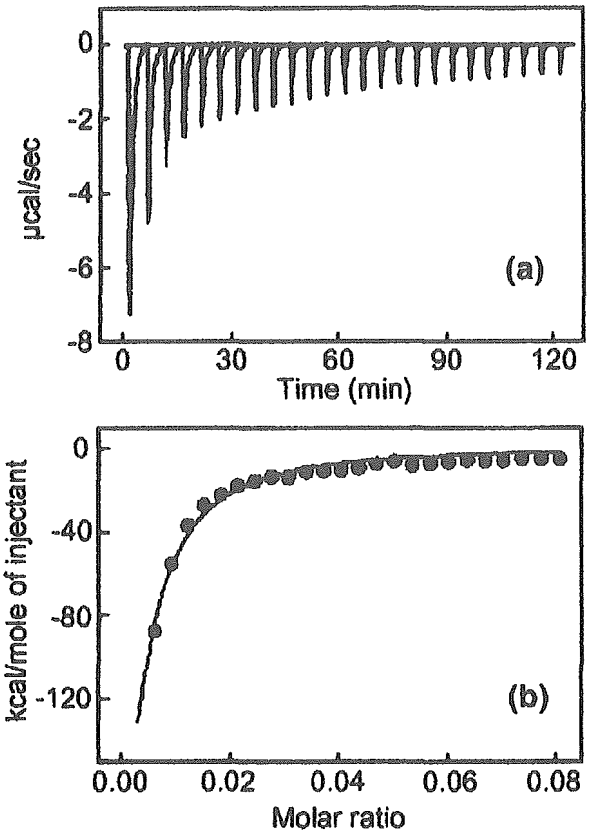

Figure 2. (a) Raw and (b) integrated data of the isothermal titration calorimetry for the incorporation of LPS (*Salmonella Minnesota* wild type) into phospholipid vesicles: [LPS] = 334 μM in a syringe (10 μL/one injection), [lipid] = 835 μM in a cell.

this absorbance decreased with the addition of the $C_{12}E_{10}$ solution, indicating the solubilization of HbV with the surfactant (Fig. 3). The complete solubilization required the addition of 2 vol% of $C_{12}E_{10}$ solution. At this concentration, the absorbance at 660 nm was identical with the pure Hb solution without vesicles. After the solubilization procedure, the color of the solution changed from red to brown, indicating the formation of methemoglobin. The concentration of $C_{12}E_{10}$ and the lipids of HbV in the resulting solution are 1.6 and 0.24 g/dL, respectively. $C_{12}E_{10}$ shares 87 wt% (86 mol%) of the mixed micelles. As for the other surfactants, $C_{12}E_9$ showed a similar ability to dissolve HbV that was evident from the profiles of the reduction of the light scattering of HbV (Fig. 3). On the other hand, Triton-X and sodium dodecyl sulfate (SDS) showed incomplete reduction of the absorbance and they could not sufficiently dissolve HbV.

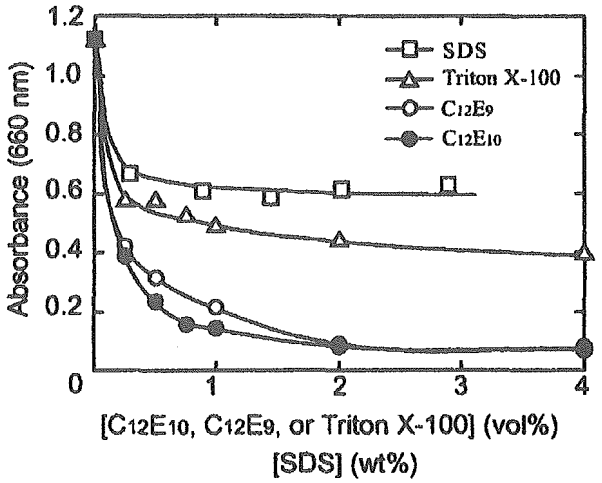

Figure 3. Absorption changes at 660 nm of HbV after solubilization of HbV by the addition of surfactants ($C_{12}E_{10}$, $C_{12}E_9$, Triton-X100, and SDS). Various concentration of a surfactant (800 μL) was added to the HbV suspension ([Hb] = 2 g/dL, 200 μL) and incubated at 42°C for 2 min.

Effect of $C_{12}E_{10}$ on Calibration Curves for LPS in LAL Assay (Method 1)

The calibration curves for Method 1 were drawn as logarithm plots of the gelation time versus the final LPS concentration (Fig. 4). Actually, the

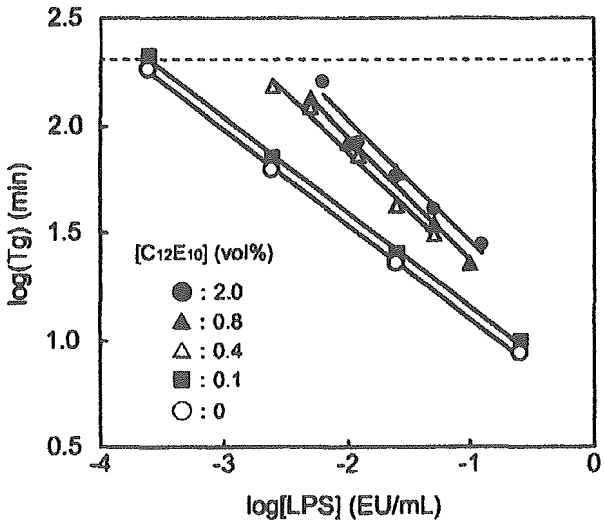

Figure 4. Calibration curves for the quantitative measurement of LPS in the presence of $C_{12}E_{10}$ at various concentrations. [LPS] is the final concentration in the test tube for turbidimetry. Dilution factors should be multiplied to obtain [LPS] in the HbV suspension at [Hb] = 10 g/dL. For example, [LPS] should be multiplied with 400 at $[C_{12}E_{10}] = 0.1$ wt%. The broken line indicates the gelation time limit for Toxinometer® (200 min).

plots show a linear relationship over a wide range of LPS concentrations (final concentration: 0.000125–0.25 EU/mL). The gelation time limit for the Toxinometer® is 200 min (log 200 = 2.3) as shown by the dashed line. Increasing the $C_{12}E_{10}$ concentration tended to retard the gelation. However, 0.1 vol% of the final $C_{12}E_{10}$ concentration did not show a significant retardation. At the LPS concentration of 0.01 EU/mL, the gelation time of ~32 min without $C_{12}E_{10}$ was prolonged to 39 min with 0.1 vol% of $C_{12}E_{10}$ and to ~107 min with 2.0 vol% of $C_{12}E_{10}$. In the case of the other surfactant, the presence of 0.1 vol% $C_{12}E_9$ prolonged the gelation time to 50 min (data not shown). Therefore, the inhibitory effect of $C_{12}E_9$ is stronger than that of $C_{12}E_{10}$.

LPS Measurement in HbV (Method 1)

To improve the LPS detection limit, the dilution factor could be minimized by increasing the HbV concentration at the solubilization process or by decreasing the amount of diluent (Table 1). The lowest detection limit (0.1 EU/mL) was obtained for entry No.1, with 0.1 vol% $C_{12}E_{10}$. The conditions of entries 2, 3, and 4 could reduce the dilution factor, however, [$C_{12}E_{10}$] at >0.4 vol% resulted in a significant inhibition of the gelation, as shown in Figure 4 and by the worsened detection limit (0.23 EU/mL).

The recoveries of the spiked LPS ranged between 92 and 124% for a wide range of LPS concentrations (Table 2, next page). The LPS measurement in an Hb solution did not require a surfactant so it was possible to detect a lower concentration (0.005 EU/mL), which was significantly better than the detection limit for HbV (0.1 EU/mL) that required $C_{12}E_{10}$. The FDA Guideline, published in 1987, requires that the inhibition/enhancement testing of spiked LPS at four times the amount of the detection limit and that the recovery should be within 100 ± 25%.[17] We tested the addition of LPS at 0.4 EU/mL (= 4 × 0.1 EU/mL) to the phospholipid vesicles and HbVs and at 0.02 EU/mL (= 4 × 0.005 EU/mL) to the Hb solution, and all the recoveries were within 100 ± 25%.

The LPS content in the LPS-contaminated vesicles, measured with the aforementioned LAL assay using $C_{12}E_{10}$, was 1.03 EU/mL (Table 3). On the other hand, the LAL assay without $C_{12}E_{10}$ resulted in 0.6 EU/mL. The recovery was calculated to be 60%.

