The Degree of Vascular Leak of Hydroxyethyl Starch in Severe Hemorrhagic Shock in Rats

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Abstract

Aim of the Study: In circulatory shock, colloid solution is beneficial to effectively maintain blood pressure. The HES solution is characterized by its mean Molecular Weight (MW), concentration, Degree of Substitution (DS) and the pattern of the substitution (C2/C6). To find the ideal character of the HES to administer to the patients in shock, we studied how the HES is retained in the vascular vessels in the shock rats using three types of HES bound with Fluorescein-Isothiocyanate (FITC-HES) with a spectrophotometer-computer detection system to determine the rate of elution of the HES from the microvascules.

Methods: We adopted the model of a severe hemorrhagic shock with a Mean Arterial Pressure (MAP) of 50 mmHg in rats. We infused one of three types of FITC-HES; HES-A (MW 150-200 kDa, DS: 0.6-0.68, C2/C6=8), HES-B (MW 175 kDa to 225 kDa, DS: 0.45 to 0.55, C2/C6=6) or HES-C (MW 550 kDa to 850 kDa, DS: 0.7 to 0.8, C2/C6=5). The FITC-HES retention rates in the image of the intravital microscope.

Results: MAP transiently increased toward the baseline value after infusion but then progressively decreased in all groups. HES-C remained in the A2 arteriole longer than HES-A (P=0.022). HES-C was eluted from the blood vessels more slowly than HES-A (P=0.028). We did not find any statistical difference in MAP among groups and the rate of disappearance from the vessels between HES-A, HES-B and HES-C.

Conclusion: HES of high MW was retained in the blood vessel longer those HES with low MW. In the hypovolemia, the retention rate was more dependent to the MW than that in the normovolemia. Other factors as DS and C2/C6 showed less effect in the severe hypovolemia.

Keywords: Decompensated hemorrhage; Hydroxyethyl starch; Rats; Shock; Intravital microscopy

Introduction

Significance: Hypovolemia in bleeding decreases cardiac output and tissue oxygen supply. Both the duration and extent of tissue hypoperfusion determine the severity of cellular damage, which should be kept to a minimum with timely volume substitution.

Background: Critical hemorrhagic shock requires most effective treatment for volume expansion in a limited time. Previous studies showed that colloid solutions are superior to crystalloid solutions for the management of blood volume [1-3]. There are hypo-, iso- and hypertonic crystalloids, and human albumin, dextran, gelatin and Hydroxyethyl Starch (HES) in colloid solutions. HES is a popular regimen for hypovolemia among the colloids [4]. The pharmacological effects of HES depend on the number of starch particles in the vascular space, which produce Colloid Osmotic Pressure (COP). The number of the starch particles relates the velocity of the decay and the elution of the particle through the vascular wall. Therefore, we think that it is helpful to observe the concentration of the HES in the blood vessel, and the leakage of the HES to the outside of the blood to assess the retention of HES in the blood circulation.

The study's aims: In this study, the model of severe hemorrhagic shock was chosen which actually occurs in severe critical injuries. We used intravital microscopy to observe the distribution and concentration of the HES particles. To observe the HES, the HES is bound with Fluorescein-Isothiocyanate (FITC-HES) to produce chemiluminescence. We adopted three types of HES with known molecular weight. The fascia in the cremaster muscle of the rat was chosen to observe the
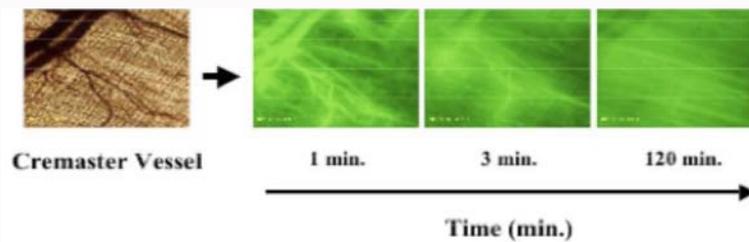
Figure 1: Color image of leaking hydroxyethyl starch particles bound with fluorescein-isothiocyanate (FITC-HES), seen under the intravital microscope over time. The particular amount of FITC-HES was shown to have leaked out from the capillary blood vessels in the cremaster muscle a few seconds after the FITC-HES administration.

vessels distributing an area of muscular fascia by using intravital microscopy [5-7]. We could evaluate macromolecular concentration and leak in the vessels in the cremaster muscle fascia [7-10].

