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Attenuated Cerebral Ischemic Injury by Polyethylene Glycol-Conjugated Hemoglobin

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Abstract − Polyethylene glycol-conjugated hemoglobin (PEG-Hb) has been proposed as a blood substitute for transfusion due to their plasma expansion and oxygen transport capabilities. The protective effect of PEG-Hb on cerebral hypoxic-ischemic injury was investigated in neonatal hypoxia model and adult rat focal cerebral ischemia model. As intravenously administered 30 min before the onset of hypoxia, PEG-Hb markedly protected cerebral hypoxic injury in a neonatal rat hypoxia model. A similar treatment of PEG-Hb largely reduced the ischemic injury ensuing after 2-h middle cerebral artery occlusion followed by 22-h reperfusion. Consistently, neurological disorder was significantly improved by PEG-Hb. The results indicate that the pharmacological blockade of cerebral ischemic injury by using PEG-Hb may provide a useful strategy for the treatment of cerebral stroke.

Keywords: Polyethylene glycol-conjugated hemoglobin, Hypoxia, Focal cerebral ischemia

INTRODUCTION

The brain is more vulnerable to ischemic insults than any other organs. The level of oxygen in brain depends on cerebral blood flow and arterial oxygen content. Normal cerebral blood flow is approximately 50 ml/100 g/min. When cerebral blood flow drops below a critical level of 10 ml/100 g/min, irreversible brain damage occurs, with its ensuing metabolic derangement (Pulsinelli, 1992). Even when cerebral blood flow falls to 25 ml/100 g/min, neuronal cells become electrically silent, although they remain potentially viable (Hossman, 1994). Therefore, augmentation of oxygen delivery into hypoxic/ischemic lesions would be beneficial to prevent further neurological deterioration in stroke.

Development of artificial blood substitutes has been motivated by insufficient blood supply, dangers of blood incompatibility reactions, and/or infectious diseases in homologous blood transfusion. Furthermore, hemoglobin-based oxygen carriers can be sterilized and stored for prolonged periods of time. For decades, free hemoglobin derived from hemolyzed blood or hemoglobin-based oxygen carriers have been designed as temporary substitutes to red blood cells (Winslow, 2008). Artificial oxygen carriers are grouped into hamoglobin-based oxygen carriers and perfluorocarbon emulsions (Spahn and Kocian, 2005). Several of hemoglobin-based oxygen carriers, including cross-linked hemoglobin, polyethylene glycol (PEG)-conjugated hemoglobin (PEG-Hb), and recombinant hemoglobin, have been demonstrated to maintain circulating volume (Hu *et al*., 2007). Previously, the synthetic modifiers of hemoglobin significantly reduced tissue injury in animal model of cerebral ischemia (Otani *et al*., 2004; Kawaguchi *et al*., 2009). PEG-Hb, which is bovine hemoglobin conjugated with polyethylene glycol, has low viscosity, high osmotic pressure and high O_2 affinity compared with a conventional plasma substitution and increases intravascular retention time by prevention of the rapid dissociation of hemoglobin tetramers (Bi *et al*., 2004). In the present study, therefore, we examined if PEG-Hb ameliorated the hypoxic-ischemic brain injury in a neonatal hypoxic model and a middle cerebral artery occlusion model of rats.

MATERIALS AND METHODS

Animals

For transient focal ischemia, male Sprague-Dawley rats

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weighing 280 and 290 g were housed in a room maintained at 23 ± 1°C with 55 ± 5% humidity and a 12 h light/dark cycle (light on at 07:00 h). Rats were fasted from food but allowed free access to water for 12-16 h before the experiment. All experimental procedures using animals were in accordance with the NIH *Guide for the Care and Use of Laboratory Animals* and were approved by the Committee of Korea University College of Medicine.

Preparation of PEG-Hb

PEG-Hb was prepared and kindly donated by SunBio Inc (Anyang, Republic of Korea). PEG-Hb was manufactured with hemoglobin extracted from red blood cells. Two anion exchange columns, Matrex PEI-1000 (crosslinked polyethyleneimine, mean particle size: 50 micron, pore size: 1000 A°, Millipore, USA) and Matrex Cellufine Q-500 (crosslinked cellulose beads, particle size: 53-125 micron, exclusion limit: >500 kD, Millipore, USA), comprise chromatographic purification of the hemoglobin. Two anion exchange chromatography processes remove phospholipids, endotoxins, and residual DNA from the hemoglobin solution. PEG was conjugated to the surface of hemoglobin according to the manufacturer's precedure. Fig. 1 shows the association-dissociation curve of PEG-Hb and oxygen.

