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ORIGINAL ARTICLE

Coagulation patterns following haemoglobin-based oxygen carrier resuscitation in severe uncontrolled haemorrhagic shock in swine

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SUMMARY. Massive blood loss due to penetrating trauma and internal organ damage can cause severe haemorrhagic shock (HS), leading to a severely compromised haemostatic balance. This study evaluated the effect of bovine polymerized haemoglobin (Hb) (Hb-based oxygen carrier, HBOC) resuscitation on haemostasis in a swine model of uncontrolled HS. Following liver injury/HS, swine received HBOC ($n = 8$), Hextend (HEX) ($n = 8$) or no resuscitation (NON) ($n = 8$). Fluids were infused to increase mean arterial pressure above 60 mmHg and to reduce heart rate to baseline. At 4 h, the animals were eligible for blood transfusions. Prothrombin time (PT), activated partial thromboplastin time, fibrinogen, thromboelastography (TEG) and platelet function analyser closure time (PFA-CT) were compared by using mixed statistical model. At 4 h, blood loss (% estimated blood volume) was comparable for HBOC (65·5 ± 18·5%) and HEX (80·8 ± 14·4%) and less for NON (58·7 ± 10·1%; $P < 0·05$). Resuscitation-induced dilutional coagulopathy was observed with HBOC and HEX, as indicated by reduced haematocrit, platelets and fibrinogen ($P < 0·05$). At 4 h, PT was higher in HEX than in HBOC groups ($P < 0·01$). In the early hospital phase, a trend to increased TEG reaction time and PFA-CT indicates that dilutional effects persist in HBOC and HEX groups. PFA-CT returned to baseline later with HBOC than with HEX (48 vs. 24 h) following blood transfusion. At 4 h, all surviving HEX animals ($n = 3$) required transfusion, in contrast to no HBOC ($n = 7$) or NON ($n = 1$) animals. In this severe uncontrolled HS model, successful resuscitation with HBOC produced haemodilutional coagulopathy less than or similar to that produced by resuscitation with HEX.

Key words: haemostasis, oxygen carriers, resuscitation, swine model, transfusion, trauma.

Acute blood loss of more than 45% of estimated blood volume (EBV) is fatal within 18 min without appropriate interventions (Champion *et al.*, 2003). Massive haemorrhage of this scale induces vasoconstriction and platelet activation by the release of thromboxane, as well as by the exposure to collagen from injured endothelium. Subsequent activation of the coagulation cascade and further activation of platelets produce an immediate hypercoagulable state (Jacoby *et al.*, 2001; Lapointe & Von Rueden, 2002; DeLoughery *et al.*, 2004). Despite the need for rapid resuscitative fluid infusions to stabilize haemodynamics and to provide for adequate tissue perfusion, fluid resuscitation itself further disturbs haemostasis, leading to predictable traumatic coagulopathy (Ledgerwood & Lucas, 2003). Clinical indicators include elevated prothrombin time (PT) and/or activated partial thromboplastin time (aPTT) (Lapointe & Von Rueden, 2002; Brohi *et al.*, 2003). Intricate events combining

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overcompensation of procoagulant and anticoagulant responses can result in disseminated intravascular coagulation. These alterations occur early after injury and correlate with clinical outcome (Heckbert *et al.*, 1998). Patients with elevated PT and aPTT on arrival at hospital are at 35 and 326% greater risk of death, respectively (MacLeod *et al.*, 2003). Therefore, initial therapeutic interventions may exert significant effects on subsequent outcome following haemorrhage.

Haemoglobin (Hb)-based oxygen carrier (HBOC) solutions have been proposed for use in traumatic haemorrhage as an 'oxygen bridge' for stabilization prior to definitive intervention (Klein, 2005). Theoretically, some HBOCs provide benefits as prehospital resuscitative fluids including intravascular volume expansion with limited haemodilution, improved tissue oxygenation, universal ABO compatibility, limited risk of disease transmission and immediate availability (Chamberland, 2002). HBOC-201, for example, has been shown to provide both oxygen transport and effective volume replacement immediately after injury (Levy, 2003; Sampson *et al.*, 2003). These properties may be of particular benefit in cases of prolonged evacuation and/or delay to definitive care (Gurney *et al.*, 2004). HBOC-201 has been studied in both animal models and human trials (Manning *et al.*, 2000; Katz *et al.*, 2002; Sprung *et al.*, 2002; Philbin *et al.*, 2005) and appears to have an acceptable risk : benefit ratio (Levy, 2003). However, the literature regarding the effects of HBOC on haemostasis, coagulation and thrombosis is limited. Potential HBOC-resuscitation-related haemostatic effects include haemodilution, decreased cellular mass and nitric oxide (NO) scavenging. Since NO is a platelet relaxant, HBOC could promote platelet activation and accelerated clot formation (e.g. *o*-raffinose polymerized Hb; Lee *et al.*, 2000), although there is no platelet aggregation or surface adhesion molecule expression (e.g. GPIb, GPIIb/IIIa could be detected *in vitro*; Toussaint *et al.*, 2003). Additionally, hypercoagulation with HBOC-201 was not observed *in vivo* in a rabbit model of stenosis (Marret *et al.*, 2004). These observations were confirmed in a swine model of moderately severe controlled haemorrhagic shock (HS) (40% EBV), in which HBOC-201 had no major clinical adverse effects on thrombosis or haemostasis during the early posthaemorrhage period (Arnaud *et al.*, 2005). However, the effect of HBOC-201 in a more severe model with significant tissue injury and disruption of endothelium has not been reported in the literature.

This study was designed to test the hypothesis that in a severe model of HS (uncontrolled haemorrhage with internal organ injury), HBOC-201 resuscitation does not lead to significant haemostatic or coagulation impairment compared to control resuscitation with HEX.

MATERIALS AND METHODS

These experiments were conducted according to the principles set forth in the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animals Resources, National Research Council, National Academy Press, 1996. The study was approved by the WRAIR/NMRC Institutional Animal Care and Use Committee. All procedures were performed in an animal facility accredited by the American Association for Accreditation for Laboratory Animal Care.

Animal procedures

A model of traumatic HS with uncontrolled haemorrhage due to grade III liver laceration/crush injury and subsequent fluid resuscitation was previously described by Gurney *et al.* (2004). Briefly, 24 Yucatan minipigs ($23{\cdot}0 \pm 8{\cdot}5$ kg) were anaesthetized (ketamine/isofluorane induction and isofluorane maintenance), intubated and allowed to breath spontaneously ($FiO_2 = 0{\cdot}21$). Rectal temperature was monitored and body heat maintained (36–37·8 °C) using a BAIR hugger device (Model 505, Arizant Healthcare Inc., Eden Prairie, MN, USA). The external jugular vein and carotid artery were catheterized via an open technique to allow monitoring of pulmonary and systemic arterial pressure. The bladder was catheterized for urine collection.

