Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue

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Erni, Dominique, Reto Wettstein, Sören Schramm, Claudio Contaldo, Hiromi Sakai, Shinji Takeoka, Eishun Tsuchida, Michael Leunig, and Andrej Banic. Normovolemic hemodilution with Hb vesicle solution attenuates hypoxia in ischemic hamster flap tissue. Am J Physiol Heart Circ Physiol 284: H1702–H1709, 2003. First published January 9, 2003; 10.1152/ajpheart.00821.2002.—The aim of this study was to test whether oxygenation in acutely ischemic, collateralized tissue may be improved by normovolemic hemodilution with a solution containing liposome-encapsulated human Hb (HbV). A skin flap model in anesthetized hamsters was used, which consisted of two parts receiving either anatomic or collateral perfusion. Microhemodynamics were investigated with intravital microscopy. Partial tissue oxygen tension was measured with a Clark-type microprobe. Hemodilution was obtained by exchanging 50% of the total blood volume with HbV suspended in 8% human serum albumin (HSA8) or 6% Dextran 70 (Dx70). The size of the vesicles was 276 nm, the P₅₀ was 22 mmHg, and the Hb concentration of the solutions was 7.5 g/dl. Colloid osmotic pressure and viscosity were 49.9 mmHg and 8.7 cP for HbV-Dx70 and 40.0 mmHg and 2.9 cP for HbV-HSA8, respectively. Hemodilution with HbV-Dx70 led to an increase in microvascular blood flow in the ischemic microvessels to maximally 158% (median, P < 0.01), whereas blood flow remained virtually unchanged after hemodilution with HbV-HSA8. In the ischemic tissue, oxygen tension was improved from 11.9 to 17.0 mmHg (P < 0.01) after hemodilution with HbV-Dx70 but remained virtually unchanged after hemodilution with HbV-HSA8. Our study suggests that the oxygenation in acutely ischemic, collateralized tissue may be improved by normovolemic hemodilution with HbV suspended in Dx70. The effect was achieved by an increase in microcirculatory blood flow related to the rheological properties of the suspending medium.

blood substitutes; artificial red blood cells; microcirculation; microhemodynamics; collateral circulation

THE OXYGENATION OF TISSUE rendered ischemic after the acute obstruction of its anatomic blood supply requires perfusion via a collateral vasculature until adequate blood supply is reestablished either by surgical revascularization or spontaneously by angiogenesis or arteriogenesis. In this scenario, it is of major clinical importance to improve the microcirculation and oxygenation in the ischemic tissue during the period of jeopardized perfusion to ensure the survival of the tissue and its functional recovery.

One therapeutic approach consists of exchanging blood with crystalloid or colloid solutions, thus reducing blood viscosity. Provided normovolemia and normotension are maintained, this results in an increase in cardiac output (27) and in red blood cell velocity at the capillary level (18). Normovolemic hemodilution has been shown to improve microcirculatory blood flow in ischemic myocardial (15), cerebral (7, 16), and peripheral tissue (5, 25). However, as long as the diluting solutions are void of oxygen carriers, the enhanced microcirculatory blood flow may be offset by a reduction in oxygen supply to the ischemic tissue.

This drawback can in principle be circumvented if hemodilution is implemented with solutions containing oxygen carriers, such as fluorocarbons or modified hemoglobins. Perfluorocarbons and at least three chemically modified hemoglobin solutions are currently in advanced clinical trials to evaluate their potential as red blood cell substitutes in blood loss (1). However, these solutions are not free of untoward effects. Ventilation with supplemental oxygen is essential to obtain the desired tissue oxygenation with perfluorocarbons, and the chemically modified hemoglobins may cause a vasopressor response, which may have devastating consequences for the compromised ischemic tissue. The vasoactivity has been attributed to circulating tetrameric hemoglobin, which may penetrate the vascular lining and thus scavenge nitric oxide (NO) (1). By reducing the concentration of the tetrameric component, a large volume infusion of polyhemoglobin solution has recently become possible without showing any discernable vasopressor response.

Another method to attenuate vasoactivity consists in the encapsulation of human hemoglobin in liposome vesicles (HbV). HbV solutions caused significantly less vasoconstriction and hypertensive response than the...
conjugated or polymerized hemoglobins currently in experimental and clinical investigation (21). HbV solutions maintained microvascular function and oxygen delivery to the tissue even at relatively high levels of blood exchange (23). Another advantage of HbV is that P50 can be modified by selecting the appropriate amount of coencapsulated allosteric effector (pyridoxal 5’-phosphate) (24), which may be essential for achieving maximal oxygen supply to the jeopardized tissue.

