liposomes administration followed by PPP infusion, and three of four (75%) rabbits with PPP infusion alone failed to stop bleeding. No rabbits receiving lactated Ringer solution stopped bleeding, resulting in decease (Figure 3B). MAP was markedly restored after RBC transfusion in the groups of administration of H12-(ADP)-liposomes followed by RBC/PPP transfusion, RBC/PRP transfusion, H12-(PBS)-liposomes followed by RBC/PPP transfusion, and RBC/PPP transfusion alone, but not groups of lactated Ringer infusion (Figure 2A). Regarding survival, H12-(ADP)-liposomes followed by RBC/PPP transfusion significantly improved rabbit survivals rather than RBC/PPP transfusion and H12-(PBS)-liposomes followed by RBC/PPP transfusion (Figure 4). At 24 h, H12-(ADP)-liposome with RBC/PPP group showed 80% survival, RBC/PRP group was 70%, H12-(ADP)-liposomes followed by PPP group: 60%, RBC/PPP group: 40%, and H12-(PBS)-liposomes with RBC/PPP: 20%. PPP alone group and H12-(ADP)-liposomes followed by lactated Ringer infusion group were lethal.

3.4 | Changes in blood cell counts and coagulation factors

Transfusion with RBC/PRP restored Hb concentration, hematocrit (HCT), and platelet counts (Figure 2B–D). RBC/PRP transfusion also tended to restore fibrinogen concentrations and PT (Tables S3 and S4), suggesting that it improved anemia and coagulopathy. Administration with H12-(ADP)-liposomes followed by RBC/PPP transfusion also tended to restore anemia (Hb concentration and HCT) and coagulopathy (fibrinogen concentrations and PT), although it did not affect platelet counts (Figure 2B–D, Tables S3 and S4), because H12-(ADP)-liposomes do not affect platelet counts. RBC/PPP transfusion similarly restored anemia and coagulopathy but not platelet counts (Figure 2B–D, Tables S3 and S4). Either treatment with lactated Ringer or PPP without RBC transfusion even following the H12-(ADP)-liposomes infusion showed marked reduction of Hb concentration (Figure 2B–D, Tables S3 and S4).

3.5 | Analyses of whole blood coagulation activity

Administration of H12-(ADP)-liposomes followed by RBC/PPP transfusion significantly shortened CT at 15 min after injury in comparison to RBC/PPP transfusion alone or lactated Ringer infusion (Figure 5A). Bolus administration of H12-(ADP)-liposomes in the