Approximately 10 min following surgical preparation and baseline observations, the liver was exposed through a midline laporotomy incision. A grade III liver laceration/crush injury was created by placing a ring clamp at approximately three-fourth length from the edge of the exposed liver lobe and incising through its width. After 1 min, the clamp was removed and the remaining tissue excised. Bleeding from the lobe was allowed without intervention.

The shed blood was removed by suction in vacuum canisters from the intraperitoneal cavity. The canister weight was measured at 5, 15, 20, 30, 60 and 240 min; additional fluid weight of sponges left in the cavity was added to the 240-min measurement. Phlebotomy blood volumes (totalling approximately 125 mL for animals surviving 240 min) were not included in the reported haemorrhage volumes. Baseline EBV was calculated as animal baseline weight $\times$ 65 mL blood kg^{-1}. Total blood loss was reported as % EBV at 240 min or at the endpoint for each animal and then averaged.

At 15-min post onset of haemorrhage (time 0, end of the initial haemorrhage phase), animals were randomized to resuscitation with HBOC-201 (HBOC; $n = 8$) or hydroxyethyl starch (Hextend, HEX; $n = 8$), or were not resuscitated (NON; $n = 8$). Resuscitation fluids were infused at 15 min, over 10 min, and at a rate of 10 mL kg^{-1}, and subsequently at 5 mL kg^{-1} at 30, 60, 120 and 180 min for mean arterial pressure (MAP) below 60 mmHg or heart rate above baseline. This was followed by a simulated hospital phase starting at 4 h that included definitive surgical repair and blood transfusions during a 3-day observation period. Following surgical repair, arterial and bladder catheters were removed, and the neck and abdominal skin and fascia closed. The jugular venous catheter was maintained for blood sampling and fluid infusions. At 4, 24 and 48 h, animals received allogenic-matched whole-blood transfusions (for Hb < 7 g dL^{-1}) or normal saline (Baxter, Deerfield, IL, USA) (for Hb >7 g dL^{-1}) at 10 mL kg^{-1} over 30 min. Animals were followed for 3 days postoperatively and then euthanized. Whole blood from matching Yucatan pigs was collected in blood bags containing standard anticoagulant Citrate phosphate dextrose-adenine (CPD-A) (Fenwal, Deerfield, IL, USA) and stored at 4 °C for potential transfusion during the simulated hospital phase (all pigs were of blood type A).

HBOC-201 is purified, filtered, stroma-free and heat-treated bovine Hb derived from an isolated herd and is certified free of pathogens including transmissible spongiform encephalopathies. HBOC-201 is polymerized by gluteraldehyde cross-linking to form polymers ranging in molecular weight (MW) from 130 to 500 Kd (Pearce & Gawryl, 1998). HBOC-201 is prepared in a buffer similar to lactated Ringer's solution (LR) containing a 50 : 50 racemic D- and L-lactate mixture (27 mEq lactate), *N*-acetyl-polycysteine (0·17%), approximately 12·5 g Hb dL^{-1}, with an oncotic pressure of 17 mmHg, an osmolality of approximately 300 mOsmol kg^{-1}, a pH of approximately 7·8 and an oxygen affinity (P_{50}) of 38 mmHg (lower than human blood). HBOC-201 does not contain glucose and is stable at 25 °C for at least 3 years (Pearce & Gawryl, 1998). HEX is 6% hydroxyethyl starch (MW = 670 Kd) prepared in balanced LR (50 : 50 racemic mixture, 28 mEq lactate), containing glucose (1 g L^{-1}), with a pH of approximately 6·6, an osmolality of 307 mOsmol kg^{-1} and an oncotic pressure of 30 mmHg (Hextend, Abbott Laboratories, Abbott Park, IL, USA). HEX has been recommended as the standard resuscitation fluid for the US Special Forces for battlefield care.

Fluid infusion was computed as the number of resuscitation fluid infusions (10 mL kg^{-1}) per surviving animal during the prehospital phase and similarly for the number of blood transfusions or saline infusions (10 mL kg^{-1}) during the hospital phase. For example, if all eight animals survived the entire 240 min, the maximum allowable number of infusions at 20, 30, 60, 120 and 180 min would have been 1, 0·5, 0·5 and 0·5, respectively (1 for 10 mL kg^{-1} and 0·5 for 5 mL kg^{-1}), and the maximum allowable number of blood transfusions or saline infusions at 4, 24 and 48 h would have been 1, 1 and 1. For each treatment group, the number of infusions was summed for all animals at each time point and then divided by the number of animals surviving at this time. This was then cumulated with time and plotted. This calculation eliminates the confounding of final fluid infusion volumes resulting from animal death. Also, these normalized data could be compared to a maximum number of theoretical infusions and then further compared to each treatment group.

Bleeding time (BT) was measured by an ear incision with a scalpel blade (no. 11) on an ear edge to create a reproducible 5-mm anterior incision at time 0 and 4 h posthaemorrhage (at 4 h, the MAP had stabilized). The time for the bleeding to stop was recorded by the paper blotting method using Whatman paper no. 1.

In vitro assays

All functional laboratory assays were performed at 37 °C, consistent with the recorded animal temperatures (36·8 ± 1·5 °C).

Thrombosis and haemostasis was assessed as previously described (Arnaud *et al.*, 2005). The following tests were carried out on blood samples collected at 0, 30, 60, 180 and 240 min and 24, 48 and 72 h (in vacutainer tubes, BD Vacutainer, Becton Dickinson, Palo Alto, CA, USA) before intervention for fluid infusion or transfusion. Complete blood count (CBC) with differential was performed using a Pentra $60C^{+}$ cell counter (ABX Diagnostics, Irvine, CA, USA). Normalized platelet to haematocrit (Hct) was calculated as follows: platelet concentration × (100 − Hct)/100. Normalized white blood cell (WBC) was computed similarly. Plasma Hb (due to HBOC) was measured with the B-Hb method (Hemocue, Angelholm, Sweden; (Jahr *et al.*, 2002).

Coagulation parameters, including PT, aPTT, thrombin time, antithrombin (AT-III) and fibrinogen, were measured using both clot-based principles and colorimetric determination on a Stat Compact (Diagnostica Stago, Parsippany, NJ, USA). AT-III was not determined for samples containing HBOC, as HBOC interferes with the test. Normalized AT-III for HEX and NON groups was calculated as AT-III × (100 − Hct)/100.

Thromboelastography (TEG) reaction time (TEG-R, corresponding to fibrin formation), kinetics of clot formation (TEG-K and TEG-α), maximum amplitude (TEG-MA) and fibrinolysis (TEG-Ly) were measured using a TEG 5000 Haemostasis Analyzer (Haemoscope Corp., Niles, IL, USA). The coagulation index (TEG-CI) was calculated as: TEG-CI = (0·0184 × TEG-K) + (0·1655 × TEG-MA) − (0·0241 × TEG-α) – (0·2454 × TEG-R) − 5·022 (Kaufmann *et al.*, 1997). The test was initiated with 340 μL whole blood recalcified with 20 μL of $CaCl_2$. Platelet-adjusted TEG-MA was calculated as TEG-MA/Platelet (PLT) adjusted.