Despite the profound clinical demand, there is a paucity of scientific data documenting the therapeutic efficacy of artificial oxygen carriers in ischemic conditions ascribed to acute vascular obstruction. The aim of this study was to test whether oxygenation in acutely ischemic, collateralized tissue may be improved by normovolemic hemodilution with HbV solutions and to assess their effect on microcirculatory hemodynamics. Studies were made using the recently described hamstring flap model, which consists of an anatomically perfused portion and an ischemic collateralized portion (4). This model provides the unique opportunity to assess the microhemodynamics directly and quantitatively in acutely ischemic, collateralized tissue with the use of intravital microscopy.

MATERIALS AND METHODS

Experiments were performed according to NIH guidelines for the care and use of laboratory animals and with the approval of the local Animal Ethics Committee. Seventy male Syrian golden hamsters weighing 65–85 g were used in this study. The animals were randomly assigned to either the control group (n = 14) or to one of five groups subjected to normovolemic hemodilution with 8% human serum albumin (HSA8, n = 13), 8.95% human serum albumin (HSA9, n = 9), 6% Dextran 70 (Dx70, n = 12), HbV suspended in HSA8 (HbV-HSA8, n = 11), or HbV suspended in 6% Dextran 70 (HbV-Dx70, n = 11), respectively.

Animal and flap preparation. Anesthesia was induced by pentobarbital injected intraperitoneally (100 mg/kg body wt, Nembutal, Abbott Laboratories; Chicago, IL). The carotid artery and jugular vein were cannulated for blood pressure and heart rate monitoring and for blood exchange and laboratory analysis, respectively. Catheterization and flap dissection were performed with the aid of an operating microscope at ×10 magnification (Wild; Heerbrugg, Switzerland). After the animal was shaved and back skin of the animal was epitomized, the vascular anatomy was identified by diaphanoscopy. An island flap measuring 30 × 20 mm was dissected free from the surrounding tissue. The animal was then placed in a lateral position on a specially designed Plexiglas stage providing a platform for mounting the flap, which was positioned with the skin lying on the platform and kept at its original size by sutures. The panniculus carnosus was meticulously removed except for a single layer of muscle tissue left in place to protect the vascular network. The flap was merely perfused via one artery and vein, which bifurcate into equal-sized branches within the flap, each of them supplying a separate vascular territory. One of the two branches was transected after being secured with microsurgical ligatures. Therefore, one vascular territory was anatomically perfused by the intact branch, whereas the other was indirectly perfused through the collateral vascular network connecting the two vascular networks. The raw surface of the flap was finally covered with a polyevinyl film to isolate the tissue from the environment. During surgery, 4 mg papaverine hydrochloride (Sigma; St. Louis, MO) dissolved in 1 ml physiological saline solution was applied to the pedicle by a soaked cotton tip to prevent vascular spasm.

Microhemodynamic measurements. Investigations were performed using an intravital microscope (Axioplan 1, Zeiss; Jena, Germany). Microscopic images were captured by a television camera (Intensified CCD camera, Kappa Mess-technik; Gleichen, Germany), recorded on video (50 Hz, Panasonic; Osaka, Japan), and displayed on a television screen (Trinitron PVM-1454QM, Sony; Tokyo, Japan). The preparation was observed visually with a ×40 objective resulting in a total optical magnification of ×909 on the videomonitor. Microvascular diameter was measured by transillumination with a green filter, which gave a well-defined image of the width of the erythrocyte column. For the assessment of centerline velocity, white blood cells (WBCs) were stained in vivo with rhodamine 6G (2 μmol/kg body wt iv, Sigma). Fluorescence was visualized with the aid of an excitation filter (530–560 nm), a dichroic mirror (580 nm), and a barrier filter (630 nm) and through epi-illumination by a mercury lamp (Ampex; Zeiss) (14). Velocity was calculated by measuring the distance covered by the WBC during one time frame (20 ms). Microvascular blood flow (Q) was calculated by means of Eq. 1

\[ Q = \pi \times (\text{WBC velocity/1.6}) \times (\text{diameter}/2)^2 \]  

The value 1.6 represents the empirically determined ratio of centerline velocity to whole blood velocity (8).