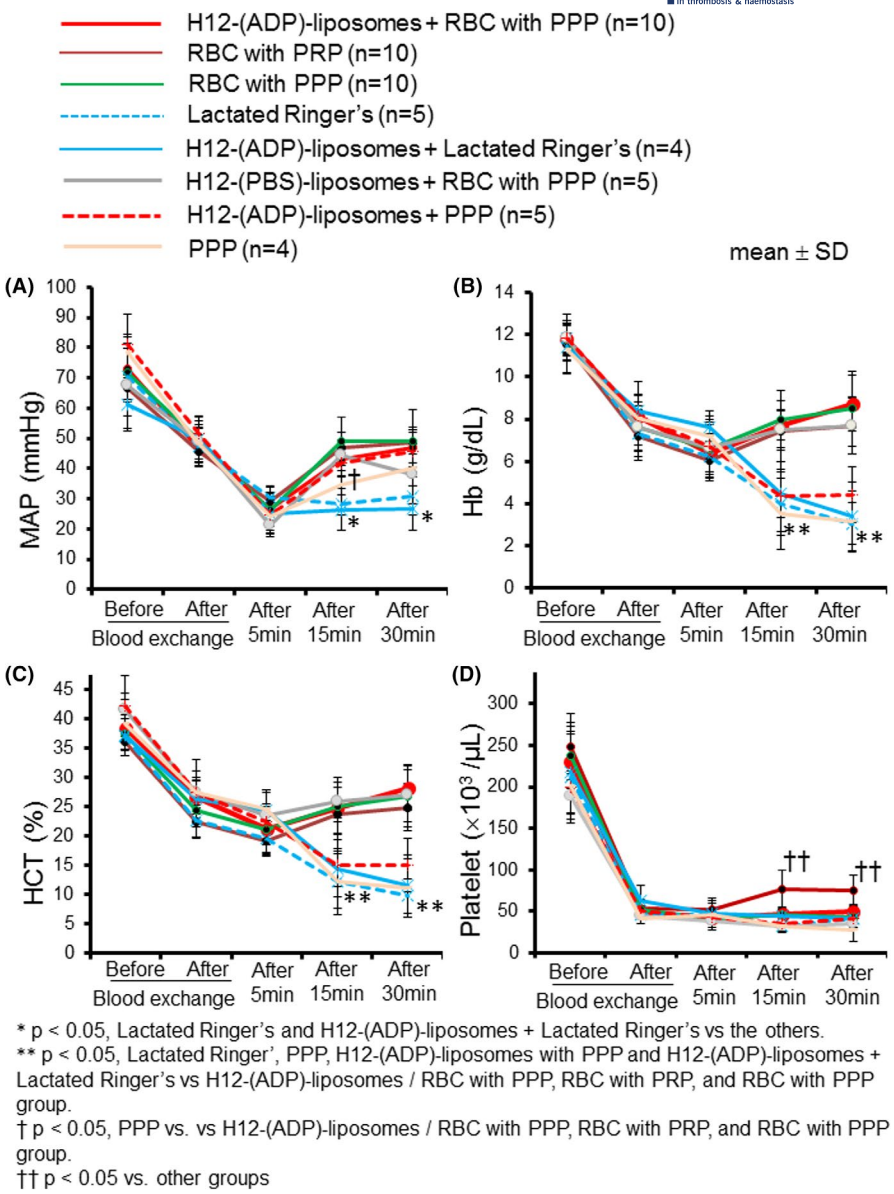

FIGURE 2 Changes in mean arterial pressure and hematological parameters in rabbits. At the initial 5 min from the bleeding (just before the first resuscitative transfusion), MAP, Hb levels, and platelet counts of rabbits further reduced to 28 ± 5 mmHg, 6.4 ± 1.0 g/dl and 45 ± 11 × 10^3/μl. These parameters were measured at each indicated time point. Hb, hemoglobin; MAP, mean arterial pressure

* p < 0.05, Lactated Ringer's and H12-(ADP)-liposomes + Lactated Ringer's vs the others.
** p < 0.05, Lactated Ringer', PPP, H12-(ADP)-liposomes with PPP and H12-(ADP)-liposomes + Lactated Ringer's vs H12-(ADP)-liposomes / RBC with PPP, RBC with PRP, and RBC with PPP group.
† p < 0.05, PPP vs. vs H12-(ADP)-liposomes / RBC with PPP, RBC with PRP, and RBC with PPP group.
†† p < 0.05 vs. other groups

initial bleeding phase may quickly improve CT. However, RBC/PRP transfusion and RBC/PPP transfusion also reduced CT at 30 min after injury but lactated Ringer infusion remarkably prolonged CT at 30 min (Figure 5A). Administration of H12-(ADP)-liposomes followed by RBC/PPP transfusion as well as transfusion with RBC/PRP or RBC/PPP alone significantly increased CR at 30 min after injury in comparison to lactated Ringer infusion (Figure 5B). H12-(ADP)-liposomes followed by PPP infusion also shortened CT and gained CR at 15 min after injury (Figure 5A,B). Although the bolus infusion of H12-(ADP)-liposomes followed by lactated Ringer infusion shortened CT at 15 min (Figure 5A), it neither reduced the bleeding volume (Figure 3B) nor maintained the hemodynamics (Figure 2A), resulting in death (Figure 4). PPP infusion alone showed the similar CT transition (reprolongation at 30 min) as H12-(ADP)-liposomes followed by lactated Ringer group. In line with this finding, survival rate of PPP infusion alone was also identical to H12-(ADP)-liposomes followed by lactated Ringer group (0% survival). H12-(PBS)-liposomes (without ADP) infusion had no shortening effect on CT at 15 min. Consistently, total blood loss of rabbits treated with H12-(PBS)-liposomes infusion followed by RBC/PPP was significantly larger than that of H12-(ADP)-liposomes followed by RBC/PPP. In all groups, clot amplitude reduced to 30%–40% levels compared with that of the previous experiment in a similar fashion (Table S5).

