The effect of tetrastarch on the endothelial glycocalyx layer in early hemorrhagic shock using fluorescence intravital microscopy: a mouse model

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Abstract

Background: This study aimed to clarify the effects of the optimal timing of infusion therapy during massive hemorrhage by measuring the glycocalyx (GCX) damage and its effect on vascular endothelial cell dysfunction.

Methods: In this randomized control animal study, we withdrew 1.5 mL of blood and administered 1.5 mL of infusion resuscitation. The mice were divided into the following six groups according to the type of infusion and administration timing: group NS-NS (Normal Saline (NS)→NS), group NS-Hydroxyethyl starch (HES) (NS→Hydroxyethyl starch 130/0.4/9 (HES130)), group HES-NS (HES130→NS), group NS-Albumin (ALB) (NS→albumin), group ALB-NS (albumin→NS), and group C (control, no withdrawal or infusion). We investigated the vascular endothelial dysfunction based on the GCX impairment, measured indirectly through the interstitial fluorescence intensity and syndecan-1 concentration.

Results: The GCX index (GCXI) of a 40 μ m artery was significantly larger in group C than in the other groups ($P<0.01$). Similarly, the GCXI for a 60 μ m artery was significantly larger compared with the NS-NS ($P\leq 0.05$), NS-HES ($P\leq 0.01$), and NS-ALB groups ($P\leq 0.05$). The plasma syndecan-1 concentration, at 7.70 ± 5.71 ng/mL, was significantly lower in group C than in group NS-NS ($P\leq 0.01$). The tetramethylrhodamine-labeled dextran (TMR-DEX40) fluorescence intensity in the ALB-NS and HES-NS groups, as well as the fluorescein isothiocyanate-labeled hydroxyethyl starch (FITC-HES130) fluorescence intensity in the NS-HES and HES-NS groups, were not significantly different from those of group C at any time point. FITC-HES130 was localized on the inner wall of the vessels in groups without HES130 infusion but uniformly distributed in groups treated with HES130.

Conclusions: In an acute massive hemorrhage mouse model, initial fluid resuscitation therapy with saline administration impaired GCX and increased the vascular permeability. Prior administration of HES130 prevented the progression of GCX damage. Early colloid resuscitation may improve endothelial cell function.

Background

Hemorrhage is responsible for >30% of trauma deaths and intraoperative cardiac arrest [1, 2]. Similarly, acute massive hemorrhage occurs in 30% of scheduled surgeries, where the anaesthesiologist's management is often deemed inappropriate [3]. Thus, appropriate infusion management for bleeding may become a question of life or death.

The type of initial infusion performed during massive bleeding may affect the patient's prognosis. Colloids, such as HES130 (hydroxyethyl starch 130/0.4/9; Voluven®) and albumin, are often administered when the blood pressure is unstable after crystalloid administration. Transfusion therapy is the first choice for patients with severe massive hemorrhage; however, transfusion products can rarely be administered immediately in clinical situations. A mixture of crystalloids and colloids is routinely used in cases of massive bleeding; however, there is insufficient evidence regarding which infusion product is the most effective.

Recently, Uzawa et al. bound a fluorescent dye to HES70 and identified its localization position in the endothelial surface layer (ESL) of the glycocalyx (GCX) [4]. The study visualized the direct protective effect of HES70 on GCX under severe conditions. However, they used HES130 for resuscitation and did not clearly demonstrate the direct effect of HES130 administration on GCX.

Circulating blood is composed of two components: a circulating and a non-circulating volume [5]. Pries [6] considered the non-circulating volume to be the immobile plasma layer. The combination of the immobile plasma layer and the GCX scaffold has been termed "the endothelial surface layer" [6]. The GCX generates a passive permeability barrier by

creating a scaffolding on which serum proteins are absorbed, forming a gel-like layer on the vascular wall [7]. GCX has garnered increasing attention due to its involvement in fluid management as it plays an important role in maintaining vascular wall integrity and preventing plasma leakage [8].

Depending on the severity of shock and the type of transfusion therapy, vascular dysfunction has been reported to progress after the loss of blood volume is recovered [9]. Endothelial dysfunction is a major cause of hemodynamic failure and secondary organ dysfunction; it is considered a target for successful resuscitation after shock [10, 11]. Therefore, minimizing GCX disruption and preserving microcirculation is necessary [12]. Hemorrhagic shock can impair endothelial function and induce hyperpermeability, leading to poor prognosis [13]. Shock-induced endotheliopathy occurs in the early stages of massive hemorrhage [13, 14]. Catecholamine-induced damage to the endothelium causes endothelial breakdown, resulting in GCX shedding and subsequent deterioration of the tight junctions causing capillary leakage [13]. Additionally, the increased expression of the disintegration markers in the GCX is associated with increased mortality in trauma patients [15]. Syndecan-1 and hyaluronic acid, which are important components of the GCX, are released into the bloodstream of patients with severe trauma due to increased permeability and low plasma colloid osmotic pressure [16, 17]. In patients with massive hemorrhage, fresh frozen plasma can be an effective treatment for suppressing GCX degradation [18, 19]; however, these transfusion products are not always readily available [9, 20].