The Pyrosep® Method to Detect LPS in $C_{12}E_{10}$-Solubilized HbV (Method 2)

The calibration curves of the Pyrosep® method in the presence of $C_{12}E_{10}$ showed a detection limit of 0.0001 EU/mL or lower (Fig. 5). The dilution factor for the HbV measurement was 25. Therefore, the LPS detection limit for an HbV suspension at [Hb] = 10 g/dL is 0.0025 EU/mL. One HbV suspension showing an LPS content of <0.1 EU/mL as measured by Method 1 was shown to have an LPS content of 0.011 EU/mL by the Pyrosep® method; this HbV was used for the inhibition/enhancement testing. The recovery of spiked LPS (Fig. 5, inset; 0.001, 0.01, and 0.1 EU/mL) from an HbV suspension was within 100 ± 25% over a wide range of LPS concentrations.

DISCUSSION

The interaction between an amphiphilic LPS molecule and a phospholipid vesicle should influence the accuracy of the LAL assay. It has been

Table 1. Solubilization Condition of HbV with $C_{12}E_{10}$ and Detection Limit of LPS[a]

	Condition of HbV Solubilization				
Entry	HbV/200 μL ([Hb] g/dL)	$C_{12}E_{10}$/800 μL ([$C_{12}E_{10}$] vol%)	Dilution Factor[b]	Final vol% [$C_{12}E_{10}$]	Detection Limit of LPS (EU/mL HbV)[c]
1[d]	2	2	400	0.1	>0.1
2	2	2	100	0.4	>0.25
3	2	2	50	0.8	>0.25
4	5	5	20	2.0	>0.23

[a]The calibration curves are shown in Figure 4.
[b]See Figure 1, from HbV ([Hb] = 10 g/dL) to gel clotting assay.
[c]At [Hb] = 10 g/dL.
[d]The optimal condition in this study.

Table 2. Inhibition/Enhancement Testing to Monitor the Recovery of Spiked LPS

Sample	Spiked LPS (EU/mL)[a]	Recovery (%)
Vesicles (6 g/dL) with $C_{12}E_{10}$	0.4	108.6 ± 4.6[b]
	1.25	92
	2.5	116
	5	103
	10	120
HbV (Hb, 10 g/dL; lipid, 6 g/dL) with $C_{12}E_{10}$	0.1	107
	0.4	111.3 ± 3.6[b]
	1.0	114
	10.0	112
Hb solution (10 g/dL) without $C_{12}E_{10}$	0.005	121
	0.02	103.9 ± 4.4[b]
	0.05	101
	0.5	124
	5	97

[a]LPS was spiked in the $C_{12}E_{10}$-solubilized vesicles and HbV, and Hb solution without $C_{12}E_{10}$. All the spiked LPS concentrations are converted to the conditions of [Hb] = 10 g/dL, or [lipid] = 6 g/dL. All the recoveries were within $100 \pm 25\%$.
[b]$n = 3$.

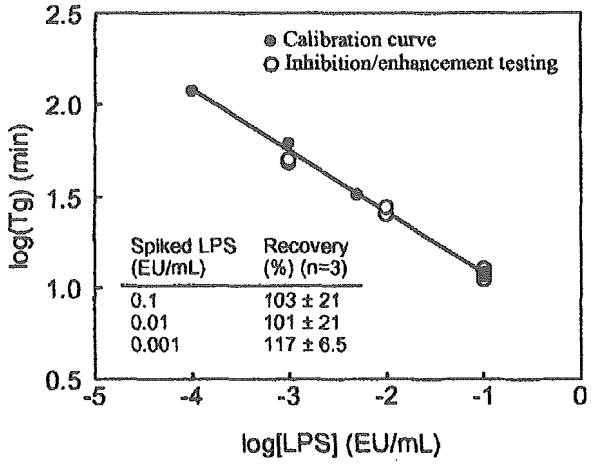

Figure 5. A calibration curve for the Pyrosep® method including the treatment of $C_{12}E_{10}$, and the results of the LPS-spiking test to the HbV suspension. Inset: The recovery of the spiked LPS in the HbV suspension. During the procedure, the HbV suspension was diluted 25 times. Therefore, the spiked LPS concentrations of 0.1, 0.01, and 0.001 EU/mL correspond to 2.5, 0.25, and 0.025 EU/mL, respectively, at [Hb] = 10 g/dL. The original LPS content in the HbV suspension was 0.011 EU/mL at [Hb] = 10 g/dL.

well documented that the biological activity of the LPS is significantly suppressed when an LPS molecule is incorporated into phospholipid vesicles.[25,26,44,45] However, there has been no report on the quantitative observation of the LPS–phospholipid vesicle interaction. Using the ITC method, we quantitatively clarified for the first time that LPS from the *Salmonella Minnesota* wild type (smooth form) was inserted into the phospholipid vesicles with an enthalpy change (ΔH) of ~ -80 kcal/mol and the maximum incorporation of ~ 7.6 mol% on the outer surface of the vesicles. To our knowledge, the ΔH value of PEG_{5000}–DSPE (MW of PEG = 5 kDa) for the same phospholipid vesicles is only -13 kcal/mol.[38]

Table 3. Detection of LPS in the LPS-Contaminated Vesicles with or Without the $C_{12}E_{10}$ Treatment[a]

Treatment	LPS (EU/mL)	Recovery (%)
Without $C_{12}E_{10}$	0.6	60
With $C_{12}E_{10}$	1.03	103

[a][lipid] = 1.2 g/dL, [spiked LPS] = 1.0 EU/mL. The higher recovery of the $C_{12}E_{10}$-solubilized vesicles indicates the necessity of the pretreatment.

This comparison indicates that LPS inserted into the bilayer membrane is thermodynamically more stabilized than PEG_{5000}–DSPE. The large difference in ΔH is probably due not only to the hydrophobic interactions by the eight alkyl chains of LPS but also to the hydrogen bonding of the amide bonds into the interface of the hydrophobic and hydrophilic regions that contribute to the interaction of LPS with the phospholipid bilayer membrane. As for the interaction between Hb and LPS, the ITC analysis was recently reported by Jurgens et al.,[46] who clarified that 3–5 LPS molecules bind to one Hb molecule.

These ITC results clearly emphasize the necessity of the pretreatment of HbV for the LAL quantitative measurement of LPS; that is, to release LPS from the phospholipid vesicles using an organic solvent[47] or surfactant.[28,29] We tested the solubilization of HbV with a surfactant because of the presence of a large amount of Hb that is insoluble in an organic solvent. We determined the optimal condition as Method 1 to detect the LPS content, with the best detection limit at 0.1 EU/mL for HbV at [Hb] = 10 g/dL. The lipid bilayer of HbV is composed of DPPC/cholesterol/DPEA/PEG_{5000}–DSPE at a molar ratio of 5:5:1:0.033, and the vesicles are quite stable due to the

high phase transition temperature (T_c) of DPPC (41°C), the improved molecular packing using cholesterol, the repulsive force between the bilayer membrane due to the presence of a negatively charged DPEA, and the steric hindrance of the PEG chains of PEG–DSPE. However, the procedure of the $C_{12}E_{10}$ addition and incubation at 42°C (near T_c) for 2 min completely diminished the light scattering of HbV, and this condition can effectively solubilize the HbV.

We confirmed that $C_{12}E_{10}$ showed a significant inhibitory effect at a higher concentration that is probably due to the inhibition of gel formation or solubilization of the gelated coagulin (Fig. 3). However, the final concentration of $C_{12}E_{10}$ at 0.1 vol% showed a moderate inhibitory effect. As for the other surfactants, nona(oxyethylene) dodecyl ether ($C_{12}E_9$) showed a more significant inhibitory effect for the LAL assay even though it showed a sufficient solubilization of HbV, and Triton-X 100 showed a lower solubilization of HbV. An anionic surfactant, SDS, did not show complete HbV solubilization. Moreover, according to the literature, SDS completely inhibits gelation only at >0.005 wt%.[29] On the contrary, nonionic surfactants, especially $C_{12}E_{10}$, moderately decrease the LAL activity in relation to the surfactant concentration. $C_{12}E_{10}$ has a sufficient ability to dissolve vesicles and a moderate inhibitory effect on the LAL assay. The solubilization of HbV with $C_{12}E_{10}$ should lead to the formation of micellar structures containing $C_{12}E_{10}$, LPS, and the lipid components. $C_{12}E_{10}$ shares 86 mol% of the components of the micelles, which is significantly higher than PEG–DSPE (0.04 mol%), and the aggregation number of the $C_{12}E_{10}$ micelle is estimated to be ~100.[41] Therefore, the LAL proteins could interact with the lipid-A moiety at the interface of the aqueous and hydrophobic phases of the micelles without the excluded volume effect of the PEG–DSPE.