3.6 | Analyses of blood gas and lactate levels

Mesenteric bleeding caused lactic acidosis in rabbits. In brief, pH levels shifted to below 7.2 at either 15 or 30 min after injury in all groups (Table S6). Base excess decreased to −20 mmol/L, HCO_3- decreased to 8 mmol/L, whereas plasma lactate levels were elevated to approximately 10 mmol/L. To compensate for acidemia, hyperventilation increased PaO_2 values to 160–200 mmHg. Isovolemic RBC transfusion maintained arterial oxygen content 8–10 vol% in

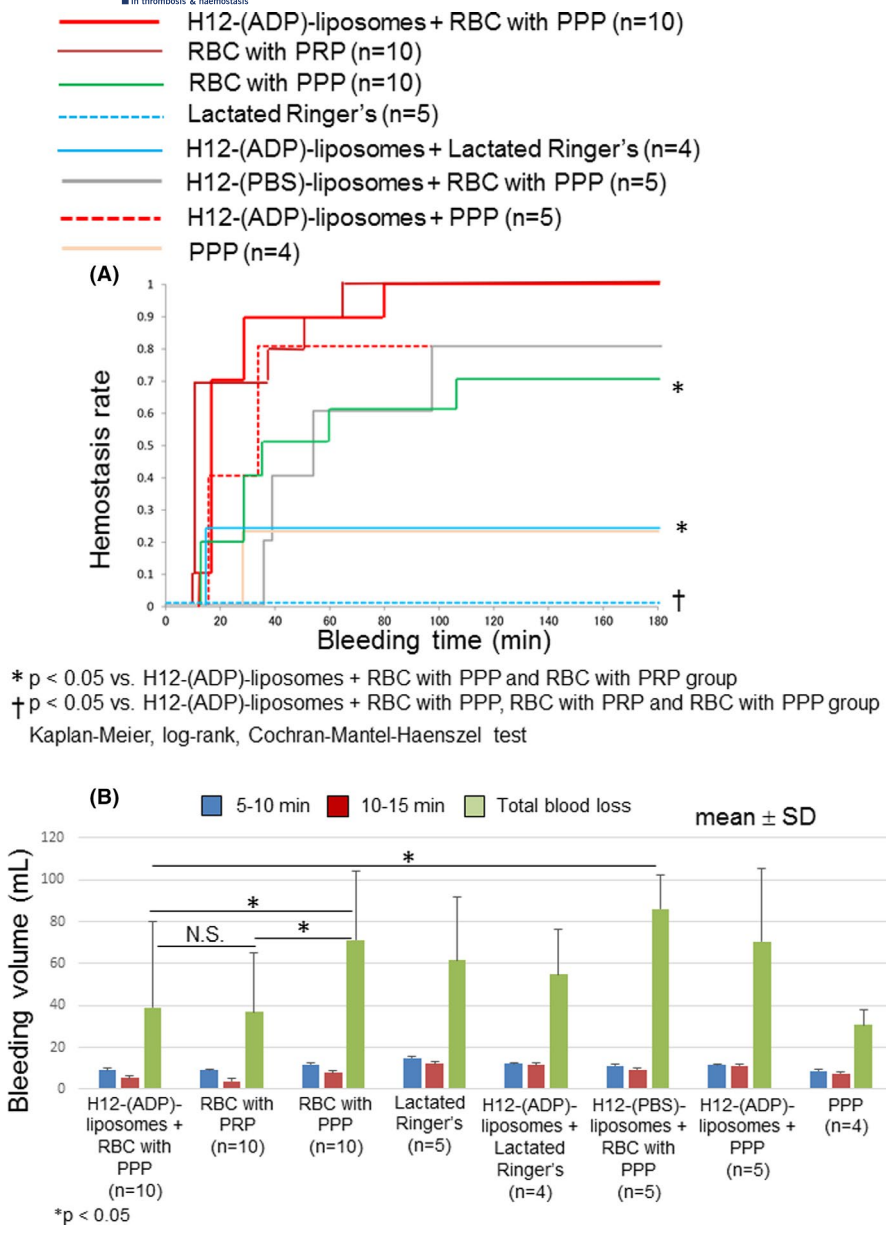

FIGURE 3 Bleeding time and blood loss volume. (A) Administration with H12-(ADP)-liposomes followed by transfusion with RBC/PPP as well as transfusion with RBC/PRP effectively stopped bleeding in all thrombocytopenic rabbits. (B) The blood loss volumes of rabbits treated with RBC/PRP, with H12-(ADP)-liposomes followed by RBC/PPP or with RBC/PPP gradually reduced. The total blood loss until hemostasis were significantly larger in rabbits treated with RBC/PPP alone than those in rabbits treated with H12-(ADP)-liposomes followed by RBC/PPP. ADP, adenosine-diphosphate; PPP, platelet poor plasma; PRP, platelet rich plasma; RBC, red blood cell

H12-(ADP)-liposomes with RBC/PPP group, RBC/PRP group, and RBC/PPP group, whereas lactated Ringer infusion and PPP infusion significantly decreased arterial oxygen content to 3–5 vol% as low as lethal levels (Table S6).

