Original Article

Response of cardiac endothelial nitric oxide synthase to plasma viscosity modulation in acute isovolemic hemodilution

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Abstract:

Background: Endothelial nitric oxide synthase (eNOS) is generally expressed in endocardial cells, vascular endothelial cells and ventricular myocytes. However, there is no experimental study elucidating the relationship between cardiac eNOS expression and elevated plasma viscosity in low oxygen delivery pathological conditions such as hemorrhagic shock-resuscitation and hemodilution. This study tested the hypothesis that elevated plasma viscosity increases cardiac eNOS expression in a hemodilution model, leading to positive effects on cardiac performance. **Materials and Methods:** Two groups of golden Syrian hamster underwent an acute isovolemic hemodilution where 40% of blood volume was exchanged with 2% (low-viscogenic plasma expander [LVPE]) or 6% (high-viscogenic plasma expander [HVPE]) of dextran 2000 kDa. In control group, experiment was performed without hemodilution. All groups were performed in awake condition. Experimental parameters, i.e., mean arterial blood pressure (MAP), heart rate, hematocrit, blood gas content and viscosity, were measured. The eNOS expression was evaluated by eNOS Western blot analysis. **Results:** After hemodilution, MAP decreased to 72% and 93% of baseline in the LVPE and HVPE, respectively. Furthermore, pO_2 in the LVPE group increased highest among the groups. Plasma viscosity in the HVPE group was significantly higher than that in control and LVPE groups. The expression of eNOS in the HVPE group showed higher intensity compared to other groups, especially compared with the control group. **Conclusion:** Our results demonstrated that cardiac eNOS has responded to plasma viscosity modulation with HVPE and LVPE. This particularly supports the previous studies that revealed the positive effects on cardiac function in animals hemodiluted with HVPE.

Key words:

Cardiac endothelial nitric oxide synthase, hemodilution, plasma expander, plasma viscosity

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Introduction

Increasing plasma viscosity with viscogenic plasma expanders provide positive effects on microvascular function in an acute hemorrhagic shock and an extreme hemodilution in unanesthetized animal models.[1-5] High-viscogenic plasma expanders (HVPE) improve capillary perfusion and sustain arterial blood pressure compared to low-viscogenic plasma expanders (LVPE) after resuscitation from hemorrhagic shock and continuous bleeding.[6] HVPE also induce vasodilatation as evidently observed in an increased blood flow while LVPE cause vasoconstriction in extreme hemodilution studies.[4] In addition, study in a hamster dorsal skinfold window chamber with an extreme hemodilution has shown that perivascular nitric oxide (NO) concentration significantly increased in animals hemodiluted with HVPE compared to that hemodiluted with LVPE.[7]

NO has been proposed as the most potent vasodilator and one of cardiac protectors. Many studies have comprehensively investigated role of NO synthesized by endothelial NO synthase (eNOS) in circulatory system.[8,9] eNOS is generally expressed in endocardial cells, vascular endothelial cells, ventricular myocytes and other cardiac cells.[10,11] De Wit *et al.* increased plasma viscosity by exchange with two different molecular weight dextrans, but maintained level of hematocrit (Hct).[12] They found that an increase in wall shear stress (WSS) by elevated plasma viscosity induced a NO-mediated arteriolar dilatation in the hamster cremaster tissues. Similarly, microvascular study by Tsai *et al.* demonstrated that the calculated WSS and aortic eNOS protein expression were higher in the animals hemodiluted with HVPE when compared to animals hemodiluted with LVPE.[7] In addition, Martini *et al.* observed no change in blood pressure in eNOS knockout mice even when blood viscosity was increased by hemoconcentration, indicating a major role of eNOS in vasodilatation.[3]

As increase in plasma viscosity, demonstrated an improved microvascular perfusion and a restored functional capillary density in shock-resuscitation and hemodilution models, it has been speculated that these beneficial effects might

occur in cardiac microvasculature and lead to positive effects on cardiac function. Heymes *et al.* reported that an increase in endocardial gene expression of eNOS and inducible NOS and an improvement in cardiac performance was observed when dilated cardiomyopathy patients were administered with substance P, an eNOS stimulator.[13] However, their study only suggested that NO production positively affects cardiac function but did not mention about plasma viscosity. Recent studies in anesthetized hamsters by Chatpun and Cabrales have provided supportive results that hemodilution with HVPE increased cardiac output, stroke volume and stroke work and decreased systemic vascular resistance compared to hemodilution with LVPE.[14,15] Although their studies have discussed the promising results of the effects of increased plasma viscosity on cardiac function, the underlying mechanism has not been experimentally studied. Furthermore, there is no experimental study elucidating the relationship between cardiac eNOS expression and increased plasma viscosity in low oxygen delivery pathological conditions such as hemorrhagic shock-resuscitation and hemodilution.