HES130 can protect GCX [4, 8, 21, 22], prevent vascular hyper-permeability [8], and reduce the syndecan-1, heparinase, and hyaluronic acid levels [21]. In 2013, the U.S. Food and Drug Administration (FDA) and European Medicines Agency (EMA) recommended caution against administering HES130 to critically ill patients due to its potential side effects (renal dysfunction and coagulation disorders). However, recent randomized controlled trials have demonstrated no adverse effects of HES130 infusion on renal function in perioperative patients [23, 24]. It is unclear whether HES130 is effective as a fluid solution in acute hemorrhage, and its suitability for this use remains contentious [25].

We predicted that prior HES130 administration would attenuate GCX injury during the early stages of acute hemorrhage. HES130 localization in the inner vessel wall can preserve GCX thickness [4, 22]. Therefore, if there is a direct protective effect of HES solutions during massive hemorrhage, as reported by Uzawa [4], prior HES administration should be more effective than its later administration in maintaining the endothelial function and GCX layer.

The present study aimed to investigate the efficacy of prior administration of HES130 on GCX under severe hemorrhagic conditions using mouse dorsal skinfold chambers (DSC) by intravital microscopy [26] with fluorescent-labeled lectin to visualize the endothelial GCX [27] *in vivo*. The primary aim was to evaluate the GCX thickness, syndecan-1 concentrations, and syndecan-1 immunohistochemical localization on GCX. The secondary aim was to evaluate the vascular permeability using tetramethylrhodamine (TMR)-labeled dextran (molecular weight, 40 kDa; DEX40) and fluorescein isothiocyanate (FITC)-labeled HES130 (molecular weight, 130 kDa; HES130). Additionally, we performed blood gas analyses (BGA) and noted the cumulative 7-day mortality of the mice.

Methods

Materials

Albumin was purified from mouse serum (Kohsin Bio Ltd., Saitama, Japan) using the modified Cohn method [4]. Albumin was eluted from the column using 25 mM acetate buffer (pH 4.5) and neutralized to pH 7.2. Albumin was

concentrated in centrifugal filter units with a nominal molecular weight limit of 50 kDa (Amicon Ultra-15 Centrifugal Filter Units; Merck, Darmstadt, Germany). Albumin purification was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration was determined using the Bradford Ultra Total Protein Quantitation Kit (Expedeon Inc., San Diego, CA, USA) and adjusted to 5% (w/v) for administration. The albumin solution was stored at -30°C until further use.

HES powder (130/0.4/9; average molecular weight: 130 kDa; Fresenius Kabi, Germany) was used. HES labeled with FITC was prepared as previously described [4]. Low molecular weight (<3 kDa) HES fractions were removed using centrifugal filters (Amicon Ultra-15 Centrifugal Filter units, Merck).

Ethics

Ethical approval for this study (Ethical Committee for Animal Experiments, protocol number: 30-006) was obtained from the Ethical Committee of the National Institute of Public Health, Saitama, Japan (Chairperson of National Institute of Public Health; Kazuya Shinmura) on 5 June 2018.

Animal preparation

Male BALB/c mice were purchased from Japan SLC Inc. (Shizuoka, Japan). The mice were fed a standard pellet diet (FR-2; Funabashi Farm Co., Chiba, Japan), and their water was acidified with hydrochloric acid ad libitum. The mice were kept in a Super Mouse 1400TM Micro-Isolator Rack (Lab Products, Inc., Seaford, DE, USA) with an artificial 12-h light cycle. All animal experiments were performed and reported in accordance with the Animal Research Reporting of In Vivo Experiments (ARRIVE) guidelines. A dorsal skinfold chamber (DSC) [26] was used to observe microcirculation in the living environment. When the mice were approximately 15 weeks old and weighed 25 g, DSCs were surgically implanted by sandwiching a double layer of the dorsal skin and then removing one side to observe the microcirculation (Supplemental Fig. 1) [26]. During surgery, mice were anaesthetized using a mixture of ketamine (90 mg/kg) and xylazine (10 mg/kg). They were acclimatized for one week before the experimental procedure. Detailed data regarding the mice are shown in Supplemental Table 1.