3.7 | Observations with transmission electron microscope

The lesion near the surface of thrombus, adjacent to the injured vessel (Figure 6A) and the lesion inside of thrombus formation (Figure 6B) were observed in toluidine blue-staining semithin section. In the former lesion, near the surface, platelets, erythrocytes, leukocytes, and fibrin were loosely contact with each other (Figure 6C). In contrast, inside of the thrombus (the latter lesion), platelets, erythrocytes, and leukocytes were densely contact with fibrin (Figure 6D). In both lesions, anhistous particles, which were approximately 200–400 nm in diameter (indicated by arrows), were observed around the platelets, erythrocytes, or fibrin deposits (Figure 6E,F). These anhistous particles were presumably considered H12-(ADP)-liposomes.[13,17] In particular, dense coagulation clots appeared to squeeze anhistous particles by clot compression (Figure 6F).

4 | DISCUSSION

Management of abdominal vascular trauma has been standardized as stated in "Western Trauma Association Critical Decisions in Trauma." In cases of mesenteric arterial injury, this guide listed five steps to control bleeding as follows: (1) approach to hematoma at laparotomy and remove it, (2) approach to hemorrhage at laparotomy with manual compression, (3) repair treatment of arterial hemorrhage at

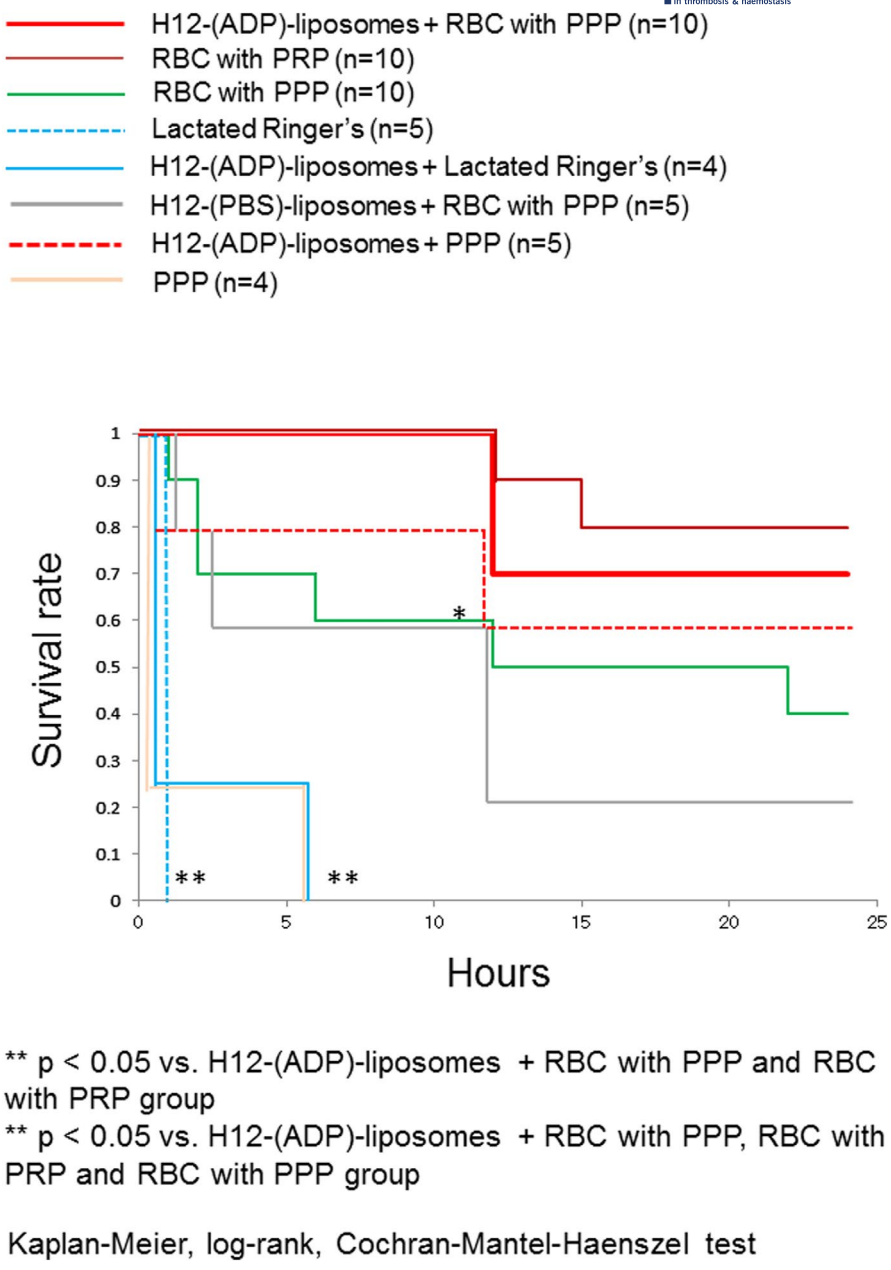

FIGURE 4 Survival rates of rabbits with induced acute thrombocytopenia/hemorrhagic shock. Three of 10 rabbits transfused with RBC/PPP failed to stop bleeding. No rabbits receiving lactated Ringer solution stopped bleeding, resulting in death. PP, platelet poor plasma; RBC, red blood cell