The objective of this study was to test the hypothesis that increased plasma viscosity increases cardiac eNOS expression in a hemodilution model, leading to positive effects on cardiac performance. Animals underwent an acute isovolemic hemodilution where 40% of blood volume (BV) was exchanged with 2% (LVPE) or 6% (HVPE) of dextran 2000 kDa. In addition, the eNOS expression was evaluated by eNOS Western blot analysis.

Materials and Methods

Animal preparation for catheterization

Experiment was carried out in 60-75 g unanesthetized male Golden Syrian hamsters from our institutional animal unit. Animal handling and care followed the National Guidelines for Care and Use of Laboratory Animals. The experimental protocol was approved by the institutional animal ethics committee (Ethical Clearance number: Ref. 05/2011). The hamster dorsal skinfold fake window chamber was performed for animal handling purpose. The surgical procedure of fake window chamber making from thin plastic sheet was similar to a real window chamber but there was no back skin tissue removal.[5] The animal was administered with pentobarbital sodium 50 mg/kg i.p. for a chamber implantation. After hair removal, sutures were used to lift the dorsal skin away from the animal and one frame of the chamber was positioned on the animal's back. A chamber consisted of two identical plastic frames. Arterial and venous catheters (polyethylene-50 tube with polyethylene-10 tube as a tip) were cannulated in the carotid artery and jugular vein, respectively. Catheters were then tunneled under the skin and exteriorized at the dorsal side of the neck. After catheterization, animal was put back in the cage for at least 1 day for recovery before starting the experiment.

Inclusion criterias

Animals with a dorsal skinfold fake window chamber were used in the experiment if there was no tissue bleeding, mean arterial blood pressure (MAP) was above 100 mmHg, heart rate (HR) above 340 bpm and systemic Hct was above 42%.

Systemic parameters

MAP was monitored continuously (MP150, Biopac System Inc., USA), except during blood exchange and blood sampling. Systemic Hct was determined from centrifuged arterial blood samples taken in heparinized capillary tube.

Test solutions

The plasma expanders were prepared using dextran 2000 kDa (Pharma-Cosmos, Holbaek, Denmark) at 6% for HVPE and 2% for LVPE in 0.9% sodium chloride. Colloid osmotic pressure was adjusted for both solutions using human serum albumin diluted in 0.9% sodium chloride.

Acute isovolemic hemodilution protocol

Forty percent of BV was exchanged with the test solution in animals, lowering the systemic Hct by 45%. Total BV was estimated as 7% of body weight. Test solutions were infused into the jugular vein catheter at a rate of 100 µl/min and blood was simultaneously withdrawn at the same rate from the left carotid artery using a syringe pump (NE-4000, New Era Pump Systems, USA). After hemodilution, animals were followed over 1 h. MAP and HR were continuously monitored and blood was collected at the end of each experiment for measurements of viscosity. Figure 1 illustrates the experimental protocol.

Experimental groups

Prior to baseline (BL) assessment, animals were randomly divided into three experimental groups: (1) A control group with no hemodilution, (2) a group hemodiluted with HVPE and (3) a group hemodiluted with LVPE. Each group had six animals involved in this study.

eNOS Western blot analysis

Heart was harvested from animal, then washed with ice-cold phosphate buffer saline and homogenized with lysis buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 0.5 mM ethylene diaminetetraacetic acid and protease inhibitor cocktail [GE healthcare]). The total protein content of sample extracted was measured using Biorad protein assay. Fifty micrograms of each sample was run separately on 12% polyacrylamide-SDS gel and then proteins were transferred to nitrocellulose membrane in glycine-methanol buffer at 4°C at 100 V for 2 h. The membranes were blocked by shaking in 5% lowfat dry milk in TBS-T (Tris buffer saline-0.1% Tween 20) for 1 h at room temperature. The membranes were incubated overnight with primary antibodies