In vitro BT was measured by the platelet function analyser closure time (PFA-CT) of an Adenosine diphosphate (ADP)-collagen-coated capillary after aspiration of 800 μL citrated whole blood using a PFA-100 (Dade Behring, Deerfield, IL, USA). Platelet-adjusted PFA-CT was calculated as PFA-CT × PLT.

Adenosine triphosphate (ATP) luminescence was measured in a limited number of samples using a whole-blood aggregometer (Chronolog, Havertown, PA, USA). The samples were adjusted to 150×10^6 platelets mL^{-1} after a 1 : 1 dilution in saline (according to the manufacturer's recommendations). In a microcuvette, 400 μL of sample was incubated at 37 °C with 40 μL of Chromolum (Chronolog, Havertown, PA, USA) for 1 min. Platelet activation was initiated by 4 μL of ADP (1 mM). Peak ATP release was measured and compared to the standard.

Electron microscopy (EM) was performed on the lungs following necropsy for the detection of microthrombi and fibrin deposition as previously described (Arnaud *et al.*, 2005; Johnson *et al.*, 2006). Briefly, lungs were fixed in 4F1G fixative (4% paraformaldehyde, 1% glutaraldehyde) overnight, post-fixed in 2% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812 (Electron Microscopy Sciences, Hatfield, PA, USA). Block sections (1 μm thickness) were examined by light microscopy, and thin (90 nm) sections were stained with lead citrate and uranyl acetate and examined with a LEO 912 AB electron microscope (Cambridge, UK).

Statistics

Results, data and figures are presented as means ± standard deviation unless otherwise stated. Animals were randomized at 10 min into the experiment via envelopes prepared by a statistician from outside. For multiple variables and for data collected over time, results were analysed by using the mixed statistical model for global inspection of continuous measurements (Proc Mixed, SAS, Cary, NC, USA). Significant group and/or time effects were indicated, and when appropriate, individual measures were subsequently compared using a two-tailed paired Student's *t*-test assuming equal variance. $P \leq 0{\cdot}05$ was considered significant. Surface under the curve tests were also performed when applicable.

RESULTS

Haemodynamics and survival results for these experiments have been extensively presented elsewhere (Gurney *et al.*, 2004). For clarity and context, they are briefly summarized below.

Twenty-four animals (23·0 ± 8·5 kg) were studied. Baseline MAP was comparable in all three groups (69·6 ± 12·2 mmHg) and came to a nadir at 15 min similarly in all groups (27·6 ± 12·0 mmHg) in response to liver crush/laceration-injury-induced haemorrhage. Upon resuscitation with HBOC, MAP was restored towards the baseline levels more rapidly than with HEX (e.g. at 40 min, MAP was 63·1 ± 28·3 mmHg vs. 40·0 ± 21·9 mmHg, respectively; $P > 0{\cdot}05$). In the NON group, only one of eight animals survived to 4 h, experiencing sustained hypotension throughout the treatment period (39 mmHg).

Preresuscitation blood loss (at 15 min) was 32·3 ± 12·7% EBV for all animals (there were no group differences). The initial rate of bleeding at 15 min was 2·15 ± 0·85% EBV min^{-1} in all animals. After 15 min, blood from the liver continued to bleed at a much lower rate. Blood loss between 15 and 30 min was also similar whether the animals received HBOC, HEX or nothing (50·1 ± 15·4% EBV). Postresuscitation blood loss at 4 h was 65·5 ± 18·5% EBV vs. 80·8 ± 14·4% EBV vs. 58·7 ± 10·1% EBV in the HBOC-201, HEX and NON groups (HBOC vs. HEX; $P < 0{\cdot}05$, *t*-test). Blood loss for surviving and nonsurviving animals is presented in Fig. 1(A and B), respectively. There was a higher blood loss in HBOC and HEX treated (combined) for survivors vs. nonsurvivors at 60 min (51·0% for survivors vs. 82·9% for nonsurvivors; $P < 0{\cdot}01$). Rebleeding was not observed after 60 min. It should be noted that part of the blood volume lost after 15 min in HBOC and HEX groups was a mixture of blood and resuscitation fluid.

Survival at 4 h was 87·5% (seven of eight) with HBOC, 37·5% (three of eight) with HEX and 12·5% (one of eight) in NON ($P = 0{\cdot}01$). Survival at 72 h was 87·5% (seven of eight) with HBOC, 12·5% (one of eight) in HEX and 12·5% (one of eight) in NON (HBOC vs. HEX; $P = 0{\cdot}01$). All deaths occurred 30–300 min posthaemorrhage. Changes in the corresponding survival rate for each treatment group are illustrated in a Kaplan–Meier plot in Fig. 2.

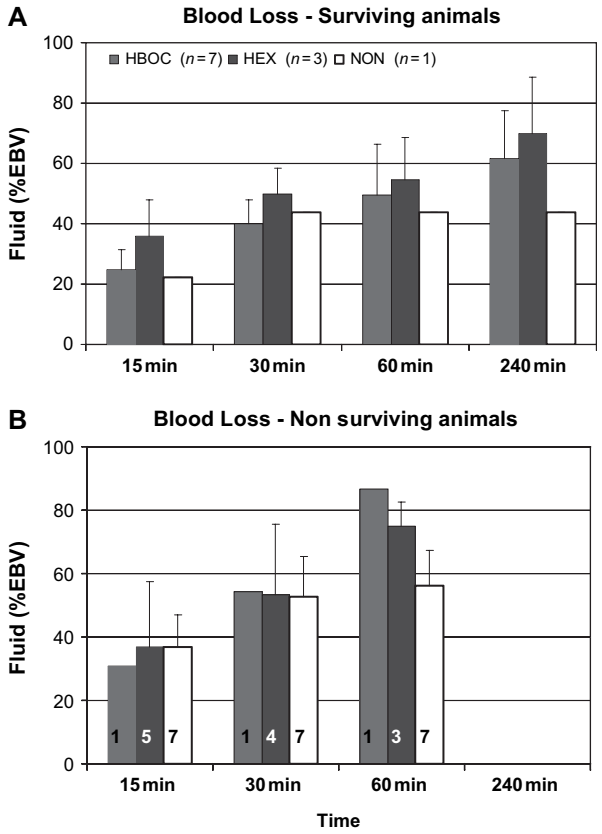

Fig. 1. Blood loss after uncontrolled haemorrhage following a grade III liver injury in surviving vs. nonsurviving animals receiving HBOC, HEX or no resuscitation fluids. Blood loss (%EBV) for the three treatment groups (grey bars, HBOC, black bars, HEX, white bars, NON) in (A) animals surviving (numbers in parentheses indicate animals that survived to 240 min) or (B) not surviving to the hospital phase at 4 h. Numbers on the bar graph indicate animals in the nonsurvivor group that survived to the indicated time. Blood loss was similar in HBOC and HEX animals throughout the experimental protocol ($P > 0{\cdot}05$).