laparotomy or after hematoma opened, (4) systemic complications (hypothermia, acidosis and coagulopathy) are corrected in the intensive care unit, and (5) "second-look" operation within 12–24 h should be always considered after such extensive reconstruction of the superior mesenteric artery.[25] In addition, DCR including MTP should be introduced before laparotomy. Maciel et al. elucidated the impact of MTP on the outcomes of patients with abdominal aortic injuries by a retrospective analysis of level 1 trauma center database.[10]

Recently, prehospital DCR has improved prognoses of trauma patients. A recent study of prehospital blood transfusion during helicopter transport showed a survival benefit to combat casualties.[26] The Prehospital Air Medical Plasma trial reported a generalizable intervention of "prehospital plasma resuscitation," which appears to improve survival in trauma patients who are bleeding and at risk for hemorrhagic shock.[27,28]

Infusion of H-12-ADP-liposomes with lactated Ringer solution was unable to stop bleeding effectively, whereas infusion of H12-(ADP)-liposomes followed by RBC/PPP transfusion effectively stopped bleeding from the mesenteric vessel injury in rabbits. This suggests that the hemostatic effect of H-12-ADP-liposomes may require supplemental augmentation by plasma components. It may contribute to prehospital DCR as a prompt hemostatic agent. Even following systemic administration, H12-(ADP)-liposomes accumulated at bleeding site.[13] H12-(ADP)-liposomes may constitute a novel therapeutic strategy for abdominal vascular injury; prehospital resuscitation using H12-(ADP)-liposomes could be useful for abdominal trauma hemorrhage caused by mesenteric injury in patients with coagulopathy, which is one of the most serious trauma conditions.

The initial focus on hemostasis is an important and effective measure at prehospital level to improve the outcomes in hemorrhagic