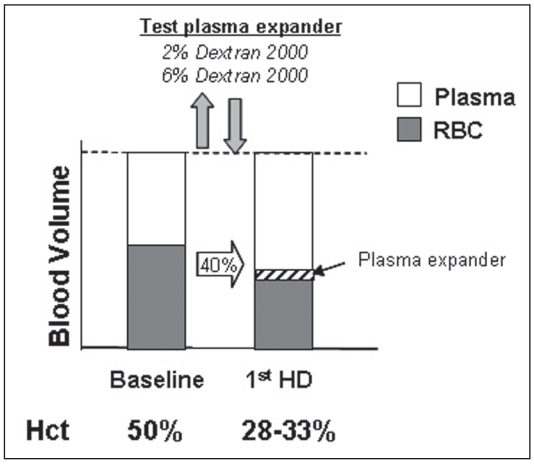

Figure 1: Schematic diagram of acute isovolemic hemodilution protocol

with gentle shaking against eNOS (1:250) and actin (1:1000) as an internal control in 5% lowfat dry milk in TBS-T, all antibodies were from Cell Signaling. The membranes were washed 3 times (5 min/wash) and then incubated with ECL anti-rabbit IgG-horseradish peroxidase (GE health care) diluted to 1:1000 (eNOS) and 1:5000 (actin) in 1% lowfat dry milk in TBS-T for 1 h at room temperature and washed 3 times (5 min/wash). The proteins were visualized using an ECL chemiluminescent detection kit (Pierce) and exposed to film.

Statistical analysis

Results are presented as mean ± standard deviation unless otherwise addressed. Data between interested time points in a same group were analyzed using analysis of variance and followed by *post hoc* analyses with the Dunnett's multiple comparison tests. An unpaired *t*-test with two-tailed was performed to compare between groups at the time point of interest. All statistics were performed with GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). Results were statistically significant when $P < 0.05$.

Results

Animals were assigned randomly into three experimental groups: Control ($n = 6$; weight 75 ± 4 g), HVPE ($n = 6$; weight 66 ± 9 g) and LVPE ($n = 6$; weight 71 ± 6 g). The measured parameters at BL were not significantly different between the groups. The viscosities of HVPE and LVPE were 6.1 cP and 1.7 cP, respectively.

Systemic and blood gas parameters

The changes of MAP after hemodilution are shown in Figure 2. Hemodilution with LVPE markedly reduced MAP compared to other groups through the experimental period. At the end of experiment, MAP in the LVPE group decreased to 72 ± 17% of BL while MAP in the HVPE group dropped to 93 ± 3% of BL. On the other hand, MAP in the control group was maintained at the BL at the end of experiment. Figure 3 shows that HR after hemodilution increased over time, whereas HR did not significantly change in the control group. Figure 4 presents the change in Hct at each time point of interest. As expected, exchange of 40% of blood volume with plasma expanders significantly decreased the amount of red blood cells and hence the Hct (Hct decreased from 44% to 29%). The pH, pO_2 and pCO_2 of blood in each group are shown in the Table 1. Animals hemodiluted with HVPE or LVPE had an increased pH while pH in animals without hemodilution was maintained at the BL. Furthermore, pO_2 increased 18%, 16% and 36% from BL in the HVPE group, control group and LVPE group, respectively. pCO_2 in the control group increased 15% from BL. In contrast, pCO_2 decreased 16% and 5% from BL in the HVPE group and LVPE group, respectively.

Rheological parameters

Changes in blood and plasma viscosities after hemodilution were particularly different from BL as presented in Figure 5. Whole blood viscosity in the HVPE hemodiluted group was lower by about 26% compared to the control group while the blood viscosity was lower by about 36% in the LPVE hemodiluted group. Plasma viscosity in the HVPE group was significantly higher than that in the control and LVPE groups.

Western blot analysis

A significantly higher cardiac eNOS protein expression was observed in hemodiluted groups via Western blot analysis compared to control group as shown in Figure 6. Although there was no statistically significant difference between the intensity ratios of the group hemodiluted with HVPE and that hemodiluted with LVPE, it showed that HVPE induced higher cardiac eNOS protein expression than LVPE.

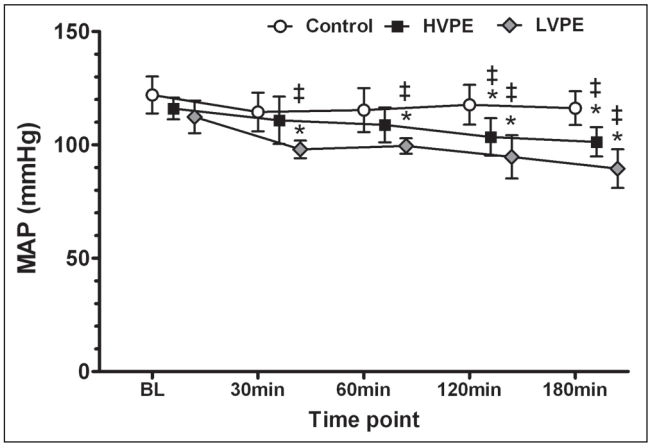

Figure 2: Mean arterial blood pressure measured at baseline (BL), at 30, 60, 120 and 180 min after hemodilution. *$P < 0.05$ compared to BL. ‡$P < 0.05$ compared to control