Improvement of the detection limit requires a lower dilution factor and lower $C_{12}E_{10}$ concentration. However, a reduction in the dilution factor is accompanied by an increase in the concentration of $C_{12}E_{10}$. When the [$C_{12}E_{10}$] was 0.1 vol%, the inhibitory effect was minimal (see Fig. 4), and we obtained the optimal pretreatment condition as entry No. 1 in Table 1. The total dilution factor of this condition is 400. The FDA Guideline defines the maximum valid dilution (MVD) depending on the maximum dose (mL/kg) and lysate sensitivity.[17] The MVD for HbV should be 1000 (= 0.25/0.00025) under the condition that the LPS limit for HbV is 0.25 EU/mL, and the detection limit in the standard curve is 0.00025 EU/mL [log(0.00025) EU/mL = −3.60; (see Fig. 4). Therefore, the dilution factor of 400 is within the MVD.

We compared the measurement of the LPS content in the vesicles with or without $C_{12}E_{10}$ and found that the solubilization of vesicles with $C_{12}E_{10}$ showed a higher LPS concentration. This result clearly demonstrates that LPS molecules in the bilayer membrane or in the inner aqueous phase of the vesicles are released and detected after the solubilization. The recovery of spiked LPS was reproducible within the range 92–120% over a wide range of LPS concentrations. The FDA Guideline requires LPS recovery in the range of 100 ± 25% to indicate no influence of solutes in a specimen.[17] This value was revised to 100 ± 50% in 1991.[48] In spite of the ITC analysis of Hb that indicated 3–5 LPS molecules bind to one Hb,[46] we did not see any interference effect by the presence of Hb. Our result is in contrast to previous reports. Levin et al.[22,49] reported that LPS bound to Hb showed activation of LAL, whereas Archambault et al.[50] reported a significant reduction of LAL activity when the LPS was bound to Hb. Jurgens et al.[46] reported that LAL activity depended on the concentrations of LPS and Hb in the assay. The reason for these discrepancies is not clear, however, there is a large difference in the LPS concentrations for the LAL assay between the reports. In our assay conditions, we measured the LPS concentration at <0.2 EU/mL (~20 pg/mL). On the other hand, other groups conducted the experiments at much higher LPS concentrations (100 pg/mL–800 μg/mL). In some cases, the LPS concentrations were above the critical aggregation concentrations (10–38 μg/mL).[40] It was reported that the LAL activity was significantly suppressed by the LPS aggregation.[51] In the presence of Hb, LPS should bind to Hb rather than form aggregates, resulting in an apparent enhancement of the LAL activity. On the contrary, in our significantly diluted assay condition, it can be speculated that LPS should not be aggregated and be more freely dispersed from Hb, and the presence of Hb should not induce any inhibition or enhancement.

The LPS measurement is often performed to monitor patients with septic shock. Because blood plasma contains some unknown elements that inhibit or accelerate the LAL assay, Obayashi et al.[52] proposed perchroic acid (PCA) treatment to inactivate or remove the interfering plasma components and to obtain a sufficient recovery of

the spiked LPS. In this method, the proteins denatured by PCA are removed as a precipitate. However, this method leads to underestimation of the LPS content because LPS strongly binds to plasma proteins, such as albumin, lipoprotein, and the LPS-binding protein,[53] which should denature and precipitate after the PCA. The new PCA method[54] includes the addition of an alkaline solution to solubilize the denatured protein so that the recovery of LPS is much improved. The dilution of plasma with water and subsequent heating at 100°C for 10 min in the presence of a weak surfactant such as Triton-X100 also releases LPS from the plasma protein.[42,45] On the other hand, our HbV does not contain plasma-derived interfering elements because it is made from ultrapurified Hb solution. During the solubilization of HbV with $C_{12}E_{10}$ at 42°C for 2 min, Hb is partly oxidized to form metHb. However, there is no further denaturation of metHb and its precipitate. Moreover, a derivative of β-1,3-glucan, which is well-known as a significant interfering element for the LAL assay,[55,56] is intentionally added to the LAL reagent from Wako Pure Chemicals Industries, so the influence of contaminated β-1,3-glucan in a specimen is eliminated.[57] For all the pretreatments, the kinetic–turbidimetric assay system can be used for the LAL clotting assay using the Toxinometer®.[43,58] In the case of Hb and HbV, the strong absorption band of the Hb molecule between 400 and 600 nm may affect not only the turbidimetric measurement but also the chromogenic measurement with detection wavelengths of 405 or 545 nm (e.g., Endospecy, Seikagaku Kogyo, Ltd., Japan). In this sense, the detection wavelength of 660 nm using the Toxinometer® should be appropriate.[58]

The Pyrosep® method (Method 2) was very effective in detecting trace amounts of LPS in the $C_{12}E_{10}$-solubilized HbV samples. The solubilization of HbV requires a dilution factor of 25 as in Method 1 (see Fig. 1). There is no additional dilution afterward because the solubilized HbV was treated with the Pyrosep® column and the LPS-bound agarose gel was directly mixed with the LAL reagent. As a result, the lowest LPS concentration for the calibration curve was 0.0001 EU/mL, with a dilution factor of only 25; therefore, the detection limit was calculated to be 0.0025 EU/mL in HbV at [Hb] = 10 g/dL. The LPS content of one HbV suspension was determined to be <0.1 EU/mL, by Method 1, whereas it was 0.011 EU/mL as measured by the Pyrosep® method. This latter method also showed a sufficient recovery of the spiked LPS for a wide range of LPS concentrations. Not only the electrostatic interaction between the cationic region of the histidine residue and the anionic region of LPS, but also the hydrophobic interaction between the spacer region of the histidine–agarose conjugate and alkyl chains of LPS should contribute to the specific adsorption of LPS on the agarose gel.[30] After the adsorption of LPS, the agarose gel was washed with LPS-free water so that all the interfering elements, such as a surfactant, were removed before reacting with LAL reagent. Because Hb was also washed out, the detection wavelength was not limited to 660 nm to avoid the absorption band of Hb. As far as we know, this is the first attempt to measure the LPS content by the combination of Pyrosep® and surfactant pretreatment.

This modified LAL assay using $C_{12}E_{10}$ and the Toxinometer® is routinely used in our production system of HbVs. Significant attention is paid to the quality control of HbVs for preclinical studies, and all the HbVs prepared under sterile conditions showed an LPS content of <0.1 EU/mL at [Hb] = 10 g/dL. Moreover, utilization of the Pyrosep® improved the detection limit to 0.0025 EU/mL. Our method enables an accurate measurement of trace amounts of LPS in HbVs as an oxygen carrier of which the dose rate should be significantly large in comparison with the conventional drugs. Of course, this method is applicable to the quality control of other phospholipid vesicles and also protein drugs such as albumin and their recombinant types that strongly bind LPS.

ACKNOWLEDGMENTS

The authors gratefully acknowledge Mr. J. Takahashi at Wako Pure Chemicals Industries (Tokyo, Japan) for discussion of the experimental instruments, and Dr. K. Kobayashi and Dr. H. Horinouchi (Keio University) for the discussion of the LPS removal and the experimental procedure. This work was supported in part by Health Sciences Research Grants (H15–Research on Pharm. & Med. Safety–011, 016, Artificial Blood Project), the Ministry of Health, Labour and Welfare, Japan, Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (B12480268, B12558112), and 21 COE "Practical Nano-Chemistry" from MEXT, Japan.