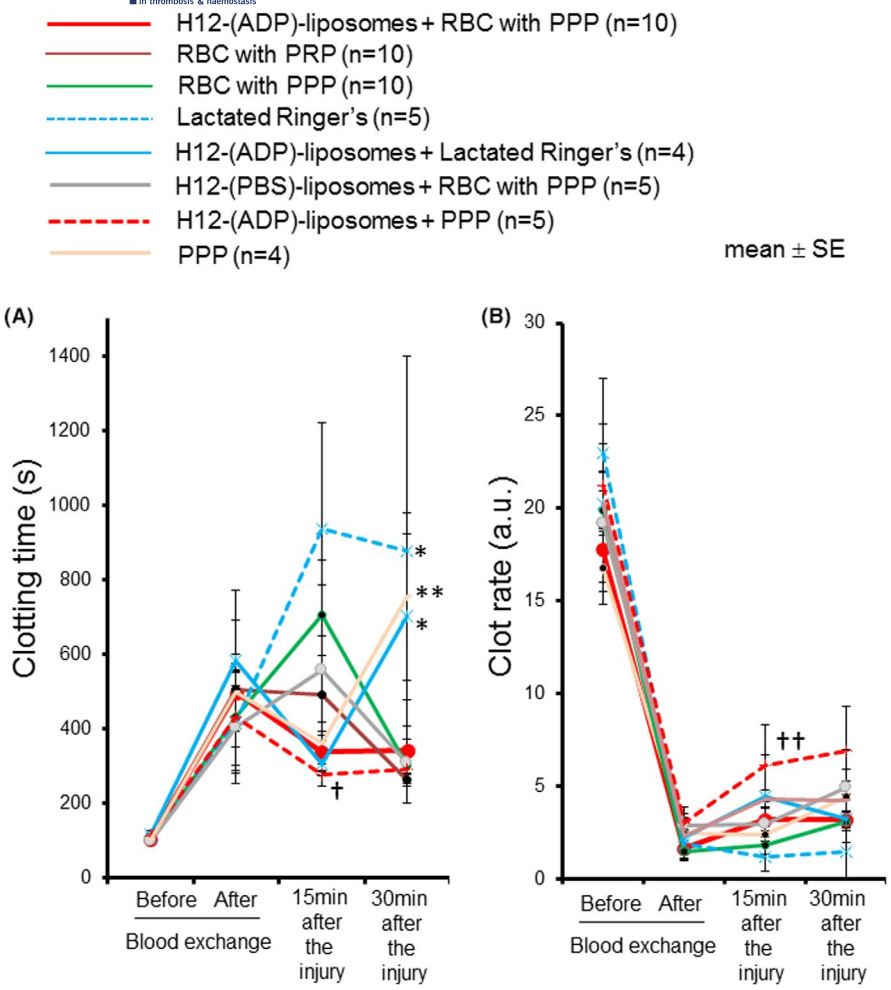

FIGURE 5 Changes in whole blood coagulation. Administration of H12-(ADP)-liposomes followed by transfusion with RBC/PPP promptly shortened CT to 338 ± 174 s at 15 min after injury, in comparison to substantial prolongation of CT in the other groups. At 30 min after injury, CT and CR were improved in all the animals except received lactated Ringer solution. ADP, adenosine-diphosphate; CR, clot rate; CT, clotting time; PPP, platelet poor plasma; RBC, red blood cell

* $p < 0.05$ vs H12-(ADP)-liposomes + RBC with PPP group and RBC with RPP group
** $p < 0.05$ vs. H12-(ADP)-liposomes + RBC with PPP group, RBC with RPP group, H12-(PBS)-liposomes + RBC with PPP group and H12-(ADP)-liposomes + PPP group
† $p < 0.05$, H12-(ADP)-liposomes + RBC with PPP vs. RBC with PPP group and Lactated Ringer's group
†† $p < 0.05$, H12-(ADP)-liposomes + PPP group vs. RBC with PPP group and H12-(ADP)-liposomes + RBC with PPP group

shock with coagulopathy. However, prehospital damage control interventions, such as platelet transfusion, exploratory laparotomy, Resuscitative Endovascular Balloon Occlusion of the Aorta, or junctional tourniquet, are usually difficult to perform in clinical settings. Administration of H12-(ADP)-liposomes could robustly and easily control bleeding even in the early phase of patient care, namely prehospital resuscitation, because it can be easily stored without shaking[18] and can be used as an intravenous bolus administration. Definitive surgery to repair damaged organ/tissues and removal of hematoma could be safely performed under controlled hemorrhage by treatment with H12-(ADP)-liposomes. Because only a small volume (2–3 ml) of H12-(ADP)-liposomes is needed to stop bleeding, this treatment could avoid the hemodilution by transfusion of RBC/PPP.

Thrombocytopenic rabbits showed more than 400 s of CT (before injury), demonstrating severe coagulopathy.[29] Administration of H12-(ADP)-liposomes with RBC/PPP rapidly shortened CT already 15 min after injury (Figure 5A), indicating improvement of coagulation function. RBC/PPP transfusion alone required two times volume as H12-(ADP)-liposomes with RBC/PPP to compensate for the blood loss volume in the current study. Consistently, Asensio et al. reported that volume overload stood behind acidosis and coagulopathy in patients with superior mesenteric arterial injury. In that report, patients had to receive a total infusion of 17 L of several blood products with colloids and crystalloid against 5.2 L of blood loss.[30]

In the current study, as the elevation of Hb concentration was 2 g/dl after transfusion, the transfusion volume of stored RBCs should be estimated as 5 units. In the same way, as the elevation of platelet counts was $35 \times 10^3/\mu l$ after transfusion, transfusion volume of platelets was comparable to 5 units. Infusion plasma volume was estimated to be 5 units because 16 ml/kg of plasma was infused