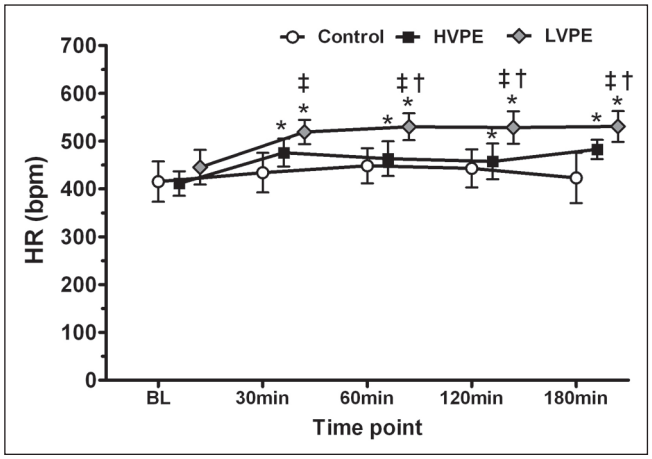

Figure 3: Heart rate measured at baseline (BL), at 30, 60, 120 and 180 min after hemodilution. *$P < 0.05$ compared to BL. ‡$P < 0.05$ compared to control. †$P < 0.05$ compared to high-viscogenic plasma expander

Table 1: Arterial blood pH, pO_2 and pCO_2 at BL and after complete hemodilution

Group	pH		pO_2		pCO_2	
	BL	180 min	BL	180 min	BL	180 min
Control	7.41±0.05	7.46±0.02	59.48±6.68	66.73±10.49	55.82±9.83	49.40±6.83
HVPE	7.38±0.02	7.45±0.03*	52.17±2.71	63.30±9.27*	60.14±3.25	52.88±7.14
LVPE	7.41±0.02	7.47±0.02*	54.12±9.58	64.27±10.28	57.46±4.25	52.55±7.81

The values of arterial blood gas analysis at the baseline and the end of hemodilution are means ± SD; *$P<0.05$ compared to baseline; BL: Baseline; HVPE: High-viscogenic plasma expander; LVPE: Low-viscogenic plasma expander; SD: Standard deviation

Discussion

The findings of this study demonstrated that the viscosity of plasma expanders have an effect on systemic physiological parameters such as mean arterial blood pressure and HR after hemodilution. Furthermore, blood gas parameters in hemodiluted group indicated that there was a regulatory mechanism to adjust physiologically when blood was diluted. Using our proposed protocol, we observed higher cardiac eNOS expression in groups hemodiluted with viscogenic plasma expanders, both HVPE and LVPE, compared to that in the group without hemodilution.

Our results showed that an increase in plasma viscosity with HVPE could maintain MAP better than LVPE which is in agreement with previous studies by Cabrales and Tsai, Cabrales *et al.*[4,16] Our result implies that blood perfusion and capillary pressure may be better maintained when hemodiluted with HVPE compared to LVPE.[17] Therefore, using HVPE has the positive effect on the circulatory system compared to LVPE. Normally, high viscosity fluid causes high flow resistance but the studies using a dorsal skinfold window chamber revealed that HVPE decreased systemic vascular resistance after hemodilution compared to LVPE due to higher shear-induced vasodilatation.[1,3,16] Hemodilution decreases the amount of red blood cells causing an increase in red cell free layer thickness in vasculature as observed in large and medium arterioles and venules.[18,19] Therefore, an increase in plasma viscosity directly affects induced WSS and vasodilator release, resulting in decreased flow resistance.

Tsai *et al.* reported that in an acute extreme hemodilution, increase in plasma viscosity, increased perivascular NO, a physiological vasodilator, by activated eNOS activity.[7] They measured the eNOS expression from aortic Western blot. Their results supported the concept that, in extreme hemodilution, high viscosity of plasma maintains functional capillary density through a NO -mediated vasodilatation. Furthermore, Chatpun and Cabrales indicated that HVPE provided positive effects on cardiac performance compared to LVPE in an anesthetized hemodilution model.[14] They showed positive results on load-dependent cardiac indices such as stroke volume, cardiac output and stroke work as well as systemic vascular resistance. Furthermore, they mentioned that the positive effects might be related to shear induced NO. Using a similar percentage of BV exchange as in Chatpun and Cabrales's work, we measured a cardiac eNOS activity with Western blot. We found the expression of cardiac eNOS in the hemodiluted groups significantly higher than the control group. Although the eNOS expression between the hemodiluted groups was not statistically significant, however, higher expression was seen in HVPE compared to LVPE. Comparing to Tsai *et al.*'s work, we used a moderate hemodilution protocol, not extreme hemodilution protocol.[7] This difference in the protocol might affect cardiac eNOS expression. Cardiac eNOS activity might be less compared to aortic eNOS activity because of the differences in blood flow interaction on endothelium.