Experimental protocol

An infusion-resuscitation model was constructed after massive bleeding. After anaesthesia administration, a catheter was inserted through the internal jugular vein and 1.5 ml of blood was withdrawn twice (70% of the total blood volume). The first dose was administered after a 0.75 ml blood withdrawal, and the second dose was administered after the second withdrawal. Mice were divided into six groups based on the type of first and second doses administered: group NS-NS (Saline (NS)→NS), NS-HES (NS→HES130), HES-NS (HES130→NS), NS-ALB (NS→albumin), and ALB-NS (albumin→NS), and group C (no withdrawal and no infusion, only anaesthesia) (Fig. 1).

GCX thickness and evaluation of vascular permeability by fluorescent dyes

After treatment, both TMR-DEX40 and FITC-HES130 were injected into the mice via a catheter to evaluate the vascular permeability. To estimate the GCX thickness, we used FITC-labeled wheat germ agglutinin (FITC-WGA) (WGA; Sigma

Aldrich). After 30 min, we randomly selected up to three arteries with inner vessel diameter of 40 ± 10 or 60 ± 10 μm from each chamber, with the walls clearly illuminated by FITC-WGA (Fig. 2). Whenever possible, overlapping and curved arteries were not included in our analysis to prevent potential staining enhancement. We drew three perpendicular lines forming four image blocks over a selected arterial wall, and their fluorescence intensities were measured using ImageJ software (Fig. 3-A). The GCX index was measured as previously reported [4,27,28]. The average of these values was defined as GCXI. Interstitial fluorescence intensity and GCXI were compared among all groups. Vascular permeability was evaluated using the average fluorescence intensities of TMR-DEX40 and FITC-HES130 in the interstitial tissue. We randomly selected three 80×80 -pixel square areas (Fig. 3-B) without any blood vessels to measure the vascular permeability over time (after 15, 30, 60, and 90 min).

Measurement of syndecan-1 blood concentration and immunostaining

Blood was collected 60 min after treatment, and the plasma concentration of syndecan-1 was measured using the sCD138 ELISA Kit (Diacclone SAS, Besançon, France). The absorbance of the final samples was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Immunohistochemical staining for CD138 in cutaneous tissue

CD138 was identified immunohistochemically in paraffin-embedded skin sections using a purified anti-mouse CD138 (Syndecan-1) antibody (BioLegend, Inc., San Diego, CA, USA). The dorsal skin of the mice (20×20 cm) was surgically excised, fixed in 4% paraformaldehyde phosphate buffer solution, and then embedded in paraffin blocks. The blocks were sliced into $4\text{-}\mu\text{m}$ thick slices on a microtome and mounted on glass slides. Following deparaffinization, antigen retrieval and blocking of endogenous peroxidase activity were performed using an antigen retrieval reagent (R&D Systems Inc., Minneapolis, MN, USA) and BLOXALL (Vector Laboratories, Burlingame, CA), respectively. Immunostaining with anti-mouse CD138 antibody was performed using an HRP anti-rat IgG polymer detection kit (ImmPRESS-HRP, Vector Laboratories) and a DAB EqV peroxidase substrate kit (ImmPACT, Vector Laboratories). All sections were counterstained with hematoxylin staining solution (Sakura Finetek Japan Co., Ltd., Tokyo, Japan) and mounted with coverslips.

Measurement of blood gas and 7-day cumulative survival rates

BGA were performed on all mice at 60 min after treatment using the i-STAT 300F Analyser EC4+, CG4+ (Abbott, Princeton, NJ, USA). The central catheter was removed after treatment without the administration of the fluorescent dye. The wound on the right internal jugular vein was sutured, and hemostasis was confirmed. The mice were then observed routinely for seven days. The seven-day cumulative mortality rate was recorded.

Data analysis

Statistical tests were performed using the JMP Statistical Analysis Software (SAS) and PRISM for Mac OS (version 8.4.3). Group C was used as the reference group for each observation. All data were analyzed by one-way analysis of variance (ANOVA) followed by Dunne's post-hoc test except for the 7-day cumulative mortality rate. The 7-day

cumulative mortality rate was analyzed using the Kaplan–Meier method followed by the log-rank test; values with $P \leq 0.05$ were considered statistically significant.

Results

The average GCXI in the 40 μm arteries in group C was significantly thicker than that in all other groups ($P < 0.01$) (Fig. 4–A, Table 1). The average GCXI in the 60 μm arteries in group C was significantly thicker than that in the groups NS-HES ($P \leq 0.01$), NS-NS ($P \leq 0.05$), and NS-ALB ($P \leq 0.05$). The GCXI of the remaining groups was not significantly thicker than that of group C (Fig. 4-B, Table 1).