The microvessels were classified according to physiological and anatomical features into conduit arterioles (connections to each other), end arterioles, and small venules (4, 10). The vessels were chosen for examination according to their optical clarity.

Tissue oxygen tension. Partial tissue oxygen tension was assessed with Clark-type microprobes consisting of polarographic electrodes and an oxygen-sensitive microcell (Revoxode CC1, GMS; Kiel, Germany). According to the manufacturer, the Revoxode CC1 provides reproducible values for several consecutive days without the need of recalibration. The length of the cell was 1 mm, and the sampling area was within 1 mm of the cell. The probes were inserted into the subcutaneous tissue in the center of each vascular territory under visual control and microscopic magnification. Care was taken to place the probes in such a way that no arterioles or large venules lay within the sampling area.

HbVs. The HbVs were prepared as previously reported (22). They consisted of isolated human hemoglobin encapsulated in a phospholipid vesicle coated with polyethylene glycol. The size of the vesicles was 276 ± 11 nm and the P50 was 22 mmHg (obtained by adding the coencapsulated allosteric effector pyridoxal 5’-phosphate). The P50 was calculated from the O2 equilibrium curve obtained with a Hemox Analyzer (TCS Medical Products). The HbVs were suspended in either HSA8 (ZLB Bioplasma; Berne, Switzerland) or Dx70 (Braun Medical; Emmenbrucke, Switzerland). These diluents were chosen because of their widespread experimental and clinical uses (3, 5, 17, 18, 22–24). The physical characteristics of the solutions are summarized in Table 1. Oncotic pressure and viscosity were measured with a colloid osmometer (model 4420, Wescor; Logan, UT) and a cone-and-plane viscometer (21, Brookfield Engineering; Middleboro, MA), respectively.

Protocol. The animals were kept under light anesthesia with a continuous infusion of 50 mg/ml pentobarbital given at a rate of ~0.5 mg·min⁻¹·kg body wt⁻¹ throughout the experiment. The depth of anesthesia was regulated by toler-
The InStat 2.03 program (Graph Pad Software; San Diego, CA) was utilized for statistical analysis. If the assumption of a normal distribution was appropriate, the data were presented as means ± SD, otherwise they were presented in median and 25th and 75th percentiles. For normally distributed data, the time-related differences between repeat measurements and the differences between the groups were assessed by paired and unpaired ANOVA, respectively, whereas the nonparametric Friedman and Kruskal-Wallis tests were used for not normally distributed data analysis. All tests were followed by the Bonferroni posttest. A value of P < 0.05 was taken to represent statistical significance.

RESULTS

Twelve animals (1 control, 3 HSA8, 1 HSA9, 2 Dx70, 3 HbV-HSA8, and 2 HbV-Dx70 animals) did not fulfill the inclusion criteria and were excluded from this study.

The systemic data are summarized in Table 2. Similar hematocrits were obtained in all hemodiluted animals. However, hemodilution with HSA and Dx70 resulted in mean hemoglobin concentrations between 6.5 and 8.2 g/dl, whereas the addition of HbV to the diluents augmented the mean hemoglobin concentration to values ranging from 9.9 to 10.7 g/dl (P < 0.01 vs. HSA8, HSA9, and Dx70). Mean arterial pressure decreased in the groups diluted with HSA solutions [P < 0.05 for HSA8 and not significant (NS) for HSA9 and HbV-HSA8]. Hemodilution caused slight to moderate increases in arterial Po2 (NS) and pH (P < 0.05 for Dx70 and P < 0.01 for HSA8 and HSA9) and a decrease in Pco2 (NS).

At baseline, diameters and centerline velocities for conduit arterioles, end arterioles, and venules in each part of the flap were similar in all groups (Table 3).

Table 1. Physical characteristics of the diluents

<table>
<thead>
<tr>
<th></th>
<th>Hb Concentration, g/dl</th>
<th>Colloid Osmotic Pressure, mmHg</th>
<th>Viscosity, cP</th>
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<tbody>
<tr>
<td>HSA8</td>
<td>0</td>
<td>40.0</td>
<td>1.0</td>
</tr>
<tr>
<td>HSA9</td>
<td>0</td>
<td>49.9</td>
<td>1.2</td>
</tr>
<tr>
<td>Dx70</td>
<td>0</td>
<td>49.9</td>
<td>2.8</td>
</tr>
<tr>
<td>HbV-HSA8</td>
<td>7.5</td>
<td>40.0</td>
<td>2.9</td>
</tr>
<tr>
<td>HbV-Dx70</td>
<td>7.5</td>
<td>49.9</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Viscosity was measured at 37°C and at 150 s⁻¹. Hb, hemoglobin; HSA8, 8% human serum albumin; HSA9, 8.95% HSA; Dx70, 6% Dextran 70; HbV-HSA8 and HbV-Dx70, Hb vesicles suspended in HSA8 and Dx70, respectively.