Infusion requirements for surviving animals are illustrated in Fig. 3(A). Overall the volumes of resuscitation fluid infused were not significantly different in HBOC compared to HEX groups, but the number of infusions per survivor at 180 min was lower with HBOC than with HEX (2·5 and 2·9 infusions, respectively, at 10 mL kg^{-1}; $P < 0{\cdot}05$). During the simulated hospital phase, HEX animals required the maximum allowable number of blood transfusions at 4 and 24 h. In contrast, HBOC and NON animals did not require early red blood cell transfusion, resulting in overall reduced transfusion requirements (Fig. 3B). In contrast to HEX animals, all HBOC and NON animals required the maximum number of allowable saline infusions up to 72 h (Fig. 3C).

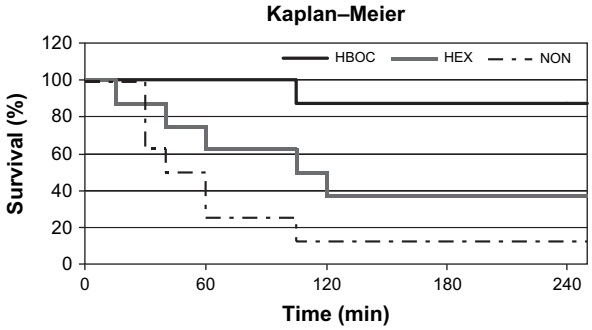

Fig. 2. Kaplan–Meier plot representing survival during the experimental course, for the three treatment groups (—, HBOC; —, HEX; ···, NON) in a severe liver haemorrhage.

In vivo BT

At 4-h post-trauma, BT was unchanged from baseline similarly in all groups (Fig. 4A). Therefore, in the context of similar overall haemorrhage volumes, HBOC resuscitation did not appear to exacerbate or diminish bleeding.

In vitro assay results

PFA-CT for HBOC and HEX groups (Fig. 4B) was significantly different from that for the NON animals (time and group differences; $P < 0{\cdot}01$). Adjustment for platelets did not change this pattern but eliminated the peak in HBOC group at 24 h (Fig. 4C). There was a trend to higher PFA-CT in HEX than in HBOC animals in the prehospital phase ($P = 0{\cdot}05$). By 48 h, PFA-CT was similar in all groups, returning to baseline by 72 h following blood transfusions.

Haematology. At 4 h, Hct (Fig. 5A) decreased similarly to a trough of approximately 13% in HEX and HBOC animals, compared to an increase of 7% in NON animals (group and time differences; $P < 0{\cdot}001$), indicating haemodilution in resuscitated animals of approximately two times EBV and haemoconcentration in NON animals. During the simulated hospital phase, NON animals experienced significantly decreased Hct ($P < 0{\cdot}01$; *t*-test) resulting from haemodilution by saline infusions. After 24 h, all groups had similar Hcts. Hb (Fig. 5B) paralleled the Hct in HEX and NON groups. Hct for HEX in the prehospital phase was different from that for the NON group (group and time differences; $P < 0{\cdot}01$). Hct remained at baseline until arrival at hospital in the HBOC group due to Hb delivery by HBOC. Platelet concentration decreased significantly and similarly in all groups during the simulated prehospital phase

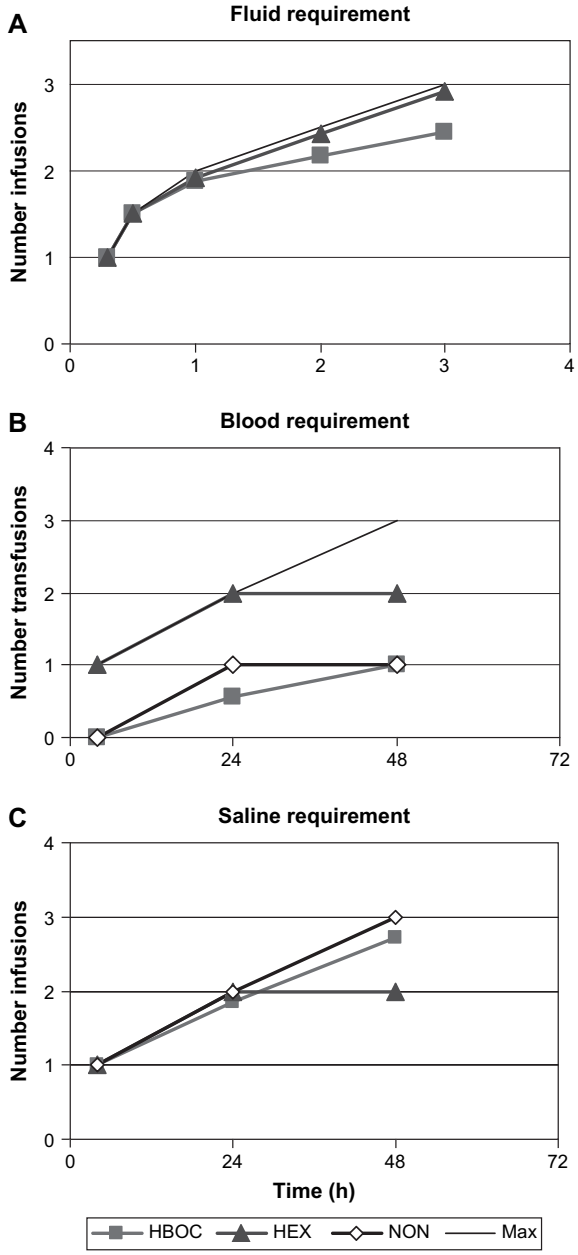

Fig. 3. Fluid requirement after uncontrolled haemorrhage. Infusion was computed as the number of resuscitation fluid infusions (10 mL kg^{-1}) per surviving animal during the prehospital phase, and blood transfusion or saline infusion (10 mL kg^{-1}) during the hospital phase. The number of infusions was computed as the total number of infusions during the given period of time divided by the number of animals that survived. This was then cumulated with time. (A) Test fluid infusion requirements during the prehospital phase; (B) RBC transfusion requirements; (C) saline infusion requirements in the hospital phase in the three treatment groups (■, HBOC; ▲, HEX; ◇, NON; —, maximal requirement). Transfusion requirements were consistently higher in HEX animals ($P < 0{\cdot}05$).

(time effect; $P < 0{\cdot}01$; Fig. 5C). This decrease in platelets remained evident when platelet concentration was normalized to Hct to reduce effects of haemodilution (Fig. 5D). Platelet numbers increased sharply after 48 h, due to blood transfusions and possibly thrombopoiesis in all groups. The WBC concentration (Fig. 5E) decreased at 30 min and increased thereafter up to 4 h. The increase was significant in the NON group (group difference; $P < 0{\cdot}01$). Normalized WBCs, corrected for haemodilution (not shown), confirmed this pattern in all groups. All three groups exhibited a similar increase in neutrophils towards 4 h, with a lower level in HBOC animals; an opposite pattern was seen with lymphocytes (Fig. 5F). Also, monocytes increased significantly in the NON group ($P < 0{\cdot}05$; data not shown).

