Use of Hemoglobin Vesicles During Cardiopulmonary Bypass Priming Prevents Neurocognitive Decline in Rats

Masataka Yamazaki, MD; Ryo Aeba, MD; Ryohei Yozu, MD; Koichi Kobayashi, MD

Background—Homologous blood use is considered to be the gold standard for cardiopulmonary bypass (CPB) priming in infants despite exposure of the patient to potential cellular and humoral antigens. However, the use of hemoglobin vesicles (HbVs), artificial oxygen carriers that encapsulate a concentrated hemoglobin solution within phospholipid bilayer membranes, for CPB priming may prevent neurocognitive decline in infants. The goal of this study was to determine whether HbV use offsets hemodilution caused by patient/priming volume-mismatched CPB and thereby prevents the development of postoperative neurocognitive deficits.

Methods and Results—CPB was established in 28 male Sprague-Dawley rats (age, 14 to 16 weeks; weight, 450 grams) after cannulation of the tail artery and right atrium. The animals were randomly assigned to 1 of 3 groups: sham surgery (n=9), HbV (−) prime (n=10), or HbV (+) prime (n=9). CPB was conducted for 90 minutes at 200 mL/kg per minute. The hematocrit during CPB was 10.0±1.2% in the HbV (+) prime group and 9.9±1.3% in the HbV (−) prime group (P=not significant). Learning and memory function were evaluated using 2 different maze tests (Maze-1 and Maze-2, in which the arrival times to the target were measured on the first, third, fifth, and seventh postoperative days). Learning and memory function were significantly better in the HbV (+) prime group than in the HbV (−) prime group (Maze-1, P=0.012; Maze-2, P=0.042); there was no difference between the HbV (+) prime and the sham surgery group.

Conclusions—The use of HbV for CPB priming may serve as a substitute for homologous blood to prevent the unacceptable hemodilution and contribute to maintenance of intact neurocognitive function. (Circulation. 2006;114[suppl I]:I-220–I-225.)

Key Words: cardiopulmonary bypass ■ hemoglobin ■ nervous system ■ pediatrics

Homologous blood use continues to be the gold standard for cardiopulmonary bypass (CPB) priming in infants and neonates despite exposure of the patient to potential cellular and humoral antigens. Neurologic morbidity after CPB has become an increasing concern ever since surgical mortality has decreased in infants undergoing repairs of simple and complex congenital heart diseases. CPB itself can cause neurologic morbidity because CPB gives rise to a systemic inflammatory response that is responsible for decreased cerebral blood flow and cerebral dysfunction. Although hemodilution during CPB increases both early neurologic complications and late neurocognitive performance, the use of homologous blood potentially exaggerates the CPB-derived inflammatory response and may contribute to post-CPB neurologic morbidity. Though miniaturization of the CPB circuit has reduced priming volume,1-3 at the present time, however, it is still not low enough to achieve an acceptable level of hemodilution in very small patients.

Hemoglobin vesicles (HbVs) have been developed for use as artificial oxygen carriers. HbV is a solution of purified Hb that is encapsulated within a phospholipid bilayer membrane. The oxygen-transporting ability of HbVs is comparable to that of blood.4 Hb-based oxygen carriers have many potential advantages over homologous blood. First, HbV has no cellular and humoral antigens, which eliminates the risks of blood-type mismatch reaction and blood-transmittable infectious disease. Second, HbVs, which have a particle diameter of only 250 nm, are small enough to circulate through blood microvessels that can become constricted during and after CPB and through which red blood cells cannot pass. Third, HbV is very stable and can be stored as a powder for a long time.5 Fourth, HbVs are captured by phagocytes in the reticuloendothelial system and are metabolized within ∼7 days, without iron or lipid deposition.6 The only concern posed by HbV for clinical use is its short half-life of only 35 hours in the circulating blood. However, its quick disappearance from the circulation could be an advantage rather than a disadvantage when using HbV as a CPB priming solution in pediatric open heart surgery, because hemodilution occurs only during and soon after CPB, which is usually <2 hours in most cases.

Thus, using HbVs as the CPB priming solution instead of a crystalloid solution or homologous red blood cells could...
improve the neurologic and neurocognitive outcomes in very small patients undergoing open heart surgery. The purpose of this investigation was to determine the effects of HbVs on neurologic and neurocognitive function after CPB using a rat model.