Methods

The study was approved by the Committee for Ethical Review of Animal Experiments at Nihon University and was conducted according to the National Institutes of Health guidelines for the use of experimental animals. Male Sprague-Dawley rats (5 ± 1 wk old, weighing 200 ± 10 g) were purchased from Sankyo Laboratory Co. Ltd (Tokyo, Japan). The rats were maintained under controlled conditions at a temperature of 23 ± 3°C with a relative humidity of 55 ± 15% and a 12:12 h light-dark cycle. They were used for experiments following acclimatization of at least 14 days to these conditions, and fasted for 24 h before the experiments, but had free access to water.

We produced Fluorescein Isothiocyanate Conjugated Hydroxyethyl Starch (FITC-HES) as described by Thomas et al. [11] from non-commercial HES for experimental use (Ajinomoto Pharm. Co., Ltd. Japan). We used three types of HES with different characters: HES-A (MW 150 kDa to 200 kDa, DS 0.6 to 0.68, C2/C6=8:1), HES-B; MW 175 kDa to 225 kDa, DS 0.45 to 0.55, C2/C6=6:1) and HES-C (MW 550 kDa to 850 kDa, DS 0.7 and 0.8, C2/C6=5:1). The volume of distribution of FITC-HES was extrapolated to Time 0 of the natural logarithms of the fluorescence derived from plasma samples obtained at the three time points.

The procedure was performed according to a previously established method, shown to produce hemorrhage shock [12,13]. Briefly, anesthesia was induced with intraperitoneal sodium pentobarbital (1 g/kg). A venous line was taken at the ventral tail vein with a 24-gauge needle to provide continuance of anesthesia with pentobarbital (40 mg/kg/h to 90 mg/kg/h). Normal saline was administered as the maintenance fluid at the rate of 2.5 ml/kg/h to 5 ml/kg/h. The infusion rate was adjusted to maintain a stable light plane of anesthesia based on the previous criteria [12]. Animals were placed on a warming pad to maintain body temperature between 37°C to 38°C, which was monitored by a rectum thermometer. The trachea was intubated with a polyethylene catheter (internal diameter: 2.1 mm) to support spontaneous breathing with the room air.

A silicone cannula was inserted into the left carotid artery to allow continuous monitoring of blood pressure *via* a pressure transducer (BP-308 ETI, OMRON Corporation, Tokyo, Japan). The femoral artery was catheterized for blood withdrawal. For the observation of intravital microscopy, the cremaster muscle was prepared [5]. When the anesthetized rats were lying with the cremaster flat on the microscope slide they were allocated into one of three experimental groups; re-infusion with HES-A, re-infusion with HES-B, or re-
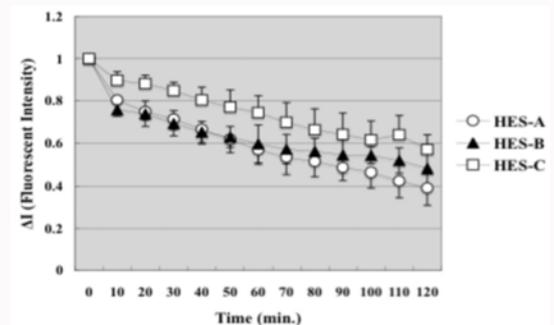