The average concentration of syndecan-1 in group C was significantly lower than that in group NS-NS (7.70 ± 5.71 ng/ml; $P \leq 0.01$). The average concentrations in groups NS-Alb, ALB-NS, NS-HES, and HES-NS were not significantly different from that of those in group C (Fig. 5, Table 1). In the syndecan-1 immunostaining images, the NS-NS group showed little staining of the inner vessel wall when compared with the other groups, with a diameter of approximately 40 μm (Fig. 6).

The TMR-DEX40 fluorescence intensity in the interstitial space was significantly increased in group ALB-NS when compared with group C (after 30, 60, and 90 min, $P \leq 0.01$), group NS-NS (after 60 and 90 min, $P \leq 0.05$), and group NS-HES (after 60 and 90 min, $P \leq 0.05$). There were no significant differences compared with group C at any time point. The TMR-DEX40 fluorescence intensity in the interstitial space was significantly higher in the NS-NS (after 60 and 90 min), ALB-NS (after 30, 60, and 90 min), and NS-HES (after 60 and 90 min) groups than in group C. There were no significant differences in the NS-ALB and HES-NS groups at any time point when compared to group C (Fig. 7). The FITC-HES130 fluorescence intensity in the interstitial space was significantly higher in the NS-NS (after 60 and 90 min), NS-ALB (after 30, 60, and 90 min), and ALB-NS (after 30, 60, and 90 min) groups than in group C. There were no significant differences in the NS-HES and HES-NS groups at any time point when compared with group C (Fig. 8). At 60 min, TMR-DEX40 uniformly stained the intravascular wall in all groups, and FITC-HES130 was localized on the inner vessel walls in the groups without HES130 infusion. Simultaneously, FITC-HES130 showed uniform staining in the blood vessels in the groups with HES130 infusion (Fig. 9) (Supplemental Movie A, B,C, D).

In the BGA (Table 2), the pH was significantly lower in all groups than in group C. In contrast, there were no significant differences in PaCO_2 and PaO_2 between group C and the other groups. The lactate level showed no significant difference in group NS-HES (1.8 ± 1.71 mmol/L) and group HES-NS (1.2 ± 0.56 mmol/L) but was significantly higher in group NS-NS (4.2 ± 0.99 mmol/L, $P \leq 0.01$), group NS-ALB (2.9 ± 1.34 mmol/L, $P \leq 0.01$), and group ALB-NS (3.9 ± 1.37 mmol/L, $P \leq 0.01$) when compared with group C (0.8 ± 0.51 mmol/L). Hemoglobin levels were lower in all other groups than in group C ($P \leq 0.01$). The 7-day cumulative mortality was significantly lower than that of group C (100%) in the NS-NS (20%, $P \leq 0.01$) and NS-HES groups (60%, $P = 0.0289$). There were no significant differences in the remaining groups when compared with group C (Supplemental Fig. 2).

Table 1
Average of FITC-WGA positive layer thickness (glycocalyx index) and syndecan-1 concentration

Group	Group C (N)	Group NS-NS (N)	Group NS-ALB (N)	Group ALB-NS (N)	Group NS-HES (N)	Group HES-NS (N)
40 μ m (Vessel diameter)	5.69 $\pm$ 1.99 μ m (7)	3.69 $\pm$ 0.91 μ m** (7)	3.31 $\pm$ 1.04 μ m** (8)	3.40 $\pm$ 1.15 μ m** (8)	2.96 $\pm$ 0.53 μ m** (8)	3.28 $\pm$ 0.66 μ m** (6)
60 μ m (Vessel diameter)	4.93 $\pm$ 1.45 μ m (10)	3.76 $\pm$ 0.80 μ m* (9)	3.77 $\pm$ 0.80 μ m* (8)	4.04 $\pm$ 1.27 μ m (7)	3.28 $\pm$ 1.07 μ m** (10)	4.82 $\pm$ 0.88 μ m (8)
Syndecan-1 ng/ml	2.54 $\pm$ 0.69 (9)	7.70 $\pm$ 5.71** (7)	3.00 $\pm$ 0.92 (5)	5.99 $\pm$ 3.04 (6)	2.11 $\pm$ 0.44 (6)	2.76 $\pm$ 1.33 (8)
Groups NS-NS (normal saline (NS) $\rightarrow$ NS), NS-HES (NS $\rightarrow$ HES130), HES-NS (HES130 $\rightarrow$ NS), NS-ALB (NS $\rightarrow$ albumin), and ALB-NS (albumin $\rightarrow$ NS), and group C (control, no withdrawal and no infusion). Data are shown as the mean $\pm$ SD. *P<0.05 and ** P $\leq$ 0.01 compared with group C						