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Acta Anaesthesiol Scand 2004; 48: 46–54
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ACTA ANAESTHESIOLOGICA SCANDINAVICA

Haemodynamic effects of volume resuscitation by hypertonic saline-dextran (HSD) in porcine acute cardiac tamponade

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Background: Hypertonic saline-dextran (HSD) has been utilized for small-volume resuscitation in acute circulatory shock. However, HSD has also been reported to induce myocardial depression. The aim of this study was to examine the effects of HSD on cardiac performance and splanchnic perfusion in a low cardiac output model based on experimental cardiac tamponade.
Methods: Seven anaesthetized, mechanically ventilated pigs of both sexes (weight 24 ± 2 kg, mean ± SEM) completed a randomized, cross-over protocol. A low cardiac output state was established by intrapericardial infusion of dextran. Animals were resuscitated by bolus infusions (4 ml kg^{-1} in 2 min) of either 7.5% hypertonic saline-dextran or Ringer's acetated solution (RAc) and then observed during tamponade (20 min) and following its release (40 min). Central haemodynamics, portal venous (QPV) and renal arterial (QRA) flows were measured together with gastric, jejunal, hepatic and renal laser-Doppler flowmetry.
Results: Resuscitation using HSD in a low cardiac output state completely restored QPV and improved gastric, jejunal, hepatic and renal microcirculation as assessed by laser-Doppler flowmetry while no significant effect was observed in QRA. No such beneficial effects could be observed when animals were resuscitated using RAc. The improved haemodynamic state by HSD was maintained following release of cardiac tamponade while perfusion in RAc resuscitated animals returned to baseline or even remained depressed (hepatic and renal microcirculation). No signs of cardiodepression by HSD were observed.
Conclusion: Resuscitation using HSD in a low cardiac output state restored splanchnic perfusion and microcirculation without any signs of cardiodepression.

Accepted for publication 10 September 2003

Key words: Cardiogenic shock; hypertonic solutions; microcirculation; splanchnic circulation.

HYPERTONIC, hyperoncotic solutions have been successfully applied for small-volume resuscitation in acute circulatory shock due to trauma (1), haemorrhage (2), sepsis (3) and burn injuries (4). A hypertonic 7.5% saline solution combined with dextran as colloid (hypertonic saline dextrane, HSD) is the most common formulation used both experimentally and clinically. The side-effects reported using HSD in acute circulatory shock include hyperchloraemic acidosis, plasma hyperosmolarity, impaired coagulation and intracellular dehydration. In addition, transient myocardial-depressive effects of HSD, observed as reductions of stroke volume, have been reported both in ischaemic and non-ischaemic isolated, perfused hearts (5, 6). Such negative inotropic myocardial effects have been ascribed the hypertonic saline component (7). Kien et al. (8) reported that hypertonic saline infusion during reperfusion following coronary occlusion worsened coronary blood flow and contractile function in the area of regional ischaemia. Early detrimental effects of HSD on cardiac performance were also reported in a model of intestinal ischaemic shock (9). The stimulatory effects of hypertonic saline reported by others may be explained by central neurogenic sympathetic mechanisms (10). Increased intracerebroventricular sodium concentrations have been demonstrated to enhance efferent sympathetic nerve activity and thus to improve cardiovascular function as well as tolerance to acute hypovolaemia (11).

Following the establishment of small-volume resuscitation by HSD in prehospital treatment protocols, this therapeutic strategy is now attracting increasing interest in hospitalized patients. Low cardiac output states are common in patients requiring intensive care and optimization of intravascular volume remains

a cornerstone in the clinical management. Persistent reduction of cardiac performance results in splanchnic hypoperfusion, which is thought to contribute to progressive circulatory deterioration or therapeutic refractoriness. This study aimed to further clarify the acute effects of HSD on cardiac function as well as regional splanchnic circulation in cardiogenic circulatory failure induced by acute cardiac tamponade.

Methods

This study was approved by the Ethics Committee for Animal Experiments at Göteborg University and the care and handling of the animals conformed to the principles set forth in the 'Guide for the Care and Use of Laboratory Animals' (National Academy of Sciences, ed. 1996, ISBN 0-309-05377-3). Nine Swedish landrace pigs (n = 9) of either sex were enrolled into the study. Two animals were excluded from the study because of technical problems in positioning the intrapericardial catheter and in placing a watertight suture. Results are reported from the remaining seven animals, weighing 24 ± 1.8 kg (mean ± SEM), that entered the study. All animals were fasted for 24 h with water ad libitum.

Anaesthesia and surgical preparation

Anaesthesia was induced by bolus injection of ketamine (Ketalar®, Parke-Davis, Solna, Sweden; 30 mg kg^{-1} body weight) intramuscularly and followed by an intravenous bolus injection of α-chloralose (Merck, Darmstadt, Germany; pH = 7.40, 100 mg kg^{-1} body weight). Anaesthesia was maintained by an intravenous infusion of α-chloralose at 25 mg h^{-1} kg^{-1} body weight. Supplemental bolus doses of fentanyl (100–200 μg, Leptanal®, Janssen-Cilag, Austria) were given during the surgical preparation. No muscle relaxants were used.

Each animal was tracheotomized and mechanically ventilated (Servo 900, Siemens, Stockholm, Sweden) with oxygen in air ($F_iO_2 = 0.40$) in a volume-controlled mode to maintain normocapnia and an arterial pH around 7.4. Core body temperature was kept around 39°C using heating blankets. Isotonic Ringer's acetated solution (RAc) was infused perioperatively at 20 ml kg^{-1} h^{-1} to maintain normovolaemia as indicated by central venous pressure (CVP) and pulmonary artery occlusion pressure (PAOP) measurements.

A central venous catheter (7F) was inserted from the left internal jugular vein, and both the femoral artery and the vein on one side were catheterized to measure central venous and mean arterial blood pressures (MAP) and to provide vascular access for volume infusions. A pulmonary artery catheter (93 A-131H-7F, Baxter Medical AB, Solna, Sweden) was inserted via the right jugular vein to measure cardiac output (CO) by thermodilution as the average of triplicate measurements within a ±10% range using a REF-1 cardiac output computer (Baxter Medical AB, Solna, Sweden). A midline laparotomy was performed and an ultrasonic transit time flowmeter probe (H16S, Transonic systems, Ithaca, NY) was placed around the portal vein to measure portal venous, and thus the total mesenteric blood flow (QPV). Laser-Doppler probes (wavelength 780 nm, 0.25-mm fibre separation; Perimed AB, Järfälla, Sweden) were placed in the corporal part of the stomach and in the jejunum 50 cm aboral from the ligament of Treitz via a small duodenotomy to measure the gastric (LDF-gastric) and jejunal (LDF-jejunum) microcirculation. A flexible, Teflon disc holding a laser-Doppler probe (wavelength 780 nm, 0.25 mm-fibre separation, type no. 421, Perimed AB, Järfälla, Sweden) was placed on the anterior surface of the right liver lobe to measure liver microcirculation (LDF-hepatic). A right subcostal incision was made and the renal artery was dissected free in the retroperitoneal space. An ultrasonic transit time flowmeter probe (H6S, Transonic systems, Ithaca, NY) was placed around the renal artery to measure renal blood flow (QRA). A laser-Doppler probe similar to the one used on the liver surface was placed on the lateral surface of the upper renal pole to measure renal microcirculation (LDF-renal).

A left thoracotomy was performed and a Foley catheter (12 Ch) was placed intrapericardially in a dorsal position and secured in the pericardial sac with a watertight purse-string suture. Warm dextran (37°C) was infused into the pericardial space to establish cardiac tamponade as previously described (12). A three-way stop-cock was connected to the catheter to allow for intermittent measurement of intrapericardial pressure.

Measurement of haemodynamic variables

All pressures were measured at the midchest level using calibrated pressure transducers (DPT6003, Peter von Berg, Medizintechnik GmBH Eglharting, Germany) and recorded using a polygraph (Grass 7D Polygraph, Grass Instrument Co, Quincy, MA). Systemic vascular resistance (SVR) and

pulmonary vascular resistance (PVR) were calculated using standard equations based on pressures and flows.

The portal and renal ultrasound transit time flow probes (for QPV and QRA, respectively) were connected to an HT207 Flowmeter (Transonic Systems, Ithaca, NY) and the calibrated signals recorded on the polygraph as above. The laser-Doppler probes were connected to two Periflux® PF-4001 laser-Doppler Flowmeter units (780 nm laser light wavelength, 12 kHz band width, 0.2 s time constant, Perimed AB, Järfälla, Sweden) and the laser-Doppler signals (in perfusion units, PU) were continuously recorded on an IBM-compatible PC 486 computer using the RS232 serial interface of the base unit. All analyses of the laser-Doppler signals were performed using the Perisoft® software (Perimed AB, Järfälla, Sweden). The laser-Doppler catheters were calibrated according to the manufacturer's instruction in a zeroing disc and in moving standard.