Figure 5(G) indicates plasma concentration of Hb (HBOC) during the course of the study. On average, the animals received 4·9 HBOC infusions (5 mL kg^{-1} per infusion, HBOC concentration 12·5 g dL^{-1}) or 32·5 g HBOC kg^{-1}. The plasma Hb concentration peaked at 4 h and was $5{\cdot}5 \pm 0{\cdot}8$ g dL^{-1}. The half-life of HBOC in plasma was calculated to be approximately $23{\cdot}5 \pm 5{\cdot}3$ h, comparable to what was reported in a controlled HS model (Hughes *et al.*, 1995; Arnaud *et al.*, 2005).

PT (Fig. 6A) was mostly unchanged throughout the simulated prehospital phase in HBOC and NON animals. However, in HEX animals, PT departed from the other groups, with a peak at 4 h (time difference; $P < 0{\cdot}01$), indicating hypocoagulation. This appeared to resolve during the hospital phase, although the data represent only one surviving animal in HEX and NON groups. aPTT (Fig. 6B) in both HBOC and HEX groups was lower at 4 h, compared to the NON group (by 23%; group difference; $P < 0{\cdot}05$). During the hospital phase, aPTT increased similarly in all three groups (time effect; $P < 0{\cdot}01$). Fibrinogen levels decreased for HBOC and HEX in the prehospital phase (time effect; $P < 0{\cdot}05$) (data not shown). When normalized to Hct to account for haemodilution (Fig. 6C), fibrinogen levels were similar for all treatments during the course of the experiment; the sharp increase at 24 h suggested similar acute-phase reactions in all groups. AT-III levels, indicative of anticoagulant activity, were reduced at 15 min independently of haemodilution in HEX and NON animals (Fig. 6D). After 1 h, this level increased in NON but continued to decrease in HEX animals. After correction for Hct, AT-III/Hct was similar in NON and HEX groups (Fig. 6E). AT-III was similar in HEX and HBOC-201 animals at 48 and 72 h (when colour did not interfere with assay performance).

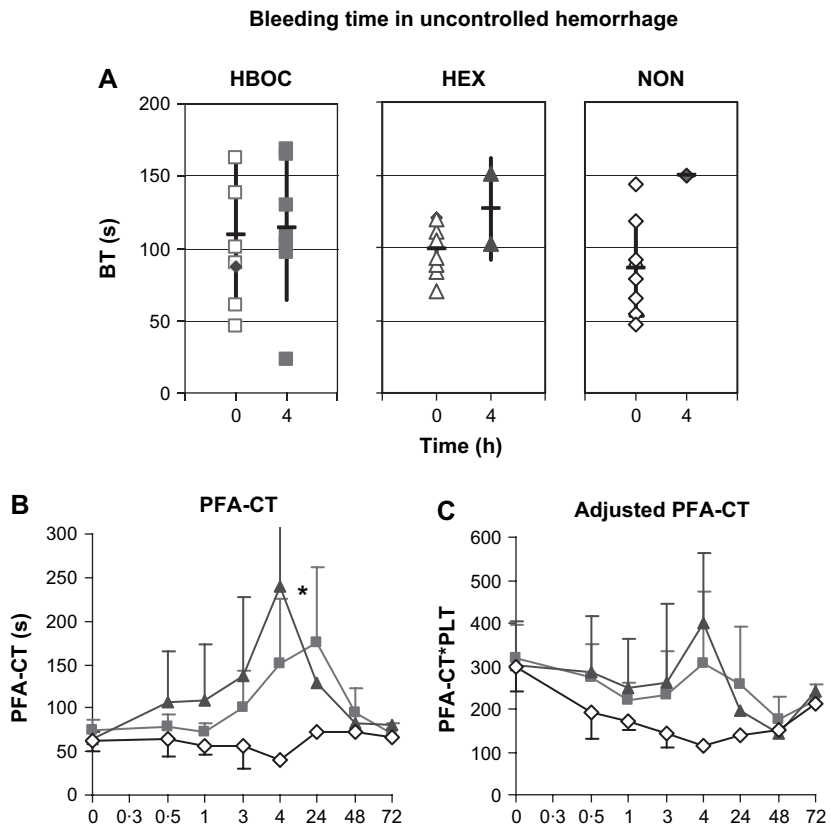

Fig. 4. *In vivo* BT in prehospital phase and *in vitro* BT measured by PFA-CT. (A) *In vivo* BT as measured by ear bleed in the three treatment groups (■, HBOC; ▲, HEX; ◇, NON) at baseline (time 0) and at 4 h at the end of the prehospital phase. (B) *In vitro* BT as measured by the platelet function analyser (PFA-CT), and (C) PFA-CT adjusted for platelets and calculated as PFA-CT × platelets during the time course of the experiment. *group and time difference ($P < 0{\cdot}01$) for HBOC and HEX. PH, prehospital phase.

TEG-R (Fig. 7A) remained unchanged during the prehospital phase for HEX and NON animals ($P > 0{\cdot}05$). A trend to increased TEG-R was seen in HBOC animals at 24 h compared to HEX animals ($P = 0{\cdot}06$). TEG-α (data not shown) expressed a mirror image of the TEG-R pattern, with a greater departure for HBOC animals compared with HEX and NON animals at 24 h (time difference; $P < 0{\cdot}01$). The TEG-MA decreased in HEX ($P < 0{\cdot}01$) and to a lesser extent in HBOC animals (group difference; $P < 0{\cdot}01$) compared to the NON animals, which remained unchanged. After normalization to platelet concentration (TEG-MA/PLT), all three time courses could be superimposed (Fig. 7C) without an apparent functional effect of resuscitation treatments. During the hospital phase, TEG-MA increased by 24 h and slowly returned to baseline similarly in all groups. The clotting index (TEG-CI) (Fig. 7B) was consistent with the other TEG parameters (TEG-R, TEG-MA). There was no significant difference for TEG-CI in NON animals throughout the 4-h prehospital period, whereas it decreased significantly for fluid-resuscitated animals; TEG-CI was lower at 3 and 4 h in HEX and HBOC, respectively, compared to baseline (time difference; $P < 0{\cdot}05$). The rate TEG-Ly remained near baseline during the prehospital phase and decreased at the onset of the hospital phase without significant differences (Fig. 7D). ATP release was tested only in the prehospital phase and was similar in all groups (data not shown).

Coagulation parameters are known to vary with temperature and pH. The temperature in the studied pigs was controlled at $36{\cdot}8 \pm 1{\cdot}5$ °C and blood pH was $7{\cdot}40 \pm 0{\cdot}04$ for the first 4 h.

Electron microscopy. EM examination of the lungs revealed marked alveolar oedema in NON animals. Small amounts of fibrin deposition were observed in all three groups. However, no platelet aggregates or microthrombi were found in any of the animals.