Materials and Methods
The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Preparation of HbV CPB Prime
HbVs were manufactured and provided by the Department of Polymer Chemistry, Advanced Research Institute for Science and Engineering, Waseda University (Tokyo, Japan). HbVs were prepared under sterile conditions as previously reported. Hb was purified from outdated donated blood provided by the Hokkaido Red Cross Blood Center (Sapporo, Japan) and the Japanese Red Cross Society (Tokyo, Japan). HbVs were suspended in a physiological salt solution at [Hb] = 10 g/dL, sterilized with filters (Dismic, Toyo-Roshi, Tokyo, Japan, pore size, 0.45 μm), and deoxygenated with N₂ bubbling for storage. Before use, the HbV suspension ([Hb] = 10 g/dL, 8.6 mL) was mixed with a solution of human serum albumin (1.4 mL; Nipro, Osaka, Japan) to adjust the albumin concentration in the vesicle suspending medium to 5 g/dL. Under these conditions, the colloidal osmotic pressure of the suspension is ~20 mm Hg (Wescor 4420 Colloid Osmometer; Wescor, Logan, Utah). As a result, the Hb concentration of the suspension was 8.6 g/dL.

Animal Model and Preparation
SD rats were purchased from Sankyo labo service Corp (Tokyo, Japan). The experimental protocol was approved by the Laboratory Animal Care and Use Committee of Keio University School of Medicine. It also complied with the Guide for the Care and Use of Laboratory Animals.

Twenty-eight male SD rats, aged 14 to 16 weeks and weighing 450 grams, were housed in cages and provided with food and water ad libitum in a temperature-controlled room with a 12-hour dark/light cycle. The animals were anesthetized with 3.0% sevoflurane-mixed air inhalation with a vaporizer. The rats were intubated (16-gauge intravenous catheter) and mechanically ventilated. The ventilator settings included FiO₂ of 0.21 and ventilatory rate of 70 cycles per minute. Anesthesia was maintained with 1.5 to 2.0% sevoflurane.

Surgery was performed using aseptic technique.

CPB in the rat was performed using the surgical techniques described by Grocott et al. Heart rate and rectal temperature were continuously monitored, and the rectal temperature was servo-regulated at 37.5°C. After systemic heparinization using 300 IU, the tail artery was cannulated with a 22-gauge angiocatheter. A 16-gauge multi-pore angiocatheter was introduced into the right internal tail artery and advanced into the right atrium. A roller pump and multi-pore angiocatheter was introduced into the right internal jugular vein and advanced into the right atrium. A roller pump and custom-made CPB oxygenator/circuit were used for all the experiments.

The animals were randomly divided into the 3 experimental groups (Figure 1): (1) the sham surgery group (n = 9); (2) the HbV (−) prime group (n = 10); and (3) the HbV (+) prime group (n = 9). In the sham surgery group, the animals were cannulated but CPB was not induced. In the other groups, the CPB circuit was primed with 60 mL of human serum albumin solution either with or without HbV (the HbV (+) prime group and the HbV (−) prime group).

Normothermic CPB with a flow of 200 mL/kg per minute was performed for 90 minutes. During CPB, 100% oxygen gas was delivered to the oxygenator at 1.0 L/min. The animals were separated from CPB without the use of any vasoactive agents. After removal of the cannula, the animals were ventilated for another 30 minutes, at which point all the blood that was left in the CPB circuit was collected and centrifuged at 2000 rpm for 5 minutes, and then the precipitates were returned intravenously. Arterial blood samples were collected after placement of the CPB cannulae (T-1), 45 minutes after CPB initiation (T-2), as well as after CPB and CPB blood return (T-3). A pH/blood gas analyzer (I-STAT; Fuso, Osaka, Japan) was used to determine arterial Po₂, PCO₂, and pH.

After the animals recovered from the effects of the general anesthetic, they were extubated and returned to their cages. The animals were observed for 7 days, during which time they had free access to water and food.

Neurologic and Neurocognitive Evaluation
Neurologic and neurocognitive outcomes were assessed by video-recording all behaviors of the animals, which a physician blinded to the groups reviewed collectively later.

Neurologic outcome was assessed on the days 1, 3, 5, and 7 after the operation using neurologic performance and functional disability scores. The neurologic performance scale consisted of a physical examination with points given for deficits. A normal examination score was 0, and the worst score was 95. The functional disability score was ranked from 1 to 5: score 1 (no disability), able to run, explore the environment, and feed from the trough; score 2 (mild disability), gait disturbances but able to ambulate, explore the environment, and feed from the trough; score 3 (moderate disability), unable to walk and required bottle-feeding, but was alert and able to crawl; score 4 (severe disability), not able to feed even with assistance and unable to crawl; and score 5, death.

To evaluate neurocognitive outcome, 2 different kinds of behavioral testing using maze tests (Maze-1 and Maze-2) were performed on days 1, 3, 5, and 7 after the operation. The Maze-1 test is generally referred to as the Morris water maze test. Briefly, the Morris water maze consisted of a 1.5-m-diameter, 50-cm–deep water pool (27°C) with a submerged (3 cm below surface) hidden platform in 1 quadrant. The time to locate the submerged platform (defined as the latency) is measured to test for impairment in visual–spatial learning and memory. The animals underwent testing in the water maze with 4 trials per testing period. Each of the trials began from a separate quadrant. Testing was performed on days 1, 3, 5, and 7 after the operation. The Maze-2 test is of the type that actually has a maze-shaped pool of water with 5.5 m of total pathway length and 11 junction points, where the animals have to swim without rest until arriving at the sole exit. The time from the departure point to the goal point was measured in a similar way to that of Maze-1.