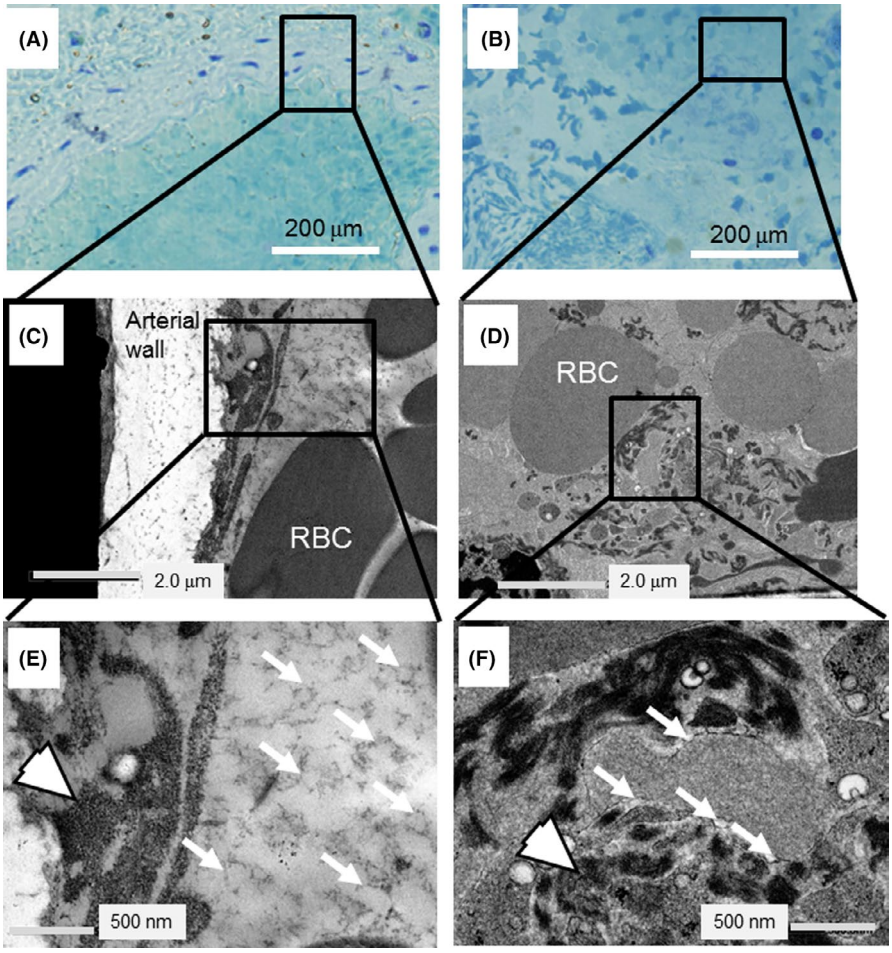

FIGURE 6 Transmission electron microscopic observation. A semithin section showed the loose coagulation clots (A) and dense coagulation clots (B) adjacent to the injured site. The clots had substantial involvement of platelets and fibrin around RBCs (C, D). Arrows indicate anhistous particles, which were presumably considered as H12-(ADP)-liposome (approximately 200–400 nm)[13,17] (E, F). Although loose coagulation clots involved anhistous particles (E), dense coagulation clots squeezed anhistous particles by clot compression (F). Arrowheads indicate platelets. ADP, adenosine-diphosphate; RBC, red blood cell

into rabbits in the current study. Therefore, administration ratio of stored-RBCs: platelets: plasma was considered to be 5:5:5 (units/units), which is comparable to the ratio of the most recent transfusion protocols based on the 1:1:1 scenario.