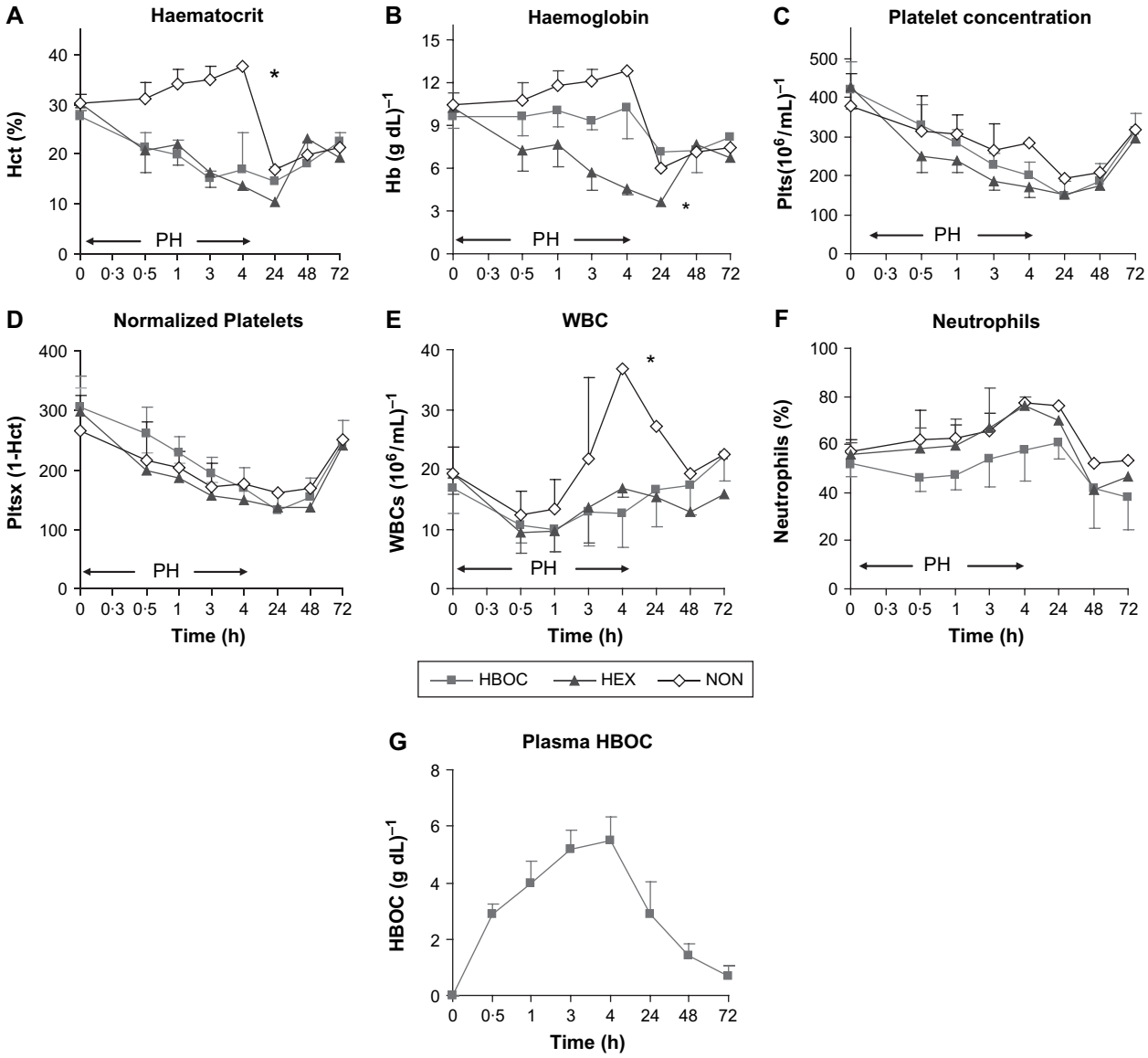

Fig. 5. Haematocrit, haemoglobin, platelets, WBC and plasma haemoglobin after uncontrolled haemorrhage following grade III liver injury in animals receiving HBOC, HEX or no resuscitation fluids. CBC in the three treatment groups (■, HBOC; ▲, HEX; ◇, NON) during the experimental period. (A) Haematocrit, *group and time difference ($P < 0{\cdot}01$) for NON; (B) haemoglobin, *group and time difference ($P < 0{\cdot}01$) for HEX; (C) platelet concentration, time difference ($P < 0{\cdot}01$); (D) adjusted platelet concentration (platelets × (100 − haematocrit); (E) WBC concentration,*group difference ($P < 0{\cdot}01$) for NON; (F) neutrophil %, and (G) plasma haemoglobin over time in HBOC animals during the course of the experiment. PH: prehospital phase.

DISCUSSION

The present study provides novel and relevant data regarding the effects of HBOC resuscitation on haemostasis and coagulation in a model of severe uncontrolled haemorrhage. Resuscitation with HBOC-201 improved survival without causing adverse effects on haemostasis. Although untreated animals experienced an anticipated posthaemorrhagic coagulation pattern with respect to PT, TEG-R and PFA-CT, allowing rapid control of bleeding, they also suffered a high rate of mortality, presumably due to insufficient haemodynamic compensation leading to acidosis and hyperkalaemia. HEX resuscitation allowed for rapid restoration of MAP to acceptable levels; however, PT and PFA-CT were negatively affected.

Furthermore, the apparent early physiological support provided by HEX did not result in improved