Neonatal hypoxia model

The neonatal hypoxia procedure was performed as described by Rice *et al*. (1981). On postnatal day 7, the right common carotid artery of the individual rat pup was exposed, isolated from veins and nerves, and permanently ligated with 4-0 surgical silk under light anesthesia with isoflurane. After recovery from anesthesia, the pups were returned to their dam for 1 h. Twenty-four hours after ligation of the right common carotid artery, pups were then placed in 500 ml airtight jars and exposed to humidified oxygen-nitrogen mixture (8% O_2 and 92% N_2) delivered at 2 to 3 L/min for 90 min. The jars were placed in a 37.5°C incubator to maintain a constant thermal environment. After the hypoxic exposure, the pups were allowed to recover in room air for 20 min before being returned to the dam. Sham-operated animals underwent neck incision and vessel manipulation without ligation and hypoxia. For PEG-Hb treatment, pups were treated with vehicle alone or PEG-Hb (0.5, 1, 2.5 or 5 ml/kg; 0.048 g/ml) intravenously into the right internal jugular vein at 30 min before hypoxia.

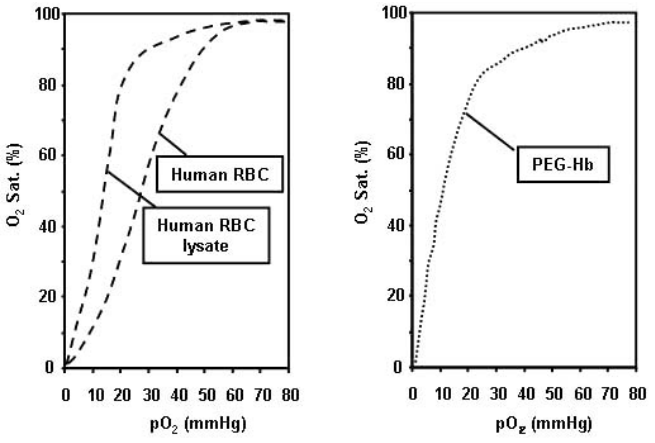

Fig. 1. Relationship between partial pressure of oxygen (pO_2) and oxygen binding.

Focal cerebral ischemia model

Rats subjected to focal cerebral ischemia were initially anesthetized with 3.0% isoflurane in a 70% N_2O and 30% O_2 (v/v) mixture via a face mask. Anesthesia was maintained with 2% isoflurane. A rectal temperature probe was introduced, and a heating pad maintained the body temperature at 37°C during whole surgery period. Focal cerebral ischemia was achieved by right-sided endovascular middle cerebral artery occlusion (MCAO) (Lee *et al*., 2005). Briefly, the right carotid arteries were exposed through a midline cervical incision. The right external carotid artery (ECA) was dissected free and isolated distally by coagulating its branches and placing a distal ligation prior to transection. A piece of 3-0-monofilament nylon suture (Ethicon, Johnson-Johnson, Brussels, Belgium), with its tip rounded by gentle heating and coated by 0.1% (w/v) poly-L-lysine, was inserted into the lumen of right ECA stump and gently advanced 17.5 mm into the internal carotid artery (ICA) from the bifurcation to occlude the ostium of MCAO. After 2 h of ischemia, the suture was pulled back and the animal was allowed to recover. For pre-treatment of PEG-Hb (1, 5 and 10 ml/kg), It was slowly administered intravenously into the right internal jugular vein at 30 min before MCAO. And PEG-Hb (5 ml/kg) was intravenously injected into the right internal jugular vein right after reperfusion for post-treatment.

Physiological parameters

A left femoral artery was catheterized for continuous blood pressure monitoring and periodic blood sampling. Physiological values were measured 5 min after PEG-Hb infusion. Mean arterial blood pressure (MABP) was monitored for 10 min using a DigiMed blood pressure analyzer (MicroMed, Louisville, KY). Blood samples were taken to measure pH, paO_2, $paCO_2$ and blood glucose using an automatic pH/blood gas analyzer (Ciba Corning Diagnostics Corp., Medfield, MA).