Histopathological Examination
After completion of the neurologic testing on day 7, the animals were euthanized with 3.0% sevoflurane inhalation. The brains were harvested and stored in 4% formalin. Paraffin-embedded brain sections were then serially cut (5-μm-thick sections) and stained with hematoxylin and eosin. A neuropathologist who was blinded to group assignment counted the total number of necrotic cells in the hippocampus (CA1–2) area.
Physiologic Data in Each Group

<table>
<thead>
<tr>
<th></th>
<th>T-1</th>
<th>T-2</th>
<th>T-3</th>
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<tbody>
<tr>
<td></td>
<td>Sham Surgery</td>
<td>HbV(−) Prime</td>
<td>HbV(+) Prime</td>
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<td>Arterial pH</td>
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<td>7.61±0.05</td>
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<td>Arterial PO₂, mm Hg</td>
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<td>Hematocrit, %</td>
<td>40.7±2.4</td>
<td>40.4±3.1</td>
<td>42.0±2.5</td>
</tr>
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</table>

Values are mean ±SD. n=9, sham surgery; n=10, HbV (−) prime; n=9, HbV (+) prime. *P<0.01 vs sham surgery; †P<0.05 vs sham surgery. PCO₂ indicates partial pressure of carbon dioxide; PO₂, partial pressure of oxygen.

Statistical Analysis
All continuous numerical data were presented as means ± SD. Intergroup comparisons were made with 1-way analysis of variance. When a significant F ratio was obtained, further analysis was performed with Scheffe F post-hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test. Statistical significance was assumed when P<0.05.

Results
All rats survived the entire period of time needed to complete the experimental protocol. The Table shows the baseline data for the 3 groups at T-1, T-2, and T-3.

The hematocrit was lower in the HbV (−) and HbV (+) prime groups than in the sham surgery group at T-2 (P<0.01). The hematocrit at T-2 was 9.9±1.3% in the HbV (−) prime group and 10.0±1.2% in the HbV (+) prime group (P=not significant). The arterial pH was lower in the HbV (−) prime group than the sham surgery group at T-2 and T-3 (P<0.01). The arterial PCO₂ was higher in the HbV (−) and HbV (+) prime groups than in the sham surgery group at T-3 (P<0.05). At T-2, the arterial PO₂ was >400 mm Hg in the HbV (−) prime and HbV (+) prime groups, whereas that in the sham surgery group was 96.8±8.9 mm Hg at T-2 (P<0.01).

The neurologic examination showed no significant differences among the 3 groups with respect to performance and disability scores (Figures 2 and 3), and none of the groups showed any distinctly abnormal neurologic behaviors. All animals were able to feed by themselves, ambulate, and freely explore their surroundings.

Neurocognitive outcome is shown in Figures 4 and 5. The HbV (−) prime group had longer maze latencies for both maze tests compared with the other groups (Maze-1, P=0.012; Maze-2, P=0.042), indicating significant neurocognitive dysfunction after hemodilution. The maze latency curves were similar in the HbV (+) prime and the sham surgery groups. On day 1, the arrival times were similar among the 3 groups. However, subsequently, the HbV (+) prime and sham surgery groups had shorter arrival times than the HbV (−) group (Maze-1, P<0.01; Maze-2, P<0.01); the differences between the HbV(+) prime and the sham surgery groups were similar for all intervals.

Figure 6 represents the swimming speed of the rats. The swimming speed was similar among the 3 groups and did not show any chronological change from days 1 through 7 after the operation (P=not significant), indicating that exercise capacities were intact even in animals subjected to CPB.

Figure 2. Neurologic performance scores after CPB on days 1, 3, 5, and 7 after the operation. Score 0 indicates no neurologic deficits and 95 represents brain death, n=9, sham surgery; n=10, HbV (−) prime; n=9, HbV (+) prime. Neurologic performance scores were not different among the 3 groups. NS indicates not significant.

Figure 3. Functional disability scores after CPB on days 1, 3, 5, and 7 after the operation. Score 0 indicates no disability; score 2, mild disability; score 3, moderate disability; score 4, severe disability; score 5, death, n=9, sham surgery; n=10, HbV (−) prime; n=9, HbV (+) prime. Functional disability scores were not different among the 3 groups.
On histology there was no difference among groups with respect to the total number of necrotic hippocampal neuron cells.