According to several guidelines and papers, recombinant factor VIIa has been used for correcting coagulopathy in surgery or trauma, which were recommended under these conditions: platelet > 50×10^3/μl, fibrinogen > 100 mg/dl, and HCT > 24%.[31-33] In the current study, H12-(ADP)-liposomes could be used in more severe conditions: platelet counts were $45 \pm 11 \times 10^3$/μl, fibrinogen level was 59 ± 23 mg/dl, and HCT was 20 ± 3% (Table 1) because H12-(ADP)-liposomes can directly promote platelet thrombi that is crucial for hemostasis in thrombocytopenic conditions.[13,23]

Consistent with the current findings, in our previous study H12-(PBS)-liposomes with PPP were applied for the liver penetrating hemorrhage in thrombocytopenic rabbits whose platelet counts decreased less than 50×10^9/L, as low as in the current study. They had no additional platelet aggregation effect in comparison with PPP alone shown in similar ACT prolongation. Consequently, ADP loading is crucial for hemostasis in thrombocytopenia.[23] Previous studies indicated that ADP is the most appropriate agonist for enhancing platelet aggregation without adverse thrombotic event and it can be sufficiently encapsulated by liposomes.[34] Adding ADP also may help constriction of injured vessels via purinergic receptors on the endothelium and smooth muscle cells besides activation of platelets.[35,36]

H12-(ADP)-liposomes have several characteristics and advantages for application to DCR and emergency situations. Transcatheter arterial embolization[37,38] or spasm-induced pharmacotherapy[39] based on invasive angiographical procedure can selectively achieve hemostasis. H12-(ADP)-liposomes could noninvasively achieve local hemostasis because they can accumulate at the bleeding site point. In addition, H12-(ADP)-liposomes are easy to store and provide for field care, owing to characteristics of artificial liposomes, which are suitable for initial treatment of urgent hemorrhage patients. As for the safety in a nonthrombocytopenic condition, we previously applied H12-(ADP)-liposomes for normal mice, whose platelet counts were kept > 400×10^9/L. Administration of H12-(ADP)-liposomes caused no remarkable change in the Sonoclot value and induced no thrombotic events in normal mice.[14]

In recent years, several hemostatic materials like Fibroplate, HAPPI polymer, platelet-like nanoparticles, platelet-like particles, RGD-coated particles, synthetic platelets (e.g., SynthoPlate, synplat), and Thrombosome have been reported as similar type of modalities to treat trauma bleeding and thrombocytopenia bleeding.[40-43] Some of them have multiple ligands toward injured endothelium, which may enhance not only platelet aggregation but also its adhesion. In contrast, the H12-(ADP)-liposomes are designed to potentially

promote local activation of residual platelets proximal to injury as well as promote platelet aggregation.

5 | LIMITATIONS

In this study, mesenteric bleeding was located on marginal branch defined as Fullen zone IV,[44] which was relatively mild trauma injury in comparison to the injury of proximal superior mesenteric artery. However, thrombocytopenic animals fell into robust hemorrhagic shock and the single bleeding site enabled to confirm the efficacy of H12-(ADP)-liposomes for hemostasis. As for mesenteric injured procedure, anatomical position was not distinctly similar in all rabbits because of their inherent vessel variability.

In thrombocytopenic rabbits whose platelet counts decreased to less than 50×10^9/L and as low as in the current study, H12-ADP-liposmes showed similar hemostatic effect beyond 20 mg/kg of lipid concentration.[34] However, optimum liposome: platelet ratios should be elucidated for different platelet count conditions.

Clinically, early platelet dysfunction may be one of the major reasons of trauma-induced coagulopathy in severely injured patients.[45] In our study, coagulopathy was mainly attributed to the hemodilution not to platelet dysfunction, and the current experimental model enabled to maintain platelet aggregating function in the subject rabbits. The control of platelet dysfunction using H12-(ADP)-liposomes should be a target of future research. In addition, future studies are necessary for the optimization of ADP loading and H12 decoration to expand the indication of this treatment.

6 | CONCLUSION

Resuscitation with H12-(ADP)-liposomes may be effective as platelet substitutes in lethal hemorrhage caused by mesenteric injury in rabbits with coagulopathy.

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RELATIONSHIP DISCLOSURE

The authors have no conflict of interest.

AUTHOR CONTRIBUTIONS

Kohsuke Hagisawa performed the planning and execution of all experiments as well as writing the article. Manabu Kinoshita performed the planning of all experiments and editing the article. Shinji Takeoka, Morihiro Hotta, and Masato Takikawa manufactured the liposome. Osamu Ishida performed execution of all experiments. Daizoh Saitoh analyzed the data. Yayoi Ichiki validated the transmission electron microscope observation. Ivo P. Torres Filho and Yuji Morimoto critically revised the article. All authors provided final approval of the version to be published.

DISCLAIMER

The views expressed in this article are those of the authors and do not reflect the official policy or position of the U.S. Army Medical Department, Department of the Army, Department of Defense, or the U.S. Government.

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SUPPORTING INFORMATION

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