Table I. Physiological values following administration of PEG-Hb

	MABP	pH	$paCO_2$	paO_2	Glucose
Saline	101.06 ± 7.74	7.35 ± 0.04	55.9 ± 5.08	172.0 ± 15.13	145.0 ± 10.1
PEG-Hb (1 ml/kg)	106.13 ± 6.39	7.32 ± 0.06	64.7 ± 7.73	161.7 ± 14.57	141.4 ± 11.1
PEG-Hb (5 ml/kg)	105.64 ± 8.25	7.29 ± 0.05	72.0 ± 7.63	149.7 ± 12.74	143.0 ± 15.5
PEG-Hb (10 ml/kg)	103.84 ± 5.54	7.25 ± 0.05	80.7 ± 8.17	149.7 ± 33.17	140.0 ± 13.7

MABP: mean arterial blood pressure, $paCO_2$: partial arterial pressure of CO_2, paO_2: partial arterial pressure of oxygen. Data are mean ± S.D. n=6.

Neurological examination

Twenty-four hours after reperfusion following MCAO for 2 h, a neurological examination was performed as previously described (Huang *et al*., 1994). Briefly, the scores were 0 (no observable neurological deficit, normal), 1 (failure to extend left forepaw on lifting the whole body by tail, mild), 2 (circling to the contralateral side, moderate) and 3 (leaning to the contralateral side at rest or no spontaneous motor activity, severe).

Measurement of infarct volume

Twenty-four hours after reperfusion, rats and pups were deeply anesthetized with chloral hydrate (300 mg/kg). The brains were cut into coronal slices of 2 mm in thickness using a rat brain matrix (Ted Pella, Redding, CA). The brain slices were then incubated in 2% 2,3,5-triphenyltetrazoliumchloride (TTC, Sigma, St. Louis, MO) at 37°C for 30 min to reveal the ischemic infarction. After TTC reaction, the brain slices were fixed with 4% paraformaldehyde (pH 7.4) in 0.1 M phosphate buffer (PB) for 1 day and subsequently cryoprotected in PB containing 30% sucrose at 4°C for 2 days. The cross-sectional area of infarction between the bregma levels of +4 mm (anterior) and -6 mm (posterior) were determined with a computer-assisted image analysis program (OPTIMAS 5.1, Optimas Inc., Edmonds, WA). Brain infarct size was measured manually by outlining the margins of infarct areas, and the infarct volume was calculated according to the slice thickness of 2 mm per section. Each side of the brain slices was measured separately, and mean values were calculated. The total volume of infarction was determined by integrating chosen sections (four sequential slices in pup, six sequential slices in rat) and expressed as percentage of the total brain volume. Because post ischemic brain edema increases brain volume in the infarct area, the corrected infarct volumes were calculated to compensate for brain edema, as previously described (Li *et al*., 1997).

Statistical analysis

Each experimental group consisted of 10 animals. The data are expressed as the mean ± standard deviation (S.D.) and analyzed for statistical significance using analysis of variance (ANOVA), followed by Scheffe's test for multiple comparisons. A value of $p<0.05$ was regarded significant.