Figure 2: Retention of three groups of HES inside V2 blood vessel. ΔI means the value of fluorescent intensity at each time point compared to that at time zero. There was no significant difference among the three groups of the experiment. Data are presented as means ± SE in each group.
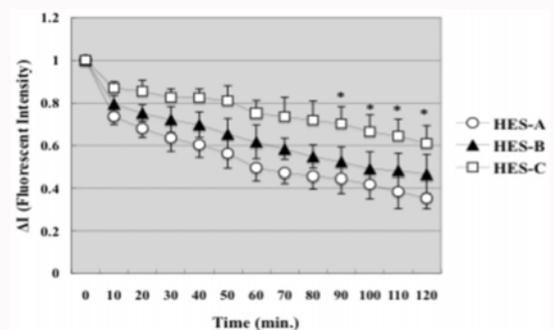

Figure 3: Retention of three groups of HES inside A2 blood vessel. *P <0.05, significant difference between HES-A and HES-C. ΔI means the value of fluorescent intensity at each time point compared to that at time zero. Data are presented as means ± SE in each group.

infusion with HES-C, all in saline solution.

In all groups based on the previous criteria as described, during anesthesia, after the Baseline (BL) readings were obtained, hemorrhage was induced by rapid withdrawal of blood for 3 to 5 minutes to obtain a Mean Arterial Pressure (MAP) of 50 mmHg [12]. Additional blood was withdrawn as required to maintain the blood pressure at 50 mmHg throughout the hypotensive period until decompensation occurred. Severe shock state was defined as the point at which blood pressure could no longer be maintained without re-infusion of previously withdrawn blood. After a 30-min stabilization period followed. During this period, areas of measurement were selected randomly to assess microcirculatory variables, and four points were fixed for observation. The first two points were the points of 100 μm from the A2 and V2 wall to the outside, who's A2 and V2 were classified according to their order of branching as previously

described [5]. The other two points were inside A2 and V2. The first sets of measurements (baseline) were recorded. The animals were randomly allocated to one of three groups, according to the type of solution used for resuscitation: 6% HES-A (n=20), 6% HES-B (n=21) and 6% HES-C (n=20) at the 1: 1 ratio of the blood volume withdrawn. Initial experiments with skeletal muscle were performed without additional blood or saline resuscitation, and observations were continued at 5-minute intervals until the animal died. In subsequent experiments, rats were further resuscitated by return of previously withdrawn blood and additional crystalloid as 0.9% NaCl as required maintaining MAP at or near BL values. Observations were then continued for additional hours or until death occurred. In the experiment with rats receiving HES, the first 5 animals died <1 minute before measurements could be made, and we aborted this protocol.

The brightness of the illumination at the four points was measured for a 30-min baseline period (t = -30 to 0 min) and for an experimental period of 120-mins (t=0 to 120 min) in all groups. The retention of HES was evaluated by the contrasting density of the brightness of fluorescence on the image stored on a PC.

For the observation of the intravital fluorescent microscopy, the animal on the warming pad was transferred to the modified stage of a BX51WI Olympus fluorescent microscope (Olympus Co. Ltd., Tokyo, Japan). This was equipped with a tungsten lamp for transmitted light and a mercury arc lamp for epi-illumination fluorescent light microscopy. A filter cube interposed into the path of epi-illumination of the cremaster microcirculation. The extravasation of FITC-HES was measured by determining the changes in integrated optical intensity by image analysis.

The FITC-HES represented relative changes in retention and permeability as previously described [9]. In brief, $\Delta I = 1 - (I_i - I_o)/I_i$, where ΔI is the change in light intensity; I_i is the light intensity inside the vessel, and I_o is the light intensity outside the vessel. Each experimental frame was digitized into a 512×512 charge-coupled device (CCD) (DP70; Olympus Co. Ltd., Tokyo, Japan).