Experimental protocol (Fig. 1)

All animals were allowed to stabilize after completion of the surgical procedures for 1 h before the experimental protocol was started. Baseline data were recorded and cardiac tamponade was established by infusion of dextran (approximately 80–100 ml) to increase the intrapericardial pressure by 10 mmHg greater than baseline. The cardiac tamponade was allowed to stabilize for 10 min, adjusting the volume of dextran to achieve constant intrapericardial pressure. The protocol was based on a randomized, cross-over design in which animals were first resuscitated by bolus infusion (during 2 min) of 4 ml kg^{-1} body weight of either 7.5% hypertonic saline with 6% dextran 70 (HSD) (Rescueflow®, BioPhausia AB, Uppsala, Sweden) or Ringer's acetated solution (RAc) (Ringer-acetat®, B Braun Medical AB, Bromma, Sweden) and then observed during 20 min. The infused dextran was then drained from the pericardial to relieve the tamponade with a return to baseline pericardial pressure and animals were observed for a further 40 min. Following an intermediate resting period of 60 min the same procedure as above was repeated using the alternate resuscitation fluid.

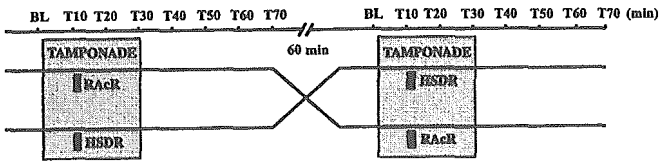

Fig. 1. Experimental protocol was based on a prospective, randomized, cross-over design. Following recording of baseline data, cardiac tamponade was established and allowed to stabilize for 10 min (T10). Animals were then resuscitated using bolus (4 ml kg^{-1} body weight in 2 min) infusion of either Ringer's acetated solution (RAc) or hypertonic saline-dextran (HSD) and observed during 20 min (T20–30). The tamponade was then withdrawn and the animals were observed for a further 40 min (T40–70). Following 1-h 'washout' period, the tamponade procedure was repeated using the alternate resuscitation fluid.

Statistical analyses

All data are expressed as mean ± SEM. Laser-Doppler blood flows (LDFs) are given in arbitrary perfusion units (PUs), both as absolute numbers and as changes relative to baseline. All statistical analyses were performed by using the Statview® version 5.0 for Macintosh software (Abacus Concepts Inc., Berkley, CA). Inter- and intragroup differences were analyzed by uni- or bivariate analysis of variance (ANOVA) for repeated measures, and Fisher's protected least-significant difference post hoc test was used to determine differences. Statistical significance was set a *P*-value less than 0.05.

Results

Baseline haemodynamic variables were similar for both the first and second tamponade procedure (Table 1). Cardiac tamponade reduced both CO and MAP in parallel to increased CVP while PAP and PCWP did not change significantly. Importantly, no differences in central filling pressures were observed in response to volume resuscitation using RAc or HSD. As would be expected from the experimental design, CO remained constant in both the RAc- and HSD-resuscitated animals throughout the tamponade period. Following release of the tamponade, an increase in CO even greater than baseline was observed in the HSD-resuscitated animals and MAP returned to baseline values in both the RAc- and HSD-resuscitated animals. No consistent, statistically significant changes in SVR were observed during the tamponade period, while SVR was transiently reduced in the initial post-tamponade period in the HSD-resuscitated animals. An increase in PVR was observed in the RA-resuscitated animals during tamponade, but this increase failed to attain statistical significance upon release of the tamponade.

Cardiac tamponade promptly reduced QPV (Table 2, Fig. 2). Resuscitation by HSD increased QPV

Table 1

General haemodynamic variables in animals (n = 7) subjected to cardiac tamponade (T10) and resuscitated (T20–30) with Ringer's acetated solution or hypertonic-saline dextran.

	Baseline	T10	T20	T30	T40	T50	T60	T70
Mean arterial pressure (mmHg)								
RAc	110 ± 6	86 ± 5*	83 ± 7*	87 ± 8*	103 ± 9	101 ± 9	97 ± 8	99 ± 9
HSD	108 ± 6	92 ± 6*	90 ± 4*	87 ± 10*	103 ± 4	100 ± 7	99 ± 7	102 ± 7
Cardiac output ($l\,min^{-1}$)								
RAc	2.94 ± 0.40	2.01 ± 0.32*	2.03 ± 0.34*	2.22 ± 0.38*	2.80 ± 0.46	2.68 ± 0.46*	2.66 ± 0.46	2.52 ± 0.41*
HSD	2.50 ± 0.33	2.06 ± 0.29*	2.04 ± 0.34*	2.07 ± 0.38*	3.00 ± 0.32*	3.08 + 0.39	3.03 + 0.43	2.56 ± 0.42
Central venous pressure (mmHg)								
RAc	5.4 ± 0.4	6.4 ± 0.5*	6.9 ± 0.6*	7.4 ± 0.5*	6.0 ± 0.4	5.4 ± 0.2	5.4 ± 0.2	5.4 ± 0.2
HSD	5.3 ± 0.3	6.7 ± 0.4*	7.0 ± 0.4*	7.9 ± 0.3*	7.3 ± 0.6*†	5.9 ± 0.1	5.6 ± 0.2	5.6 ± 0.2
Pulmonary artery pressure (mmHg)								
RAc	15.6 ± 1.0	15.1 ± 1.4	18.3 ± 2.1	16.1 ± 1.2	15.9 ± 1.9	17.1 ± 1.6	16.4 ± 1.8	16.7 ± 1.9
HSD	15.9 ± 1.7	14.9 ± 0.8	15.4 ± 1.3	14.7 ± 1.0	18.9 ± 1.5	16.9 ± 0.7	16.3 ± 0.7	15.9 ± 1.1
Pumnonary capillary wedge pressure (mmHg)								
RAc	6.6 ± 0.4	7.3 ± 0.5	7.6 ± 0.4	7.7 ± 0.5	7.0 ± 0.3	6.7 ± 0.4	6.7 ± 0.3	6.4 ± 0.2
HSD	6.6 ± 0.3	6.7 ± 0.2	7.1 ± 0.3	8.0 ± 0.7	8.9 ± 1.2*	7.6 ± 0.2	6.6 ± 0.4	6.9 + 0.4
System.ic vascular resistance ($dyne.s.cm^{-5}$)								
RAc	3121 ± 363	3574 ± 471*	3325 ± 363	3200 ± 349	3091 ± 344	3186 ± 348	3146 ± 336	3314 ± 317
HSD	2995 ± 239	3343 ± 367	3021 ± 347	2681 ± 380	2506 ± 269*†	2594 ± 260*†	2713 ± 338	2873 ± 343
Pulmonary vascular resistance ($dync.s.cm^{-5}$)								
RAc	301 ± 80	401 ± 109	530 ± 152*	420 ± 132*	384 ± 157	440 ± 166	460 ± 184	470 ± 180
HSD	282 ± 65	327 ± 48	302 ± 56†	256 ± 47	267 ± 36	276 ± 51	304 ± 65	292 ± 67

*Significant difference from baseline ($P < 0.05$).
†Significant difference between resuscitation fluids ($P < 0.05$).
Values are mean ± SEM.
RAc = Ringer's acetated solution; HSD = hypertonic saline-dextran.

Table 2

PorA venous and renal arterial (blood flows in animals (n = 7) subjected to cardiac tamponade (T10) and resuscitated (T20–30) with Ringer's acetated solution or hypertonic saline-dextran.

	Baseline	T10	T20	T30	T40	T50	T60	T70
QPV (ml min^{-1})								
RAc	596 ± 95	496 ± 70*	490 ± 73*	503 ± 74*	584 ± 94	584 ± 89	574 ± 87	567 ± 86
HSD	564 ± 93	493 ± 75*	561 ± 89†	564 ± 107†	647 ± 95*†	621 ± 91	607 ± 89	599 ± 89
QFA (ml min^{-1})								
RAc	217 ± 29	196 ± 38	201 ± 46	213 ± 48	246 ± 52	256 ± 53	259 ± 53	254 ± 53
HSD	280 ± 50	241 ± 52	249 ± 50	243 ± 54	266 ± 57	269 ± 47	267 ± 45	274 ± 43

*Significant difference from baseline ($P < 0.05$).
†Significant difference between resuscitation fluids ($P < 0.05$).
Values are mean ± SEM.
QPV = Portal venous; QRA = renal arterial; RAc = Ringer's acetated solution; HSD = hypertonic saline-dextran.

to baseline values even during maintained tamponade conditions and resulted in a transient increase above baseline when the tamponade was released. No increase in QPV was observed in the animals resuscitated with Rac, and QPV returned only to baseline, with no overshoot, in the post-tamponade period. No significant changes in QRA were observed throughout the protocol in the Rac-resuscitated animals while an intermediate decrease was observed in the HSD-resuscitated animals during tamponade (Table 2, Fig. 2).