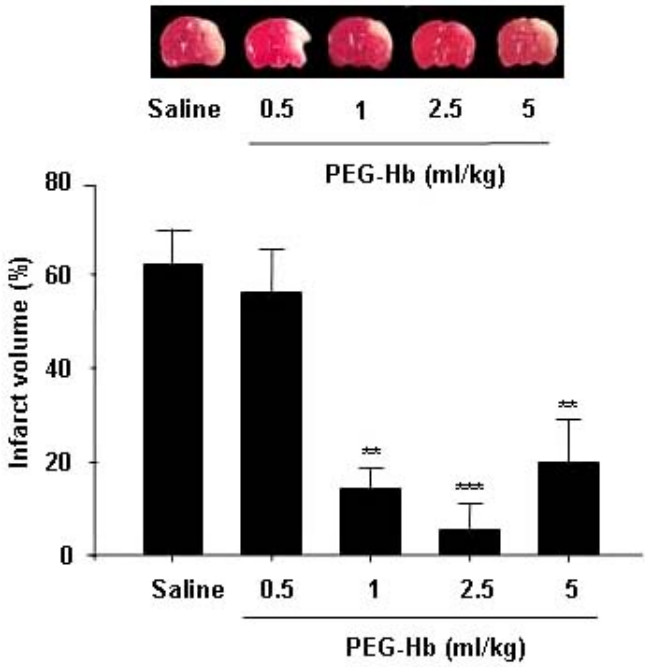

Fig. 2. PEG-Hb decreases infarct volumes in neonatal hypoxia. PEG-Hb was intra-arterially injected at indicated doses. Infarct area was calculated as a percentage of the contralateral hemisphere. Photographs showed the representative TTC-stained coronal brain sections with six slices (2 mm-thick) each between 4 and 16 mm from the frontal pole. Data are expressed as the mean ± SD (n=10). $^{**}p<0.01$, $^{***}p<0.001$, (Sheffe's test) compared with saline-treated group.

RESULTS

Physiological parameters were not significantly changed by treatments with PEG-Hb (Table I). The neonatal hypoxia procedure resulted in severe brain tissue damage in the ipsilateral hemisphere (Fig. 2). Intravenous administration of PEG-Hb prior to hypoxia markedly reduced in-

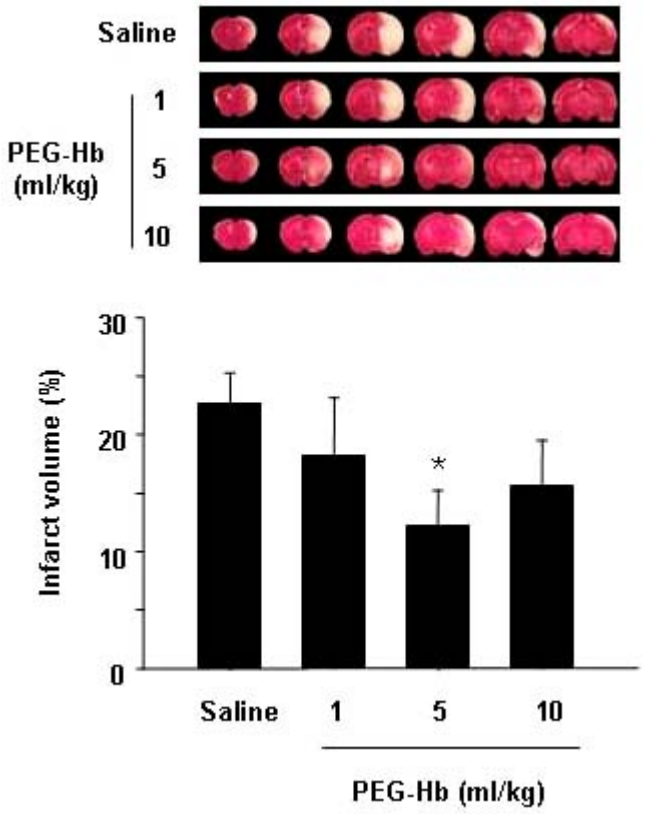

Fig. 3. Injection of PEG-Hb before MCAO decreases infarct volumes. PEG-Hb was intravenously injected at indicated doses. Infarct area was calculated as a percentage of the contralateral hemisphere. Data are mean ± S.D. (n=10). **$p < 0.01$, (Sheffe's test) compared with saline-treated group.