Table 2
Blood gas analysis parameters

Group Number	Group C (N)	Group NS-NS (N)	Group NS-ALB (N)	Group ALB-NS (N)	Group NS-HES (N)	Group HES-NS (N)
pH	7.172±0.058 (10)	6.980±0.070** (8)	7.068±0.052** (8)	7.037±0.069** (7)	7.088±0.051* (8)	7.077±0.055** (8)
pCO ₂ (mmHg)	83.0±11.73 (10)	85.3±9.85 (8)	82.7±7.19 (8)	86.0±12.10 (7)	85.0±9.96 (8)	87.9±8.41 (8)
pO ₂ (mmHg)	46.1±18.23 (10)	42.3±9.04 (8)	37.6±5.78 (8)	35.7±7.70 (7)	43.0±12.47 (8)	47.8±11.45 (8)
BE (mEq/L)	1.8±1.48 (10)	-11.5±4.21** (8)	-6.1±3.44** (8)	-7.4±4.04** (7)	-4.4±1.77** (8)	-4.5±2.00** (8)
Lactate (mmol/L)	0.8±0.51 (10)	4.2±0.99** (8)	2.9±1.34** (8)	3.9±1.37** (6)	1.8±1.71 (8)	1.2±0.56 (8)
Hb (g/dL)	13.7±1.06 (10)	6.5±1.36** (8)	4.3±1.45** (8)	7.3±0.98** (6)	5.6±1.18** (8)	6.7±0.56** (8)
BE base excess, Hb haemoglobin group NS-NS (normal saline [NS] →NS), NS-HES (NS→HES130), HES-NS (HES130→NS), NS-ALB (NS→albumin), and ALB-NS (albumin→NS), and group C (control, no withdrawal, and no infusion). Data are shown as the mean±SD. *P<0.05 and ** P≤0.01 compared with group C						

Discussion

This study aimed to clarify the relationship between HES130 expression and endothelial function in vivo. Additionally, we investigated the effect of different infusion resuscitations with prior administration of HES130 on endothelial dysfunction. Plasma syndecan-1 is a marker for GCX shedding that strongly correlates with reduced GCX thickness and increased microvascular permeability when various fluids are used for resuscitation after hemorrhage [10]. Intravital microscopy has shown that endothelial GCX thickness is reduced after hemorrhage in the skeletal muscle and mesenteric venules [14]. The degradation of endothelial GCX is pertinent to increased GCX fragmentation and fluid resuscitation vascular permeability [9].

In this study, prior administration of HES130 into the 40 µm arteries resulted in a significantly lower GCXI among all groups. However, the GCXI of 60 µm arteries after prior administration of HES130 and albumin was not significantly different when compared with group C (Fig. 4-A, -B). The prior administration of HES130, unlike that of albumin, did not result in significantly higher fluorescence intensity of TMR-DEX40 and FITC-HES130 in the interstitial tissue at 30, 60, and 90 min (Fig. 7, Fig. 8). Prior administration of HES130 suppressed vascular hyperpermeability and was associated with significantly lower lactate levels (Table 2) and no decrease in the 7-day survival period (Supplemental

Fig. 2). This suggests that prior administration of HES130 during the early stage of massive hemorrhage may inhibit GCX disruption and reduce endothelial dysfunction.

GCX exists as an ESL with a water-rich gel layer on the superior surface of the inner vessel wall in vivo, and its volume can exceed 1000 mL in humans [6]. Therefore, when observing GCX in a biological environment, it is necessary to include this water-rich gel form. Ebong et al. reported that the thickness of the GCX ranged from 3.6–12.5 μm , measured from flash-frozen specimens without dehydration [29]. The thickness of GCX observed in vivo depends on the vessel diameter and pathologic environmental factors, such as sepsis, obesity, and hypertension [30]. Uzawa also reported that the in vivo GCX thickness of 20 μm thick arteries was approximately 4 μm [4]. The GCXI in this study was 4–6 μm (Fig. 4-A, -B). Therefore, the GCXI of small arteries (40–60 μm thick) observed in this study is likely to represent the actual in vivo GCX thickness.