Discussion

For infants and neonates undergoing reparative and palliative surgery for simple and complex congenital heart disease, CPB techniques and treatment strategies have been rapidly evolving in the last decade. This has undoubtedly contributed to improved surgical outcomes. However, CPB still results in an inflammatory systemic reaction, which can, in turn, cause dysfunction in many end organs. In the brain, CPB decreases cerebral endothelial function\textsuperscript{13,14} and blood flow, a phenomenon known as "no reflow," which appears to be highly linked to postoperative neurologic morbidity.\textsuperscript{15,16}

Thus, there is a dilemma in the use of homologous blood for CPB priming in infants. The priming volume for infants and neonates has been significantly decreased by the miniaturization of commercially available CPB circuits, which include tubing, bubble filter, and oxygenator.\textsuperscript{3} This progress has decreased the ratio of CPB priming volume to circulating blood volume. However, this has helped only rather large infants who could have bloodless priming without unacceptable hemodilution and/or cases that require only a very short CPB duration. Otherwise, homologous blood is mandatory to prevent unacceptable hemodilution that leads to suboptimal oxygen supply, even though there is exposure to potential cellular and humoral antigens. In common with CPB, blood transfusion by itself stimulates systemic inflammatory cytokine production.\textsuperscript{17,18} Thus, homologous blood use for CPB priming may also be a risk to cerebral blood flow and function.\textsuperscript{19}

Artificial oxygen carriers could be a breakthrough that helps solve this dilemma. In the past, Fluosol was used in a pig CPB model to investigate its capability to augment myocardial perfusion.\textsuperscript{20,21} However, a critical adverse effect developed; there was an increased level of ionized calcium that was associated with increased myocardial contractility and anaerobic metabolism. Therefore, the clinical use of Fluosol was aborted. Izumi, who is associated with our institute, has previously found that HbV has an equivalent oxygen transporting capability to red blood cells during CPB in a dog model.\textsuperscript{22} HbV has no side effect of microvascular vasoconstriction, as commonly noted in many other Hb-based oxygen carriers. This background compelled us to perform the current study.
The clinical use of HbV in CPB priming of infants and neonate could ostensibly give rise to a variety of adverse effects during the several days before the reticuloendothelial system deals with the molecule. However, previous studies using rats found that a bolus large-dose HbV infusion was associated with minor and transient deterioration in major organ function. Furthermore, these potential adverse effects could be minimized by using modified ultrafiltration after CPB, which would eventually eliminate most of the HbV from the serum.

In our rat CPB model, the hematocrit during CPB was ≈10%, which in clinical practice should have been treated by blood transfusion. The animals had a lower pH when HbV was not added to the CPB priming solution. However, given the hypocarbia policy with ventilation and CPB during the entire period of anesthesia, the pH was maintained at ≥7.4 even in the HbV (−) prime group, and the rats all survived during CPB and for 7 days after CPB without any neurologic morbidity. On neurohistology 7 days after surgery, the findings were similar among all 3 groups. These results indicate that the rats in the HbV (−) prime group were over-hemodiluted in terms of oxygen supply, but this hemodilution was “subclinical” when surgical outcome was evaluated by gross observation and by neurohistology. Our model may be highly sensitive in detecting subtle injury of cerebral function. Learning and memory function is one of the highest levels of cerebral function and was evaluated by 2 maze tests. It may not be surprising that mild deficits in oxygen supply during CPB were only detected by impairment of neurocognitive function without any other findings.

Our model has several limitations with respect to extrapolating to the human clinical setting. The age of the animals was not matched to that of human neonates and infants. CPB was established using peripheral access with internal jugular venous and tail arterial return without opening of the chest, induced cardiac arrest, hypothermia, and circulatory arrest. All of these led to invasive CPB cannulation techniques may have contributed to the 100% animal survival rate without neurologic morbidity and allowed successful neurocognitive evaluation after CPB. However, such an approach may not necessarily mimic the procedures used for human infants and neonates undergoing open heart surgery. Another concern is species difference. To prioritize the survival of the small animals we abandoned serial measurement of intracerebral oxygen tension and cerebral blood flow, as well as repeated blood sampling for lactate extraction, inflammatory cytokine concentrations, and serologic markers of brain injury, which might have provided important information to support our hypothesis. Finally, the current study lacked a control group with homologous blood priming because of technical issues involving blood-banking in rats.

Nevertheless, the results of the current study clearly indicate that HbV can serve as a substitute for homologous blood in CPB priming to prevent the unacceptable hemodilution caused by a large difference between circulating blood and CPB priming volume. The use of HbV for CPB priming may even be potentially superior to homologous blood priming with respect to the maintenance of intact neurocognitive function. These data also provide a rationale for further studies investigating the effect of HbV on cerebral oxygen metabolism and the inflammatory response in a larger animal CPB model.

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Disclosures
None.

References


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