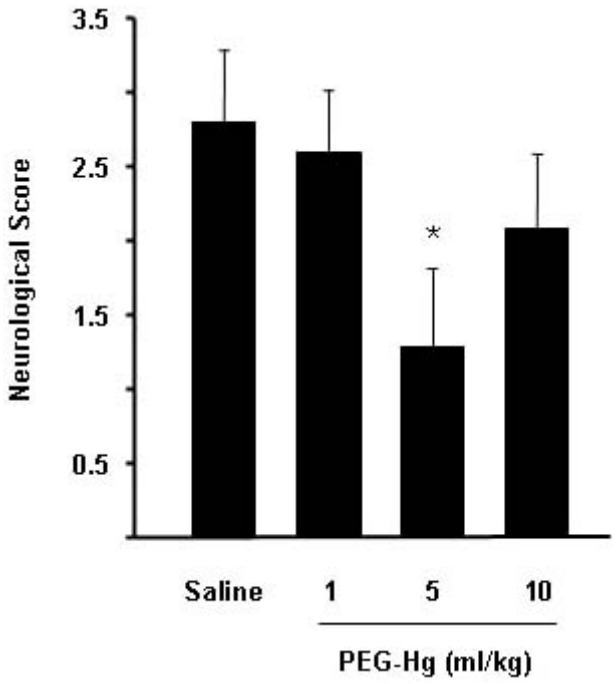

Fig. 4. Neurological examination. PEG-Hb was intravenously injected at indicated doses. Twenty-two hours after reperfusion following 2-h MCAO, a neurological examination was performed as described in the Materials and Methods section. Data are mean ± S.D. (n=10). *$p < 0.05$, (Sheffe's test) compared with saline-treated group.

farct volumes. Thus, 2.5 ml/kg of PEG-Hb reduced the infarct volume by 91.4%. However, the dose-response relationship showed a U-shaped pattern (Fig. 2). Treatment with PEG-Hb (5 ml/kg) also diminished the infarct volume in an adult rat focal ischemia model (i.e., 2 h MCAO followed by 24 h reperfusion) in a U-shaped dose-response relationship (Fig. 3). We further found that the neurological scores were well correlated with the sizes of ischemic injury. The neurological score in saline-treated rats was 2.8 ± 0.48 (Fig. 4). Treatment with PEG-Hb (5 ml/kg) before ischemia decreased the neurological score to 1.3 ± 0.53. At doses of 1 or 10 ml/kg, expectedly, PEG-Hb did not improve the neurological scores. As administered during the first several minutes of reperfusion, however, PEG-Hb did neither significantly decrease the infarct volume (Fig. 5) nor improve the neurological score (data not shown).

DISCUSSION

A distinctive feature of cerebral ischemia is a reduction of oxygen delivery from the penumbra to the ischemic core. Short interruption of oxygen delivery for only 5 minutes will result in neuronal death in corresponding brain regions. A variety of experiments to increase oxygen delivery have been carried out in animal models of ischemia by therapies including manipulation of perfusion pressure (Rossi *et al*., 2000), improvement of cerebral blood flow (Chileuitt *et al*., 1996; Ito *et al*., 2002), and enhancement of blood oxygen-carrying capacity (Bell *et al*., 2002). Hemoglobin-based oxygen carriers including PEG-Hb would be effective not only as an artificial blood substitutes in the loss of the whole blood but also as a therapeutic agent for the treatment of various diseases induced by hypoxic tissues.

In the present study, intravenous injection of PEG-Hb substantially improved neurological function markedly reduced the volume of cerebral infarction in both neonatal hypoxic and adult focal ischemic rat models. In the present study, one single injection was chosen because of the drug's prolonged half-life (t½). The half-life of PEG-Hb in blood is approximately 3 days, which covers the period