The establishment of cardiac tamponade reduced LDF-gastric and LDF-renal laser-Doppler signals while LDF-jejunum values remained unchanged (Table 3, Fig. 3). Resuscitation by HSD increased LDF-gastric and LDF-renal to baseline values while RAc was ineffective in this respect, also considering the prolonged decrease in LDF-hepatic observed in the post-tamponade period.

Discussion

The main findings of this study were that acute volume resuscitation using hypertonic saline-dextran solution (HSD) during constant diastolic dysfunction maintained by cardiac tamponade did not result in any myocardial depression but rather improved splanchnic blood flow and microcirculation as compared with isotonic saline volume resuscitation.

Resuscitation using HSD has commonly been reported to increase cardiac output by intravascular volume expansion, thus increasing cardiac preload, and by reduced cardiac afterload due to vasodilation of resistance vessels, resulting in an apparent improvement of left ventricular systolic function (13, 14). This study was designed to standardize reduced cardiac preload by a constant restriction of ventricular diastolic expansion through an increased intrapericardial pressure. No reduction of arterial pressure was observed during the HSD infusion, similar to the animals resuscitated with RAc, and taken together cardiac output remained reduced at a stable level throughout the tamponade period.

It has been argued that HSD exerts direct positive inotropic effects on the heart (15) while other studies have on the contrary demonstrated that HSD may actually induce myocardial depression through a negative inotropic effect (5–7) or by cholinergic-like effects when hypertonic solutions are rapidly infused (16). While indices of cardiac contractility were not specifically assessed in the present study, it seems highly unlikely that any negative inotropic effects by HSD would not have resulted in a reduced cardiac output. A positive inotropic effect of HSD would be expected to reduce end-systolic volume, thus increasing stroke volume despite a constant diastolic impairment, resulting in an increased cardiac output. This could not be observed during the tamponade period. The increase in cardiac output following HSD resuscitation and relief of the tamponade coincides with increased filling pressures (CVP and PAOP) and would thus indicate the effect of more effective immediate intravascular volume expansion compared with the Rac-resuscitated animals, rather than any positive inotropy *per se*.

The improvement in splanchnic perfusion during HSD resuscitation occurred despite a constant low cardiac output state. In fact, HSD resuscitation restored QPV to baseline values during the tamponade, demonstrating an effective redistribution of cardiac output towards the splanchnic region. No such beneficial effect could be observed during RAc resuscitation or in the renal circulation (QRA). The increase in QPV even above baseline values following

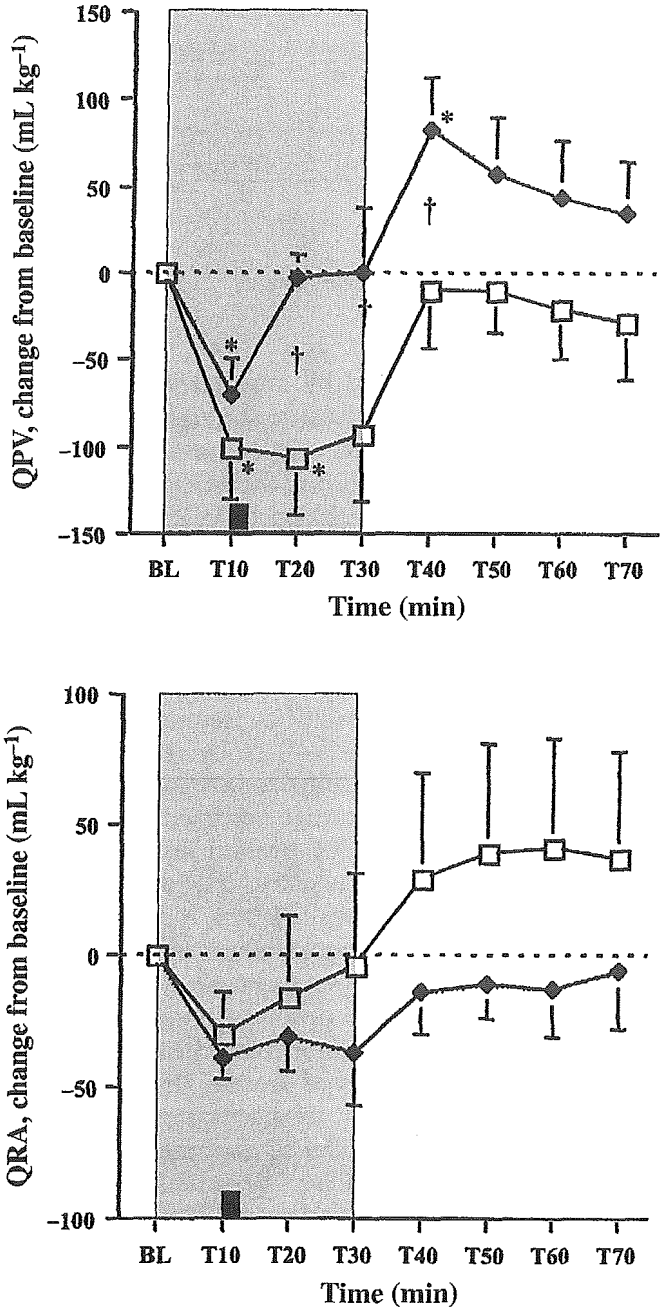

*Fig. 2. Changes in portal venous blood flow (QPV; A) and renal arterial blood flow (QRA; B) from baseline (ml min^{-1}). Cardiac tamponade was stabilized to T10, and animals resuscitated using bolus infusions (4 ml kg^{-1} body weight during 2 min) of either hypertonic saline-dextran (HSD; ◆) or Ringer's acetated solution (□), after which the tamponade was withdrawn. *Significant difference compared with baseline ($P < 0.05$). †Significant difference between resuscitation fluids ($P < 0.05$). Values are mean ±SEM, $n = 7$. The shaded area indicates the cardiac tamponade period and the black block indicates the infusion of either HSD or Ringer's acetated solution (RAc).*

the release of the tamponade can be attributed to a concomitant increase in cardiac output. The selective increase in QPV during tamponade by HSD resuscitation might relate to changes in mesenteric vascular resistance. The mesenteric vasculature is a major contributor to systemic vascular resistance, particularly in hypotensive states. It has previously been reported that HSD increases vascular conductance by deswelling of endothelial and blood corpuscular cells as well as by direct vasodilation (2, 17), possibly mediated by prostacyclin (18), which selectively increases splanchnic blood flow (19). Cardiac tamponade increased central venous pressure and consequently hepatic venous pressure, which would acutely impede portal flow blood across the liver that normally occurs along a portal to hepatic venous pressure gradient of a few mmHg. Another explanation for the selective increase in portal blood flow observed in this study could be that HSD, by intravascular volume expansion, increased portal venous pressure to the extent that a normal pressure gradient was re-established, leading to a waterfall-like effect on portal blood flow. The less potent intravascular volume expander RAc would not be as effective in this respect.

The lack of changes in QRA during tamponade and resuscitation indicates the effective renal autoregulation that is well documented in the arterial pressure range observed in this study. Resuscitation using either HSD or RAc did not result in any significant differences in QRA, to some extent related to the variation in the flow response to RAc. The similar trends observed do not give any support to beneficial effects of HSD resuscitation on QRA.

Microcirculatory changes were assessed by laser-Doppler flowmetry. This technique is well established to measure capillary perfusion (20). Although the laser-Doppler probes were calibrated to provide absolute values in perfusion units (Table 3), the Doppler principle is inherently relative, and the results will mainly be discussed in terms of changes from baseline (Fig. 3).