GCX has various functions, such as the regulation of coagulation and vascular permeability, transduction of chemical signals, transportation of biochemical substances in and out of cells, and the maintenance of electrochemical barriers [31, 32]. Various factors, such as barrier size, barrier charge and concentration gradient, are intricately related to vascular permeability, and it was reported as revised Stirling's law because it considers the structure of GCX [33]. We focused on the barrier size [34], including the "large pores and small pores" system. We used TMR-DEX40 (40 kDa), which is slightly larger than small pores but smaller in molecular weight than large pores, as a tracer of vascular permeability during endothelial dysfunction. Since HES130, which is gradually degraded by amylase [35], may be inappropriate as a tracer for vascular permeability, we prepared FITC-HES130 labeled with different fluorescent dyes from TMR and administered both simultaneously to investigate the behavior of HES130 during massive hemorrhage and captured these changes over time.

The fluorescence intensity of TMR-DEX40 in the interstitial tissue among the NS-ALB and HES-NS groups was not significantly different after 60 and 90 min compared to that in group C (Fig. 7). The fluorescence intensities of FITC-HES130 in the NS-HES and HES-NS groups in the interstitial tissue were not significantly different at any time point when compared with that of group C (Fig. 8). HES130 administration had a strong effect on maintaining FITC-HES130 in the vessel lumen. Unlike DEX40, which is difficult to degrade in plasma, HES130 is gradually degraded. The HES-NS group had a lower interstitial fluorescence intensity with the two tracers, suggesting that the group HES130-NS may have maintained even smaller molecules, such as the degraded material of HES130, that pass through the small pore system in the vessel lumen. In a report by Uzawa [4], the interstitial distribution of FITC-HES70 was suppressed by HES130 administration, and the same tendency was observed in our study. Furthermore, this effect may have been enhanced by the prior administration of HES130. It has also been reported that FITC-HES70 is localized on the inner wall of the vessel [4]. In this study, FITC-HES130 localization on the vessel inner wall was observed in images acquired 30 min after HES130 administration (Fig. 9, Supplemental Movie A, B, C, D). However, in the group with HES130, no such localization was observed, and it was uniformly present in the vessel lumen (Fig. 9). We speculated that this was probably due to HES130 already being attached to the vessel wall due to HES pre-administration and that FITC-HES130 was subsequently unable to attach to the vessel wall. The localization of HES130 on the vessel wall may have a direct protective effect on the GCX or a sealing action on the vessel endothelial surface, which may also be considered as physical protection against an impaired GCX. This study may be insufficient to accurately clarify the effect of HES130 on the small pore system and its protective effect on GCX.

In terms of the GCXI, the plasma concentration of syndecan-1, and the cumulative 7-day mortality rate, group NS-NS showed worse results than that of group C in all parameters. The syndecan-1 immunostaining images showed little staining of the inner vessel wall in the NS-NS group. Similar to previous reports [36], resuscitation with only saline

during massive hemorrhage is likely to induce GCX damage and worsen the prognosis. However, the administration of colloidal solutions would be beneficial.

Numerous reports on the effects of HES130 in patients with massive hemorrhage [4, 37–39] showed that its administration shortened the recovery time for shock [40], improved the peripheral circulation and vascular permeability, and reduced the concentration of GCX degradation products (syndecan-1, heparinase, hyaluronic acid) [7]. In a clinical trial of HES130 administration in trauma patients, improvements in the base excess and lactate levels have also been reported [41]. Prior HES130 administration may help improve the endothelial function by inhibiting vascular hyperpermeability, improving the prognosis, and reducing the risk of circulatory failure as it localizes on the GCX.

Our study has three limitations. First, we did not compare the efficacy of HES and albumin. The results of our study provide only one therapeutic strategy: prior administration of HES130 for initial fluid resuscitation in acute severe hemorrhage to prevent hyperpermeability due to endothelial dysfunction. Since albumin exists as a component of ESL [31], the fluorescent labeling of albumin is necessary to elucidate its mechanism of action and compare its relationship with HES130. Second, the side effects of HES, such as renal dysfunction, coagulation disorders, and allergies, were not investigated. We were unable to measure blood pressure; therefore, we do not know the actual duration or severity of shock in our study. Lastly, it is possible that the infusion volume in the NS-NS group was lower than that in the other groups regarding the volume expansion effect. However, considering the context-sensitive volume effect [42] and crystalloid fluid volume of large clinical studies [43, 44], which is approximately 1.2 times that of colloid fluid, the fluid volume in the NS-NS group was not extremely small. Therefore, the findings of this study may not be applicable to clinical practice.

Conclusion

Saline administration as an initial fluid resuscitation method caused GCX impairment, increased vascular permeability, and worsened the prognosis in a mouse model of acute massive hemorrhage. However, prior administration of HES130 prevented the progression of GCX damage and increased vascular permeability. Thus, prior administration of colloids at the early stage of massive hemorrhage may protect endothelial function and improve the prognosis.