However, mean centerline velocities were significantly reduced in the ischemic vessels compared with the anatomically perfused vessels (P < 0.01).

The microhemodynamic features in the anatomically perfused portion are shown in Fig. 1. Diameters decreased to 90% (84–100%) from baseline in the conduit arterioles in the HSA8 group (P < 0.05 vs. Dx70) and to 84% (81–89%) in the HSA9 animals (P < 0.01 vs. baseline and Dx70). In the venules, hemodilution with HbV-HSA8 resulted in a gradual reduction of diameter to 95% (80–99%, P < 0.05 vs. baseline). Microvascular blood flow showed the maximal increase in all microvessels after hemodilution with Dx70, to 167% (118–203%, P < 0.05 vs. control and HSA8 and P < 0.01 vs. baseline, HSA9, and HbV-Dx70).

In the ischemic tissue, diameters in the conduit arterioles decreased to 93% (87–96%) after hemodilu-
tion with HSA8 and to 93% (89–97%) after hemodilution with HSA9 (both P < 0.01 vs. baseline and Dx70), respectively (Fig. 2). Venular diameters became progressively smaller in animals diluted with HSA8 and HbV-HSA8 compared with Dx70 and HbV-Dx70, respectively (NS). Microvascular blood flow was increased to 189% (93–237%) after hemodilution with HSA8 (P < 0.05 vs. baseline), to 191% (118–312%) with HSA9 (P < 0.01 vs. baseline and control), to 219% (145–287%) with Dx70 (P < 0.01 vs. baseline and control), and to 158% (110–220%) with HbV-Dx70 (P < 0.01 vs. baseline). Blood flow was consistently higher in the HSA9 and Dx70 groups than in the HSA8 group (P < 0.05) and in the HbV-Dx70 animals compared with the HbV-HSA8 animals (P < 0.05).

Oxygen tension in the ischemic tissue was significantly reduced compared with the anatomically perfused part (P < 0.01; Fig. 3). In the control group, oxygen tension in the ischemic tissue slightly decreased from 13.0 ± 4.2 to 11.8 ± 4.8 mmHg over time (NS), whereas it was substantially improved from 11.9 ± 4.3 to 17.0 ± 5.6 mmHg after hemodilution with HbV-Dx70 (P < 0.05 vs. HSA8, P < 0.01 vs. baseline). No significant changes were seen in the other groups.

**DISCUSSION**

The principal finding of this study was that hypoxia in the ischemic, collateralized flap tissue was substantially attenuated after normovolemic hemodilution with HbV-Dx70 but not with the other diluents.

The effect was correlated with a significant increase in both hemoglobin concentration and microvascular blood flow. Apparently, the augmentation of oxygen carrying capacity per se was not sufficient to improve oxygenation in the ischemic tissue. Furthermore, our findings are in contrast to the concept that oxygen may be delivered to tissues with low-flow conditions by artificial oxygen carriers that, unlike red blood cells, are able to bypass intraluminal obstructions (sludged red blood cells, and endothelial blubs) due to their small size (11), because both solutions included the identical, submicrometer-sized oxygen carrier. Much more likely, the efficacy of HbV is related to the rheological properties of the diluent, including colloid osmotic pressure and/or viscosity.