We started the infusion of FITC-HES throughout the tail vein to the blood circulation. FITC-HES are normally retained in vasculature; therefore, epi-illumination allows the cremaster muscle microcirculation to be clearly visualized [2]. Images of the preparation were monitored by using a CCD camera (DP70; Olympus Co. Ltd., Tokyo, Japan), displayed on a high-resolution monitor (Olympus Co. Ltd., Tokyo, Japan), and the images saved to a Hard Disk Drive (HDD) (Olympus Co. Ltd., Tokyo, Japan) for later off-line analysis.

Four areas of cremaster muscle microcirculation (random location) were recorded by using epi-illumination to include two A2 (30-70 μm) arterioles and V2 (35-120 μm) venuls in each animal [5]. FITC-HES leakage from the A2 and V2 vessels were recorded every 5 minutes and assessed off-line by using an Olympus personal computer (Olympus Co. Ltd., Tokyo, Japan) and image analysis software package (MetaMorph®, Molecular devices, Sunnyvale, CA, USA). Each of the 3-5 areas selected were intermittently exposed to fluorescent light for a maximum of 15s at each 5-min interval to present photodynamic effects [2]. Macromolecular leakage was assessed based on the contrasting density of the brightness of FITC-HES fluorescence on the image stored on a computer. Permeability was measured by light intensity taken at two different sites, one within the vessel and the other adjacent to the vessel, within the same area.
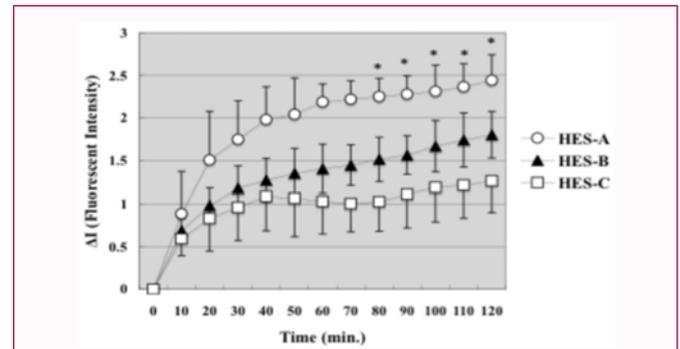

Figure 4: Fluorescent Intensity of HES outside V2. Fluorescent Intensity of HES-A (HES with the lowest molecular weights) increased the most rapidly outside the venula. The increase of HES-C was the smallest. The increase of HES-B was between HES-A and HES-C. *P<0.05 significantly different between HES-A and HES-C (statistical method ANOVA). Data are presented as means ± SE for 20 rats in each group.

All data are expressed as mean ± SE. One-way analysis of variance was used to compare the means of different treatments. If significance was identified, individual comparisons were subsequently made by Tukey-Kramer test to determine the site of significance within the data sets. The differences were considered significant when P <0.05 in use of the two tails of the test.

Results

We used 20 rats in the experiment with HES-A, HES-B and 21 rats with HES-C. We could successfully observe that FITC-HES leaked out from the blood vessels. The interstitial space of the cremaster muscle became bright after the start of re-infusion. FITC-HES leaked out from the blood vessels within one minute after the FITC-HES re-infusion (Figure 1).

The rates of retention of FITC-HES in the arteriolar and venular blood vessels were different among groups (Figure 2 and 3). Figure 2 is a graphic presentation of the change of the retention of FITC-HES inside V2 blood vessel after the FITC-HES infusion over a 120-min period. HES-C was found to have decreased steadily. The fluorescent intensity of HES-C became two third of that at zero point in two hours. The retention rates of HES-A, -B and -C at 120 minutes point were 39% ± 6.8%, 48% ± 8.9% and 57% ± 9.2% of baseline values, respectively. However, there were no significant differences among the retention rates of three groups.