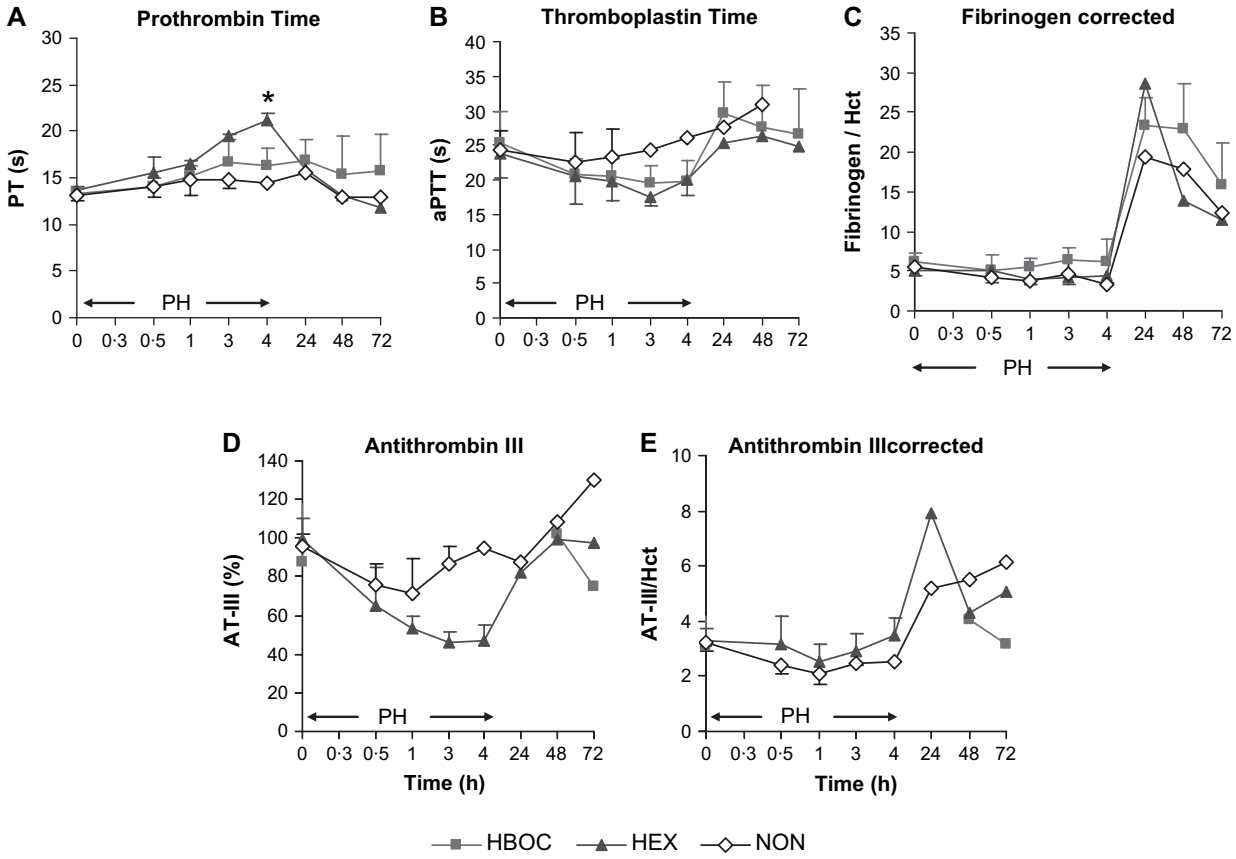

Fig. 6. Coagulation indices: PT, aPTT, fibrinogen and AT-III after uncontrolled haemorrhage following grade III liver injury in animals receiving HBOC, HEX or no resuscitation fluids. Fibrinogen and antithrombin were corrected for haematocrit. Coagulation for the three treatment groups (■, HBOC; ▲, HEX; ◇, NON) during the experimental period. (A) PT, *group difference ($P < 0{\cdot}01$) for HEX; (B) aPTT; (C) corrected fibrinogen (fibrinogen/Hct); (D) AT-III; and (E) corrected AT-III/Hct. PH: prehospital phase.

long-term survival. In contrast, HBOC resuscitation provided for rapid restoration of MAP and significantly improved survival with less impairment of coagulation than HEX, despite mild and reversible dilutional coagulopathy.

Overall, these findings are consistent with the results obtained by this laboratory in a previously reported 40% EBV-controlled-haemorrhage swine model (Arnaud *et al.*, 2005). In the present uncontrolled haemorrhage, the lower initial blood loss at 15 min (32% EBV) compared to the 40% controlled-haemorrhage model continues to increase to more than 60% at 4 h. Nonetheless, laboratory data and pattern were comparable in the two models. In the present uncontrolled-haemorrhage and the previous controlled-haemorrhage model, HBOC-treated animals experienced decreased blood transfusion requirements at 4, 24 and 48 h compared to HEX-treated animals. HBOC restored total Hb to above 9 g dL^{-1} with an infusion dose of 3·06 g HBOC kg^{-1} in the uncontrolled model compared to 2·35 g kg^{-1} in the 40% controlled-haemorrhage model, bringing plasma Hb levels to 6 and 5·5 g dL^{-1}, respectively. In each of the models, we observed a similar decrease in platelet concentration, regardless of fluid infusion or haemoconcentration. Alterations in Hct following haemorrhage and resuscitation also followed similar patterns in both models. Haemoconcentration observed in NON animals was likely due to autotransfusion via splenic contraction as the animals were not splenectomized (Hannon *et al.*, 1985). It is reasonable to assume that this effect occurred in all animals but was undetected in resuscitated animals because of resuscitation-related haemodilution. It is noteworthy that both HEX-treated and HBOC-treated animals experienced similar degrees of haemodilution in the present uncontrolled-haemorrhage model, in contrast to more marked haemodilution observed in HEX-treated animals in the 40% controlled-haemorrhage model. This is not surprising, as prehospital fluid infusion

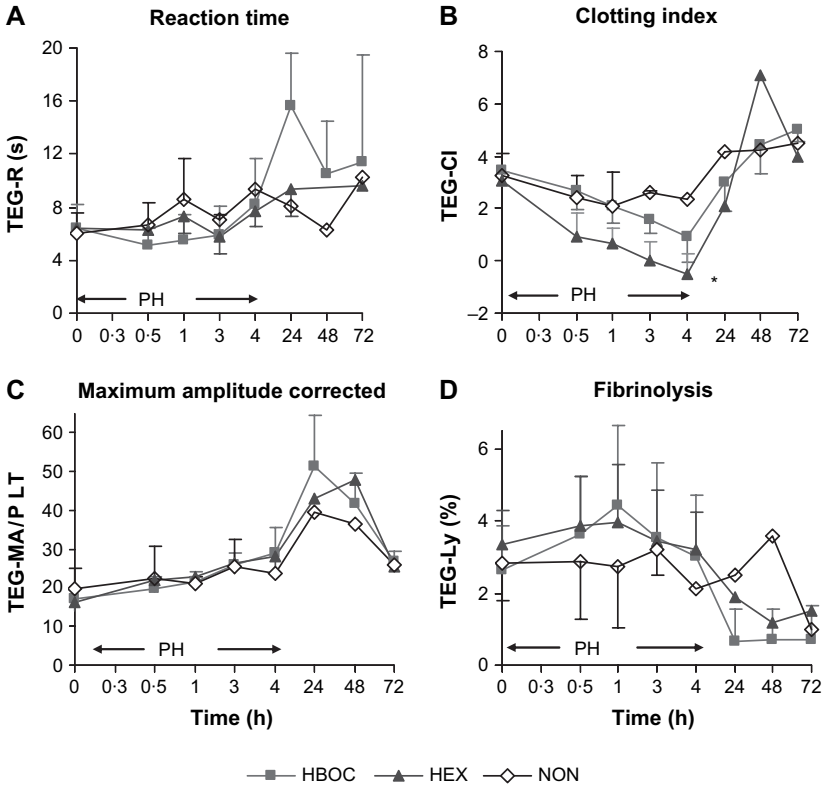

Fig. 7. Indices of TEG in animals receiving HBOC, HEX or no resuscitation fluids after uncontrolled haemorrhage following grade III liver injury. TEG for the three treatment groups (■, HBOC; ▲, HEX; ◇, NON) during the experimental period. (A) Reaction time (TEG-R); (B) clot strength (TEG-CI), * time and group difference ($P < 0{\cdot}05$) for HBOC and HEX; (C) maximum amplitude (TEG-MA) corrected for platelet concentration (TEG-MA/PLT); and (D) TEG-Ly representative of fibrinolysis. PH: prehospital phase.

volumes were similar in the present model, but relatively lower in HBOC animals in the controlled-haemorrhage model.