The colloid osmotic pressure of the HbV-Dx70 solution was higher than that of the HbV-HSA8 solution. Because both solutions are hyperoncotic (colloid osmotic pressure of hamster blood = 17.5) (26), normovolemic hemodilution leads to a fluid shift from the extravascular space into the intravascular space, thus resulting in volume expansion and subsequently in an increase in preload, cardiac output, and mean arterial pressure. This mechanism is accomplished within the first 30 min after hemodilution (28), and the hematocrit values obtained in our study suggest that the blood volume was kept constant throughout the experiments. The influence of colloid osmotic pressure may be reflected by the behavior of venular diameter and mean arterial pressure.
Fig. 1. Microhemodynamics in the anatomically perfused tissue at baseline and after 50% blood exchange with 8% human serum albumin (HSA8), 8.95% human serum albumin (HSA9), 6% Dextran 70 (Dx70), and hemoglobin vesicles (HbV) suspended in HSA8 (HbV-HSA8) and Dx70 (HbV-Dx70). Data are given as a percentage of baseline and presented in box plots reflecting the 10th percentile, 25th percentile, median, 75th percentile, and 90th percentile. *P < 0.05 and **P < 0.01 vs. baseline; #P < 0.05 and ##P < 0.01 vs. Dx70.

pressure, which were better maintained in animals receiving Dx70 or HSA9 solutions. The effect of colloid osmotic pressure on the compromised microcirculation in the ischemic tissue was most evident in the HSA9 group, which revealed significantly higher blood flow values compared with the animals receiving HSA8. Furthermore, hyperoncotic solutions may attenuate fluid extravasion in ischemic, collateralized tissues, which may contribute to the impairment of oxygenation, as shown in a porcine model (6). The benefit that a high colloid osmotic pressure may exert on the oxygenation and recovery in focal ischemia during hemodilution has been emphasized for a variety of tissues and solutions, including solutions containing oxygen carriers (2, 9, 12).

The viscosity was higher for Dx70 than HSA and was further augmented by adding HbV. In hamsters, plasma viscosity was significantly increased from 1.2 to 1.4 cP after severe hemodilution with Dx70 (26). Therefore, augmented plasma viscosities can be expected in the Dx70 group as well as in animals diluted with HbV-HSA8 and HbV-Dx70, which have equal or higher viscosities than Dx70. However, plasma viscosity may be decreased after hemodilution due to volume expansion, which may account for a net viscosity reduction in animals receiving HSA8 or HSA9. This hypothesis is supported by the vasoconstriction found in the conduit arterioles in the HSA8 and HSA9 groups, because reduced plasma viscosity negatively affects shear stress on the vascular lining, thus resulting in a lack of NO-mediated relaxation of the vascular tone in large arterioles and small arteries (13).

Moreover, NO-mediated vasorelaxation may be attenuated by free hemoglobin scavenging NO (1, 21). Thus the addition of HbV to the diluent may not only result in NO-mediated vasorelaxation due to viscosity increase but also in vasoconstriction due to NO scavenging, which may explain why the flow values were consistently lower in the HbV-Dx70 group than in the Dx70 group. However, the microvascular diameters...
were similar in both groups. It can therefore not be excluded that some of the NO-mediated effects on microvascular flow may be generated on a more upstream level than that investigated in our experimental model. In the hamster skinfold model, Sakai et al. (20) ascribed the principal role of NO-mediated regulation of microvascular blood flow to the small resistance arteries, which were also the most sensitive for the vaso-
pressor effect of acellular hemoglobins (21). On that vascular level, minor vasoactivity was caused by HbV, which, however, was far lower than that of chemically modified hemoglobin solutions. Most importantly, the hypothesized vasoconstriction in the small resistance arteries was not sufficient to inhibit the improvement of microvascular blood flow in the ischemic tissue after hemodilution with HbV-Dx70 in the present study.

Despite the substantial improvement of oxygen tension in the ischemic tissue after hemodilution with HbV-Dx70, its efficacy in ensuring tissue survival may be questioned because this was not tested in this study. However, the potential benefit of maintaining tissue oxygen tension above a specific threshold is suggested by the results from a study of Pickelmann et al. (19), who used similar techniques for measuring tissue oxygen tension in a hamster skinfold model. It was reported that a reduction of tissue oxygen tension over several hours coincided with histomorphological signs of ischemic tissue damage. The threshold was found to be 10 mmHg, which corresponds to the level of tissue oxygen tension obtained in the ischemic tissue after hemodilution with HbV-Dx70 in the present study. This indicates that the survival of this tissue is compromised, a situation that may be corrected by normovolemic hemodilution with HbV-Dx70.

In conclusion, we were able to demonstrate that it is possible to substantially improve the impaired oxygenation in ischemic, collateralized tissue by normovolemic hemodilution with HbV solutions. The efficacy of the HbV was dependant on an increase in microcirculatory blood flow, which was related to the oncotic and rheological formulation of the diluent the HbV was suspended in. Our results advocate solutions with high colloid osmotic pressure and high viscosity.

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