The retention rate of FITC-HES inside A2 blood vessel after a 120-min shock period is shown in Figure 3. In the arteriole, too, the FITC-HES decreased gradually in all groups, although the degrees were different. HES-A decreased most remarkably in the arteriole. The retention rates of HES-A, -B and -C at 120 minutes point were 35% ± 10.2%, 46% ± 9.8% and 60% ± 7.2% of baseline values, respectively. The decrement of fluorescent intensity of HES-C (P=0.038) was significantly smaller than HES-A. The retention rate of HES-C was significantly larger than HES-A after 90 minutes point.

The detection of the FITC-HES outside the V2 venules was also examined (Figure 4). The fluorescent intensity of HES-A outside the V2 venules increased the most rapidly. Apparently the increase of HES-C was the smallest, and the increase of HES-B was between HES-A and HES-C. The increments of fluorescent intensity of HES-A (P=0.039) was significantly larger than HES-C.

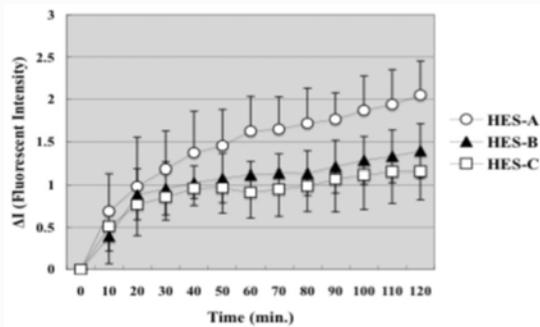The detection of the FITC-HES outside the A2 arteries is shown

Figure 5: Fluorescent Intensity of HES outside A2. Fluorescent Intensity of HES-A outside arteriole increased the most rapidly. The increase of HES-C was the smallest. The increase of HES-B was between HES-A and HES-C. However, no significant difference was found anywhere among the groups in the figure. Data are presented as means ± SE in each group.
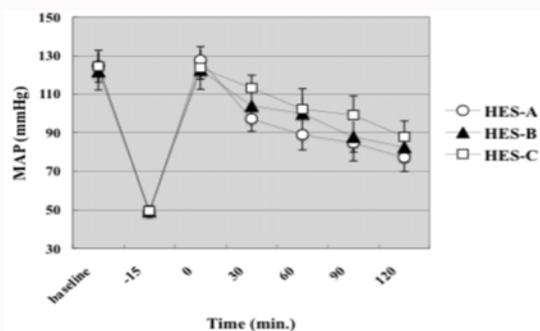

Figure 6: The change of Mean Arterial Pressure (MAP). The MAP transiently increased toward baseline after the start of infusion after the shock. But the MAP progressively decreased again down to about 65% of the baseline in all groups. There was no significant difference among three groups at any time point in the study. Data are presented as means ± SD in each group.

Figure 5. The fluorescent Intensity of HES-A apparently increased the most rapidly. However, no significant difference was found anywhere in the figure among the groups in this study.

The change of MAP of each group is shown in Figure 6. No important difference was noted among Baseline (BL) values in groups receiving HES-A, HES-B and HES-C solution. The blood withdrawal caused similar decrement of blood pressure in all groups. The blood withdrawal decreased MAP to 42.3% ± 1% of Baseline (BL) in HES-B and 40.9% ± 1% of the BL in HES-A and HES-C. After infusion of HES-A, HES-B and HES-C solution, MAP transiently increased to the BL value, but then progressively decreased again to about 65% of BL in all groups. No significant difference was found anywhere in the Figure between groups.

Discussion

Each HES solution is characterized by its concentration, MW, DS and C2/C6, where DS means the percentage of hydroxyethyl residue in the glucose subunits, and C2/C6 means the pattern of substitution of the hydroxyethyl residue [1]. Hydroxyethylation is mandatory because the natural starches are unstable and are rapidly hydrolyzed in the circulating amylase. The hydroxyethyl residues, especially when bound at the C2 carbon position of glucose, hinder the plasma-amylase to degrade the glucose polymers, hence increasing the intravascular half-life of the HES particle. As a result, a higher MW range, extensive DS value and high C2/C6 ratio result in slower elimination.