Unchanged *in vivo* BT indicated haemodilution although this is a highly subjective test and may be of limited clinical value. TEG time course profiles in the treatment groups were also similar in both the uncontrolled- and controlled-haemorrhage models, demonstrating similar acute-phase reactions at 24 h. Although HEX and HBOC treatment resulted in PFA-CT elevation, we observed a consistently lower PFA-CT during the simulated prehospital phase following HBOC resuscitation.

Furthermore, the PFA-CT peak in HEX-treated animals occurred at 4 h, as opposed to 24 h in HBOC-treated animals, presumably influenced by platelets as in both groups, resolution of PFA-CT coincided with blood transfusion and thus concurrent platelet and coagulation factor replacement. Although not statistically significant, this trend was consistent in both uncontrolled- and controlled-haemorrhage models. Regardless of treatment, PFA-CT returned to baseline by 48 h. This is suggestive of mild early hypocoagulation, which was reversed after transfusion during the hospital treatment phase. The pattern for coagulation indices was also similar for the uncontrolled- and controlled-haemorrhage models. PT was consistently higher in HEX-treated compared to HBOC-treated animals. This may relate directly to the nature of hydroxyethyl starch, known to impair haemostasis (Huttner *et al.*, 2000). aPTT was suppressed below baseline for the treated animals and remained near baseline for NON animals. Based on human clinical findings, one would have expected the opposite effect. However, this is consistent with reports by other investigators using swine models (Via *et al.*, 2001); furthermore, these results could be specific to porcine coagulation (Kostering *et al.*, 1983).

Platelet activation in trauma patients has been related to increased mortality (Boldt *et al.*, 1994). It has been suggested that HBOC might exacerbate this effect, as a result of NO scavenging, potentially leading to the stimulation of clot formation, elevated P-selectin expression and reduced closure time on PFA (Lee *et al.*,

2000; Lapointe & Von Rueden, 2002). The present findings contradict this hypothesis and are consistent with the results of Toussaint *et al.* (2003), who demonstrated that HBOC did not induce platelet activation *in vitro*. In our model, HBOC resuscitation did not activate platelets during the prehospital resuscitation phase, as measured by normalized PFA-CT, normalized TEG-MA or ATP-release after ADP stimulation. There was no demonstrable increase in thrombus formation as measured by either pulmonary EM or a reduction in BT. Furthermore, accumulation of platelets and thrombus formation was not observed histologically in the liver (Johnson *et al.*, 2006). The increase in PFA-CT during the prehospital phase in treated animals was likely reflective of a low platelet count due to haemodilution. However, one might argue that the use of anaesthesia during the prehospital phase could also have reduced platelet activity and may partly account for the observed alterations (Undar *et al.*, 2004). In fact, haemodilution alone cannot fully explain the observed reduction in platelet concentration. Platelet sequestration in the lung, liver or spleen, a reaction to haemorrhage, may also partially explain this observation as similar platelet decreases were seen in all groups (Hannon *et al.*, 1985; Blomquist *et al.*, 1989).

In this study, we introduced adjustment for assays strongly influenced by Hct (platelet concentration, AT-III) or platelets (PFA-CT, TEG-MA). Normalization of measured variables to platelet concentration or Hct allowed distinguishing pure haemodilution and functional abnormality.

Normal pressure resuscitation in uncontrolled haemorrhage may trigger the disruption of an immature clot, leading to rebleeding (Sondeen *et al.*, 2003). Thus, interventions that independently influence vascular tone may enhance this risk. HBOC solutions have been criticized as being vasoactive due to NO scavenging and thus could theoretically increase bleeding in models of uncontrolled haemorrhage. Interestingly, we found that HBOC animals experienced a consistently higher MAP than HEX animals (64 vs. 52 mmHg at 60 min, respectively) yet lower blood loss.

Circulating inflammatory mediators are integrally involved in the maintenance of normal haemostasis (Aird, 2005). It was reasonable to assume that if HBOC-201 has pro-inflammatory activity, it may exert an effect on haemostasis. In the present study, elevation of WBC at 4 h in all groups indicated an inflammatory response that was similar in both treatment groups and more profound in NON animals. This is consistent with the response seen in the previous controlled-haemorrhage model (Dong *et al.*, 2006). It is noteworthy that despite findings by McFaul *et al.* (2000) that free Hb can activate leukcocytes, HBOC-201 treatment did not result in excessive inflammatory stimulation.

Coagulation was restored to baseline during the hospital phase for most indices in all groups, likely related to blood transfusions. Since the requirement for blood transfusion during the hospital phase was based on Hb level, HEX animals received the earliest and highest number of transfusions and therefore early coagulation factor replacement. NON and HBOC-treated animals received early saline infusions but did not require blood transfusions until 24 h. Thus, coagulation factor replacement was delayed in these animals. Restoration of blood cellular mass has been reported to be an important factor in the restoration of haemostasis (Feffer, 1994). Although in the short term, HBOC-201 resuscitation may provide for adequate haemodynamic restoration and tissue oxygenation as a result of increased plasma Hb, clinical decisions should take into consideration the lack of cellular mass restoration, as well as the lack of platelet and coagulation factor replacement, during early resuscitation with HBOC. Decisions regarding blood transfusions and/or need for nonspecific factor replacement in patients receiving HBOC should also be based on Hct (which may be more reflective of unmeasured coagulation factor levels). Likewise, Hb and Hct in the nontreated patients can be misleadingly normal, and for these, blood transfusion on arrival at hospital would be better than saline, which would trigger haemodilution.

Normal liver function following trauma is critical to adequate haemostasis since the liver plays important roles in the control of coagulation by the production of most coagulation factors, control of fibrinolysis and clearance of activated clotting factors from the circulation. The acute-phase reaction observed in surviving animals, as indicated by fibrinogen elevation at 24 h and subsequent resolution towards baseline, suggests normal liver synthesis activity (Wada *et al.*, 2003). The present findings suggest that there are no significant negative effects of HBOC on liver function and coagulation activity following resuscitation.

Based on these haematologyl, TEG, coagulation and bleeding results, HBOC-201 appears to be a safe resuscitative fluid for use in traumatic HS after severe haemorrhage. It does not appear to exert harmful haemostatic effects, and furthermore, compared to standard colloidal resuscitation, produces similar haemodilution but better survival.

In conclusion, in this study of coagulation in a swine model of uncontrolled bleeding after liver injury, HBOC-201 resuscitation did not induce significant hypo- or hypercoagulation during early resuscitation.

The dilutional coagulopathy that was observed during the simulated hospital phase was reversed as a result of blood transfusion; however, this requirement was deferred compared to HEX treatment. HBOC-201 appeared to be a superior resuscitation fluid compared to HEX, as it led to significantly better survival, with only minimal delayed effects on coagulation due to diminished blood cellular components. In the event that HBOC-201 is used to treat HS patients, it may be advisable to consider both Hct and Hb for transfusion triggers to minimize the potential dilutional effect of HBOC-201 resuscitation on haemostasis. Nonetheless, further study is necessary to substantiate these findings in other model and human clinical trials.

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