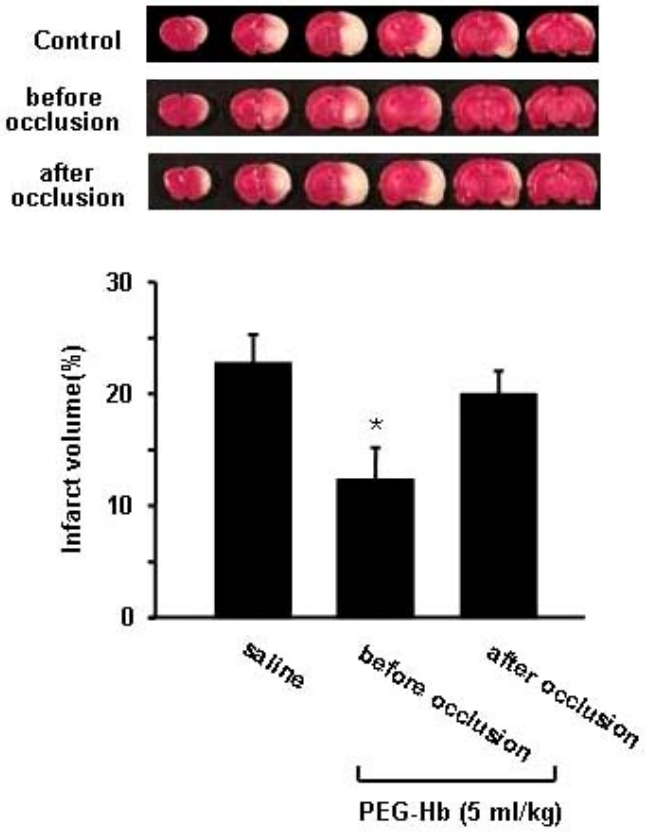

Fig. 5. Injection of PEG-Hb after MCAO did not reduce the infarct volumes. PEG-Hb was intravenously injected right after reperfusion. Infarct area was calculated as a percentage of the contralateral hemisphere. Data are mean ± SD (n=10).

(less than 30 h) of our observation (informed by the manufacturer, Sun-Bio Inc.).

Of hemoglobin-based oxygen carriers, diaspirin cross-linked haemoglobin (DCLHb) has been most extensively studied. DCLHb is produced using 3,5-dibromosalicylate to form a four-carbon fumarate bridge between the two α-subunits of the hemoglobin molecule. DCLHb has a slightly lower affinity for oxygen than fresh blood, which allows it to release oxygen more readily to tissues compared with red blood cells. PEG-Hb has high O_2 affinity, compared with a conventional plasma substitution, and increases intravascular retention time by prevention of the rapid dissociation of hemoglobin tetramers (Bi *et al*., 2004). The p50 value of oxygen at which 50% of hemoglobin is saturated (p50) is approximately 10 mmHg on PEG-Hb, compared with that of human red blood cell (Fig. 1). Thus, PEG-Hb may increase oxygen availability at the hypoxic/ischemic lesion by enhancing the release of oxygen from hemoglobin at the tissue level. Tissue acidosis rapidly develops during ischemia (Isaev *et al*., 2008). Such an acidosis would cause a rightward shift of p50, perhaps increasing the oxygen delivery-and-release efficacy from PEG-Hg. Our unpublished data indicate that the p50 of PEG-Hg is shifted rightward by slight acidosis (data not shown).

However, some problems associated with the hemoglobin-based oxygen carriers have been revealed from both *in vitro* and *in vivo* studies (Alayash, 1999; Buehler and Alayash, 2004). One of the problems is to increase the production of free oxygen radicals, particularly during at least several hours after reperfusion from an ischemic insult during reperfusion of post-ischemic tissues (Agardh *et al*., 1991; Buehler and Alayash, 2004). Ischemia leads to alterations in metabolic reactions producing hypoxanthine and activating the enzyme xanthine oxidase. The level of hypoxanthine increases with the duration and severity of ischemia. When the tissue is reperfused with oxygen carrying fluid, xanthine oxidase converted oxygen and hypoxanthine into superoxide anion. By several mechanisms, superoxide anion results in the formation of oxygen radicals that can cause tissue injury (Dröge, 2002). In clinical trials, therefore, co-administration of some antioxidants may be beneficial for the elimination of the oxygen radicals produced during the initial reperfusion period. Another problem is the volume expansion effect of PEG-Hb. Injection of PEG-Hb may increase the volume of whole blood, leading to the increased mean blood pressure. However, it may not be the case in the present experimental condition. Our present experiments showed that the systolic and diastolic blood pressures were not changed by PEG-Hb (Table I).

In the present study using the adult ischemic model, post-ischemic administration of PEG-Hb was not effective for the reduction of ischemic injury. One possibility is the complete blockade of delivery of PEG-Hb in our experimental model. Maintenance of some residual blood flow would be necessary to obtain benefit from enhanced release of oxygen from hemoglobin. Further studies are, however, needed to find out other possible reasons.

In summary, treatment of PEG-Hb may be beneficial for the therapy of hypoxia/ischemia-induced brain injury. PEG-Hb produced statistically significant reduction in ischemic brain damage and improvement in neurological disorder following transient focal cerebral ischemia in rats. Co-administration of certain kinds of antioxidants may be beneficial for better treatment. PEG-Hb may represent a target for the development of new treatments for stroke.

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