In the previous our study in normovolemia, however, the HES with middle MW with a molecular degradation at a certain rate was thought to keep the molecular concentration higher, and was retained within the blood vessel longer than other HES with lesser degradation in microcirculation. It was not necessary to infuse high MW HES, and necessary to consider DS and the pattern of the substitution. Another study showed compared with HES 200/0.5, the use of HES 130/0.4 could significantly improve internal organ perfusion and tissue oxygenation in patients undergoing liver surgery with a relatively large amount of blood loss [14]. Two recent meta-analyses concluded that a goal-directed approach to maintaining tissue perfusion reduces mortality, postoperative organ failure and surgical complications in high-risk surgical patients [15,16]. In this study, the elimination of HES from the blood vessel in hypovolemic condition was elucidated. Our current result was simple because the larger HES particles could stay within the blood vessel longer [17,18]. The MW of HES-A and -B were 175 and 200 kDa on average, respectively. Their DS and the pattern of the location of hydroxyethyl substitution (C2/C6 ratio) were 0.64 and 0.5, and 0.5 and 0.6, respectively. In the previous study, the possible explanation why HES-B was retained within the vessel space significantly longer was the difference of MW of 2.5 kDa although it is small, the difference of DS values and the difference of the C2/C6 ratio [1,3,17-23]. We hypothesized on the fact that the certain amount of medium sized HES with low DS can be divided rapidly but at a certain rate so as to keep a constant osmotic pressure in order to keep it longer in the space. The relatively decreased DS value may have led to an increased metabolic degradation, which might have been counteracted by the increased C2/C6 ratio, preventing a too rapid decrease in the plasma. Anyway, in the previous study in normovolemia, we found a difference in half lives in the vascular space between the two HESs of middle MW. Although the reason should be elucidated in the future, the fact is that we obtained a result where the HESs was retained in the vessel for a time length which related to the molecular weight. We can only assume that the vascular integrity was different between the states in normovolemia and in hypovolemia [24]. Otherwise, the titer of circulating amylase would have been diminished by the dilution after the large amount of rapid re-infusion. Further investigation is required. There are several types of HES in the world. The properties of HES were very widely. In all research, HES of high MW showed long term volume effect. Although the HES of high MW have a beneficial effect, it is also reported that any HES causes several side effects. There are reports of tissue storage, plasma accumulation, and renal dysfunction [25-28]. HES of high MW are also reported to produce decreased hemostatic conditions [29-32]. Large HES with high DS induce significant increase in prothrombin time and a greater decrease in factor VIII [13]. In 2013, In the statement from the Co-ordination Group for Mutual Recognition and Decentralized Procedures–Human (CMDh), healthcare professionals are informed to consider that 'HES solutions should be used at the lowest effective dose for the shortest period of time. Treatment should be guided by continuous hemodynamic monitoring so that the infusion is stopped as soon as appropriate hemodynamic goals have been achieved.' and 'HES solutions should only be used for the treatment of hypovolemia due to blood loss when crystalloids alone are not considered sufficient' [33]. The therapeutic target is to achieve normovolemia, not infusing beyond.

To solve the hemostatic derangement, the some studies report on the new synthesis of a middle sized HES with low SD and high C2/C6 ratio [34-38]. In our study, one of the two compounds of middle sized

HES was retained in the vascular space statistically not different from HES with high MW. We recognized that middle size HES might exert a similar volume effect as the large HES does.

Conclusion

The retention of the HES in the blood vessel in the hypovolemia simply depended on its molecular weight. The largest-sized HES of the three stayed in the blood vessel the longest. The middle-sized HES of mean MW 200 kDa with DS 0.5 and C2/C6=6 stayed midway between the other two. The smallest HES of mean MW 175 kDa with DS 0.65 was found to be eliminated the quickest. The retention rate of the middle-sized HES was different in our previous study of normovolemia.

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