This study adds up to the information for the strategy of volume replacement, as well as transfusion medicine, that higher plasma viscosity is beneficial to bring back physiological response of cardiovascular system in case of blood dilution. Therefore, in the event of acute normovolemic hemodilution such as perioperative hemodilution, HVPE might be an alternative cell-free fluid to conserve autologous blood.

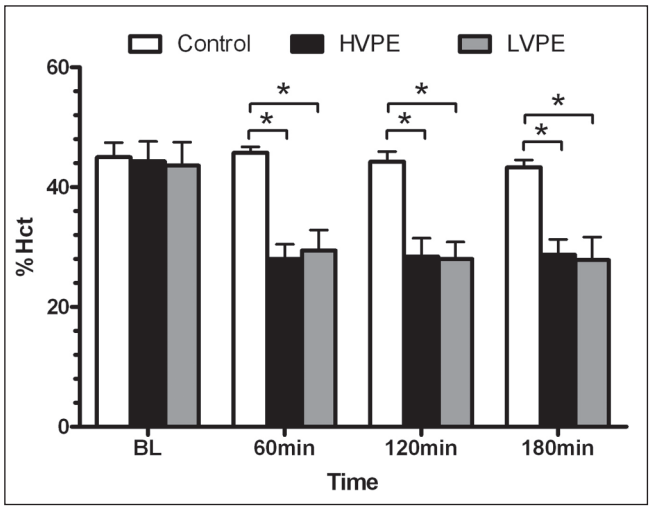

Figure 4: Hematocrit measured at baseline (BL), at 30, 60, 120 and 180 min after hemodilution. *$P < 0.05$ compared to BL

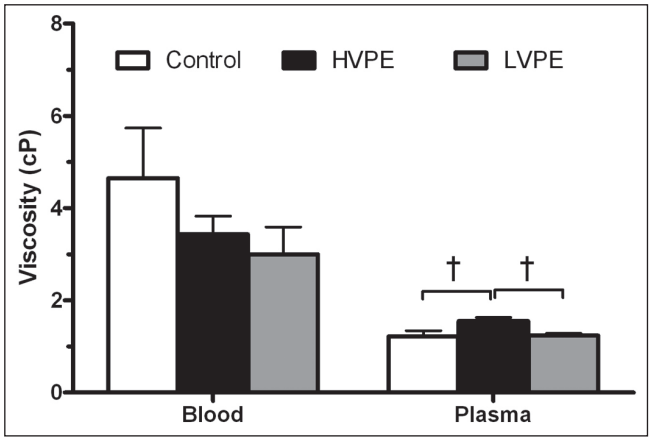

Figure 5: Blood and plasma viscosities at the end of experiment. †$P < 0.05$ compared to high-viscogenic plasma expander

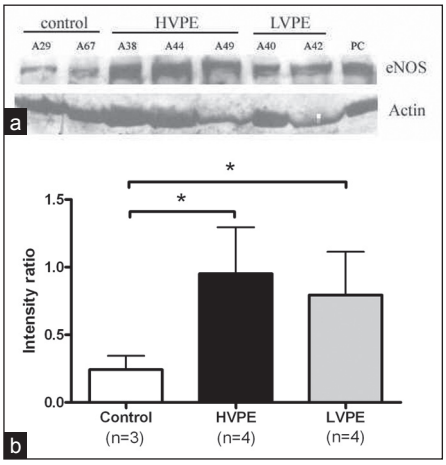

Figure 6: (a) Western blot analysis of endothelial nitric oxide synthase (eNOS) expression after treated with high-viscogenic plasma expander and low-viscogenic plasma expander; PC was lung tissue used as a positive control. (b) The band intensity ratio of eNOS-specific and β-actin bands in each group. *$P < 0.05$ compared to control

Conclusion

Increasing plasma viscosity in a moderate hemodilution in an awake animal model positively affects systemic circulation by maintenance of blood pressure with increased HR. Our results demonstrated that cardiac eNOS has responded to plasma viscosity modulation and may play a role in positive effects on cardiac performance when hemodiluted with HVPE as found in the previous studies. However, further studies need to carry out to investigate the underline causes which provide the positive effects on cardiac function in anesthetized animals hemodiluted with HVPE greater than that in awake animals.

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