As would be expected from the increase in QPV by HSD resuscitation, both gastric and jejunal microcirculation improved in those animals, while RAc resuscitation failed to support the microcirculation. Interestingly, no significant decrease was observed in the jejunal mucosa during tamponade, as opposed to the gastric mucosa. This illustrates the redistribution of the microcirculation towards the mucosa in low flow states (21). Resuscitation by HSD fully prevented the decrease in hepatic and renal microcirculatory

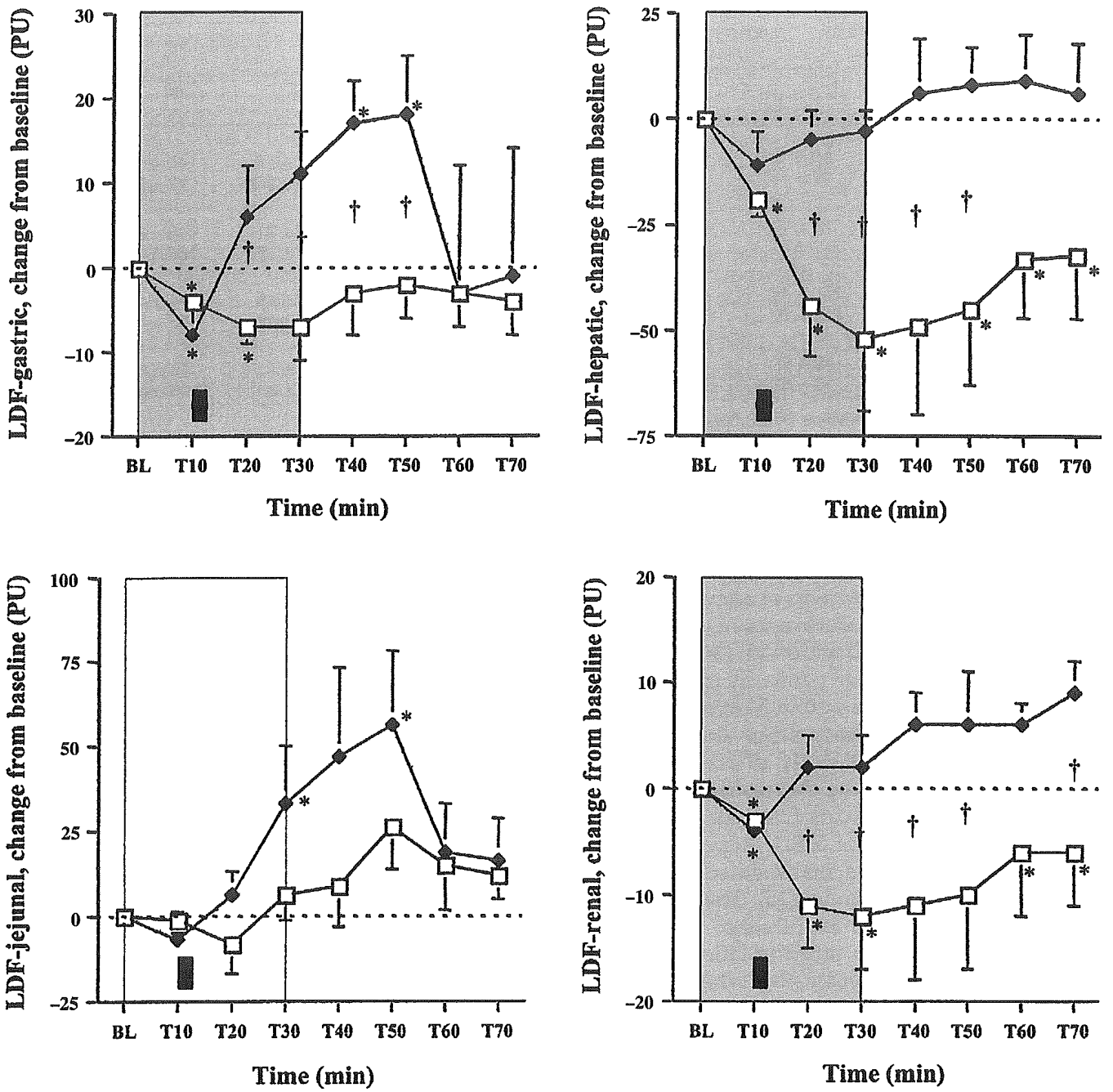

*Fig. 3. Changes in laser-Doppler flows from baseline (PU). Cardiac tamponade was stabilized to T10, and animals resuscitated using bolus infusions (4 ml kg^{-1} body weight during 2 min) of either HSD (◆) or Ringer's acetated solution (□) (T20–30), after which the tamponade was withdrawn. *Significant difference compared to with baseline ($P < 0.05$). †Significant difference between resuscitation fluids ($P<0.05$). Values are mean ± SEM, $n = 7$. The shaded area indicates the cardiac tamponade period and the black block indicates the infusion of either HSD or Ringer's acetated solution (Rac).*

flows seen in the Rac-resuscitated animals. This clearly demonstrates the benefits of HSD to support capillary perfusion. Interestingly, the reductions in hepatic and renal microcirculations persisted even after restoration of portal venous and renal arterial flows when the tamponade was withdrawn from the Rac-resuscitated animals. The particular susceptibility of hepatic and renal capillary perfusion in low flow states has been previously reported (22, 23).

Table 3

Laser-doppler flows in animals (n = 7) subjected to cardiac tamponade (T10) and resuscitated (T20–30) with Ringer's acetated solution or hypertonic saline-dextran.

	Time (min) Baseline	T10	T20	T30	T40	T50	T60	T70
LDF gastric								
RAc	73 ± 10	69 ± 10*	66 ± 11*	67 ± 11	70 ± 13	72 ± 12	71 ± 11	69 ± 10
HSD	80 ± 21	72 ± 19*	86 + 26	91 ± 25	97 ± 24*	98 ± 24*	77 ± 9	79 ± 8
LD17jejunal								
RAc	128 ± 16	127 ± 15	119 ± 12	133 ± 16	136 ± 19	153 ± 21	143 ± 22	139 ± 14
HSD	148 ± 22	141 ± 17	155 ± 20	181 ± 38*	195 ± 45	204 ± 36*	167 ± 30	164 ± 27
LDF hepatic								
RAc	274 ± 48	255 ± 49*	231 ± 47*	222 ± 48*	225 ± 46	229 ± 45	241 ± 46*	242 ± 45*
HSD	247 ± 34	236 ± 37	242 ± 36	244 ± 34	253 ± 43	255 ± 40	256 ± 42	253 ± 43
LEW renal								
RAc	52 ± 8	49 ± 8*	41 ± 8*	40 ± 8*	41 ± 7	42 ± 7	46 ± 6	46 ± 6
HSD	42 ± 4	38 ± 5*	44 ± 6	44 ± 6	48 ± 6	48 ± 8	48 ± 6*	51 ± 6*

*Significant difference from baseline ($P < 0.05$),
†Significant difference between resuscitation fluids ($P < 0.05$).
Values are mean ± SEM.
LDF = laser-doppler flow; RAc = Ringer's acetated solution; HSD = hypertonic saline-dextran.

It might be argued that the resuscitation protocol using equal volumes of HSD and RAc is hampered by the fact that the proportion of administered RAc that will remain in the intravascular compartment will be less than for HSD within 2 h (24). This study, however, was designed to primarily investigate the haemodynamic changes within 20 min of resuscitation during a low cardiac output state. The most significant results of the study all occurred within this time frame. Moreover, this resuscitation protocol adheres to previous work from our institution in which equal volumes of HSD, hypertonic saline and normal saline were investigated in an acute myocardial infarction model (25). The rate of infusion has been reported to influence the effect of HSD on cardiovascular stability. Kien et al. (26) reported an initial hypotension when HSD was infused rapidly (3 ml kg^{-1} body weight during 1 min) that was transient within 5 min. Twice the infusion time was used in this study and no signs of cardiovascular depression were observed. The validity of the crossover design is supported by the fact that no baseline values for any parameter was significantly different. Moreover, with very few exceptions, no significant differences were found between the values at baseline and 70 min later, and those that disappeared within the 60-min resting period in between the tamponade procedures. Finally, the statistical evaluation based on ANOVA for repeated measures failed to detect any significant carry-over effects.

To summarize, resuscitation using HSD at 4 ml kg^{-1} body weight in a low cardiac output state induced by constant reduction of cardiac preload by tamponade, fully restored splanchnic perfusion without any cardiodepressive effects. No such beneficial effects could be observed when animals were resuscitated using an equal volume of RAc. The efficacy of HSD to restore regional, splanchnic microcirculatory flows might be particularly interesting when volume expansion is used to treat a low cardiac output state. Speculatively, such beneficial effects could be valuable to reduce the risk of multiple organ failure that might develop when splanchnic hypoperfusion persists following resuscitation with isotonic solutions.