Abbreviations

NS: Normal Saline

ALB: Albumin

HES: Hydroxyethyl Starch

HES130: Hydroxyethyl Starch 130/0.4/9

GCX: Glycocalyx

GCXI: Glycocalyx Index

FITC-HES130: Fluorescein Isothiocyanate-Labeled Hydroxyethyl Starch

TMR-DEX40: Tetramethylrhodamine-Labeled Dextran

FITC-WGA: Fluorescein Isothiocyanate-Labeled Wheat Germ Agglutinin

FDA: Food And Drug Administration

EMA: European Medicines Agency

BGA: Blood Gas Analyses

SAS: Statistical Analysis Software

ANOVA: One-Way Analysis Of Variance

Declarations

Ethics approval and consent to participate

All animal experiments were performed in accordance with the Animal Research: Reporting of In Vivo Experiments guidelines. Ethical approval for this study (Ethical Committee for Animal Experiments, protocol number: 30–006) was obtained from the Ethical Committee of the National Institute of Public Health, Saitama, Japan (Chairperson of National Institute of Public Health; Kazuya Shinmura) on 5 June 2018.

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article and its supplementary information files.

Competing interests

The authors declare that they have no competing interests. Corresponding Author, Kohji Uzawa received a powder of Voluven® (hydroxyethylated starch: 130/0.4/6) from Fresenius Kabi (Homburg, Germany) without charge. Kyorin University Hospital has received funding for research from Otsuka Pharmaceutical Factory Inc. (Tokushima, Japan) which sells Voluven® in Japan.

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Authors' contributions

Tadao Ando and Kohji Uzawa conceived and designed this study, secured competitive funding, and wrote the manuscript. Tadao Ando, Kohji Uzawa, and Takahiro Yoshikawa conducted all animal studies in the direction of Akira Ushiyama. Tomoko Yorozu helped to acquire both Tadao Ando's grant, a Grant-in-Aid for Young Scientists (B) (Grant Number JP18K16496) and Kohji Uzawa's grant; Grant-in-Aid for Scientific Research (C) (Grant Number JP16K10973). Tadao Ando and Kohji Uzawa contributed to data analysis and main manuscript writing. Tadao Ando, Takahiro Yoshikawa, and Mituda Shingo performed the experiments and created the figures and tables. Tomoko Yorozu proposed a research draft and developed an experimental plan for the entire study. Akira Ushiyama performed the experiments, data analysis, and image processing and analysis.

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Presentation:

Preliminary data were reported as a Research Snapshot Presentation at the 45th Critical Care Congress in Orlando, Florida, United States of America, 20-24 February 2016 and as an oral presentation at the American Society of Anaesthesiologists Annual Meeting 2016 in Chicago, United States of America, 22-26 October 2016.

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Figures

Figure 1

Experimental protocol

A dorsal skinfold chamber (DSC) is surgically implanted into approximately 15-week-old male BALB/c mice under anaesthesia. At least 1 week after the DSC operation, the mice are anaesthetized again, and the acute hemorrhage experiment is performed as follows: 0.75 mL blood is withdrawn via the jugular vein at a rate of 0.1 mL per 3 s, after 5 min, an equal amount of the allocated fluid in each group is administered. This procedure is then repeated once for a total blood loss of 1.5 mL, with 1.5 mL fluid resuscitation. The mice that underwent blood withdrawal and resuscitation are divided into six groups according to the type of infusion and the timing of administration as shown in the flow chart. After 5 min, the mice are injected with tetramethylrhodamine-labeled dextran (TMR-DEX40) and fluorescein isothiocyanate-labeled hydroxyethyl starch (FITC-HES130), and their fluorescence intensities are measured at regular intervals from 15–90 min (Experiment A). Fluorescein isothiocyanate-labeled wheat germ agglutinin (FITC-WGA) is administered to separate groups of mice treated using the same protocol to calculate the glycocalyx index (GCXI) 30 min after administration (Experiment B). The same treatment is performed on all mice. Thirty minutes after treatment, the blood concentration of syndecan-1 is measured (Experiment C), and a blood gas analysis is performed (Experiment D). The prognosis of the mice is observed for seven days (Experiment E).

NS, normal saline; ALB, albumin; HES130, hydroxyethyl starch 130 (Voluven®)

Figure 2

Glycocalyx illumination by FITC-WGA (wheat germ agglutinin)

After withdrawal and fluid resuscitation, FITC-WGA is administered through the internal jugular vein and observed 30 min later using an intravital microscope. The inner vessel walls are illuminated as white. Four image blocks are drawn perpendicular to the arterial vessel wall with diameters of approximately 40 and 60 µm. **A:** group C, **B:** group NS-NS, **C:** group NS-ALB, **D:** ALB-NS, **E:** group NS-HES130, **F:** group HES130-NS.