Acknowledgements

This study was supported by the LUA-project at Sahlgrens University Hospital (Anders Åneman, Hengo Haljamäe) and the Swedish Research Council (K2003-04X-14032-03 A). The authors would like to thank Dr Mats Dahlqvist for assistance in performing the experiments.

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S-Nitrosylated Polyethylene Glycol-conjugated Hemoglobin Derivative as a Candidate Material for Oxygen Therapeutics

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1. INTRODUCTION

Several hemoglobin (Hb)-based derivatives have been studied for use as artificial oxygen carriers. However, they have several limitations[1]. The major side effects include vasoconstriction, abnormal gastrointestinal constriction and platelet stimulation. The most plausible mechanism for these side effects is the nitric oxide (NO) scavenging by acellular Hb itself, since the heme of Hb has a high affinity to NO. A universal problem among all Hb derivatives is their short plasma residence time. The half life time of Hb derivatives in the circulation ranges from 6 to 24 h in animals, and these values are very much shorter than the mean residence time of 120 days for human red blood cells. The hope, therefore, is that the new products will have better applicability in clinical situations where the short-term use of an oxygen carrier is essential.

Recently, it was proposed that the Cysβ93 of Hb is covalently bound with NO, and that this *S*-nitrosylated Hb (SNO-Hb) retains EDRF/NO-like bioactivity[2,3]. Since *S*-nitrosothiols do not react with heme of Hb, SNO-Hb can provide a protected way of delivery of bioactive NO to the tissues. Indeed, SNO-Hb induces relaxation of pre-capillary vessels and inhibits platelet aggregation[3,4]. These insights suggest that SNO-Hb can release NO preferentially where pO_2 is low, dilating small vessels, thus providing more blood to the ischemic tissues. In the circulation, SNO-Hb may still have vasoconstrictive activity because Fe(II)-Hb, which binds oxygen, also has an

Polymer Drugs in the Clinical Stage, Edited by Maeda et al.
Kluwer Academic/Plenum Publishers, New York, 2003

affinity to NO, though SNO-Hb can compensate for this vasoconstricting effect by releasing NO. These dual functions as both a scavenger and donor of NO not only contribute to avoidance of Hb-induced vasoconstriction, but increase the therapeutic potential of SNO-Hb for use in the area of oxygen therapeutics.

2. S-NITROSOHEMOGLOBIN

Hb is a tetramer composed of two α- and two β-subunits. In human Hb, the β-subunit contains one highly reactive sulfhydryl group (Cysβ93). This sulfhydryl residue has been reported to be *S*-nitrosylated to form SNO-Hb within red blood cells[2]. The authors showed a dynamic cycle in which the binding of oxygen to heme iron promotes the binding of NO to the sulfhydryl residues, and deoxygenation is accompanied by an allosteric conformational change that releases the NO group. In this context, Hb is *S*-nitrosylated in the lung when red blood cells are oxygenated, and the NO group is released during arterial-venous transit dependent on the oxygen gradient in the tissues[3]. SNO-Hb, therefore, can release an NO-group to induce vasorelaxation and increase regional blood flow, and then deliver oxygen more efficiently to the tissues with oxygen requirements.

2.1 Preparation of SNO-PEG-Hb

To utilize SNO-Hb as an oxygen carrier, we have developed a pyridoxalated and pegylated SNO-Hb derivative having low oxygen affinity and an optimum plasma residence time. The preparation steps are schematically illustrated in Figure 1. The detailed preparation method is described elsewhere[5]. Briefly, human Hb purified from outdated hman red cell products was mixed with pyridoxal-5' phosphate and pyridoxalation was started by the addition of sodium borohydrate under anaerobic conditions. For the pegylation of pyridoxalated Hb, the activated ester of PEG-bis(succinimidyl succinate) was added very slowly with stirring. *S*-nitrosylation of PEG-Hb was then performed with addition of *S*-nitrosoglutathione. The yield of *S*-nitrosylation was estimated by using a high-performance liquid chromatography (HPLC) coupled with flow reactors of metal and Griess reagent (Fig. 2)[6]. In human Hb, Cysβ93 is

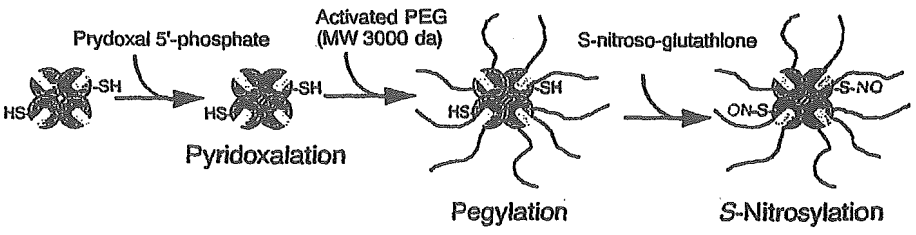

Figure 1. SNO-PEG-Hb preparation. Hb was pyridoxalated, pegylated and then *S*-nitrosylated.

highly reactive and the prefered target for *S*-nitrosylation. The content of NO in SNO-Hb reported in the text was, therefore, expressed on the basis that a fully s-nitrosylated Hb (100% SNO-Hb) contains two NOs because one tetramic Hb is constituted from two β-subunits. The yield of *S*-nitrosylation was usually set to 30-37%.

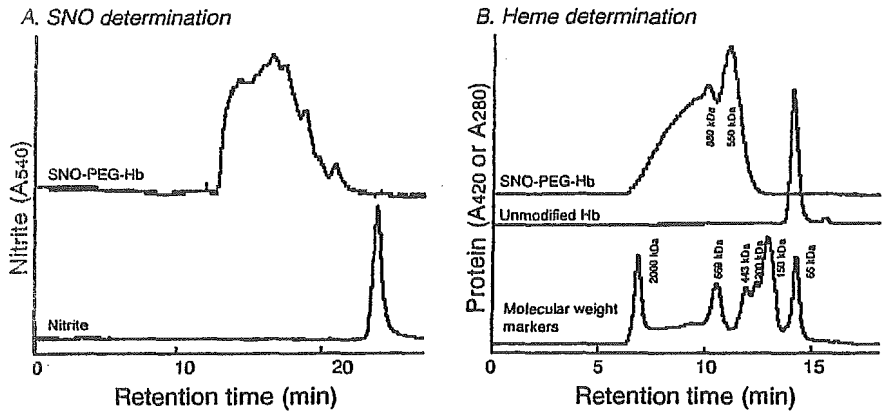

Figure 2 HPLC characterization of (A) Hb-bound NO and (B) heme of SNO-PEG-Hb. (A) Samples were separated on a gel-filtration column (8 x 300 mm, GFC-200, Eicom, Kyoto, Japan) eluted with 10 mM acetate buffer, 0.1 mM EDTA, 100 mM sodium chloride, pH 5.5, at the flow rate of 0.55 mL/min. The eluate was mixed with 1.75 mM mercury chloride at the flow rate of 0.20 mL/min to decompose S-nitrosylated protein, and further mixed with Griess reagent at the flow rate of 0.22 mL/min. The red azo-dye formed was determined by the absorption at 540 nm. (B) For the characterization of molecular weight distribution, proteins were separated on a gel-filtration column (7.6 x 300 mm, TSK G3000SW, Toyo Soda Co. Ltd, Tokyo, Japan) in 10 mM sodium phosphate buffer, 100 mM sodium chloride, pH 6.9, at the flow rate of 0.9 mL/min. Proteins were monitored at 420 nm for heme and at 280 nm for molecular weight markers.

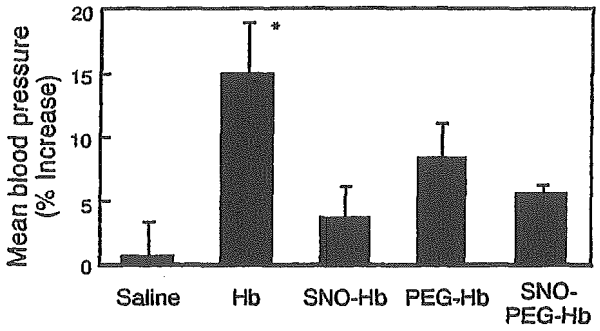

Figure 3 Changes in mean arterial blood pressure 5 min after a bolus injection of Hb materials (125 mg Hb/kg, Hb 10% solution) into male Wistar rats. Relative increase was calculated from the data before and after the injection. Each value represents the mean±SEM of 6-9 animals. *p<0.05 vs saline control by ANOVA followed by Scheffe's test.