Figure 3

Method for measurement of the glycocalyx index (GCXI) and the fluorescence intensity of interstitial tissue

A: Method used to investigate GCXI. To identify curved or overlapping arteries, we drew three perpendicular lines every 50 pixels against one side of the GCX-illuminated vessel wall. The fluorescence intensity on each line is measured, and the pictograms are constructed. The maximum fluorescence intensity is defined as (a), the left and right inflection points are calculated, and the midpoint of these two points is the baseline (c). The distance between two intersections of a straight line (b) and a curve in the upper 40% of (a)–(c) is defined as the GCXI (d). The average of the three GCXIs obtained from a single artery is defined as the GCXI of the individual values. **B:** Quantification of the interstitial fluorescence intensity. After withdrawal and resuscitation treatment, TMR-DEX40 (tetramethylrhodamine-labeled dextran, 40 kDa) and FITC-HES130 (fluorescein isothiocyanate-labeled hydroxyethyl starch 130 kDa) are administered. TMR-DEX40 and FITC-HES130 are stained red and green, respectively. We chose areas without surrounding blood vessels and randomly selected three 80 $\times$ 80 pixels square areas (boxes 1, 2, and 3). We then measured the fluorescence intensity of TMR-DEX40 and FITC-HES130. The average of the three fluorescence intensity

data is defined as the fluorescence intensity at that time, and the changes in fluorescence intensity in the interstitial tissue after 15, 30, 60, and 90 min are observed over time.

Figure 4

Comparison of the glycocalyx index (GCXI) of each group

The fluorescence intensity of the FITC-WGA-positive layer is defined as the GCXI to determine changes in the thickness of the GCX compared with control group C. Fig. 4-A shows the GCXI of an artery with a diameter of approximately 40 μm ; Fig. 4-B, shows the GCXI of an artery with a diameter of approximately 60 μm . Values are expressed as the mean $\pm$ SD. The data are analysed by one-way analysis of variance (ANOVA) followed by Dunne's post hoc test. * $P<0.05$, compared to group C.

Figure 5

Blood concentration of syndecan-1

Values are expressed as the mean $\pm$ SD. The data are analysed by one-way analysis of variance (ANOVA) followed by Dunne's post hoc test. * $P < 0.05$ and ** $P \leq 0.01$ compared with C.

Figure 6

Antibody immunostaining images for syndecan-1

Images of antibody immunostaining for syndecan-1 in all groups. In all images except group NS-NS (**B**), the vessel wall exhibited a strong black stain (black arrows). **A**: Group C (syndecan-1 concentration: 2.580 ng/ml). **B**: NS-NS group (syndecan-1 concentration: 6.301 ng/ml). **C**: NS-HES130 group (syndecan-1 concentration: 1.376 ng/ml). **D**: HES-NS group (syndecan-1 concentration: 0.773 ng/ml). **E**: NS-ALB group (syndecan-1 concentration: 3.042 ng/ml). **F**: ALB-NS group (syndecan-1 concentration: 0.453 ng/ml).

Figure 7

Fluorescence intensity of TMR-DEX40

Changes in fluorescence intensity of TMR-DEX40 (tetramethylrhodamine-labeled dextran: mean molecular weight 40 kDa) in the interstitial tissue are measured over time at 15, 30, 60, and 90 min and compared with those of group C (n=7 for each group). Values are expressed as the mean±SD. The data are analysed by one-way analysis of variance (ANOVA) followed by Dunne's post hoc test. *P<0.05 and **P≤0.01 compared with C.

Fluorescence intensity

Figure 8

Fluorescence intensity of FITC-HES130

Changes in fluorescence intensity of FITC-HES130 (fluorescein isothiocyanate-labeled hydroxyethyl starch: mean molecular weight 130 kDa) in the interstitial tissue are measured over time at 15, 30, 60, and 90 min and compared with group C (n=7 for each group). Values are expressed as the mean±SD. The data are analysed by one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. *P<0.05 and **P≤0.01 compared with C.

Figure 9

FITC-HES130 localisation

Representative images at 60 min after treatment in each group. Images A-F are obtained 60 min after FITC-HES130 administration. FITC-HES130 is localised to the intravascular wall (white arrows) in the untreated group C (A), NS-NS (B), NS-ALB (C), and ALB-NS (D) groups but is uniformly distributed in the NS-HES (E) and HES-NS (F) groups. Images G-L are acquired 60 min after TMR-DEX40 administration. All groups (G-L) show uniform intravascular wall staining.

Supplementary Files

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