

# Experimental Study of Pegylated Liposomal Hemoglobin on Norepinephrine Release and Reperfusion Arrhythmias in Isolated Guinea Pig Hearts

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**Purpose:** Under myocardial reperfusion conditions, hemoglobin (Hb)-based artificial blood showed effectiveness for post-ischemic dysfunction. However, there are no studies about the effects of this product on reperfusion arrhythmias (ventricular fibrillation, VF) associated with norepinephrine (NE) release. This study was to evaluate the effects of the timing of the administration of pegylated liposomal Hb (LHb, P<sub>50</sub>=40–45 mmHg, 1 mg/mL) on NE release and VF.

**Materials and Methods:** Isolated guinea pig hearts (*n*=6 in each group) were randomly divided into four groups in Krebs-Henseleit solution being supplemented or not with LHb as follows: pre-ischemia (PRE), reperfusion (REP), or PRE+REP groups. The hearts were perfused for 30 min (preischemic period) and then subjected to 30 min of global ischemia, followed by 30 min of reperfusion with a normothermic Langendorff apparatus at 30 mm Hg aortic pressure in a constant pressure model.

**Results:** No differences were documented among the four groups in heart rate, left ventricular-developed pressure, or coronary flow rate. However, the REP group significantly decreased the duration of VF and NE release, but it did not inhibit the incidence of VF.

**Conclusion:** These results suggest that the administration of LHb, especially with the timing of reperfusion, might prevent reperfusion arrhythmias linked to the inhibition of NE release. (*Ann Thorac Cardiovasc Surg* 2007; 13: 391–395)

**Key words:** ischemia, liposomal hemoglobin, norepinephrine, reperfusion, ventricular fibrillation

## Introduction

Norepinephrine (NE) release was mainly determined by two mechanisms: Ca<sup>2+</sup>-dependent exocytotic release and Ca<sup>2+</sup>-independent carrier-mediated release with the activation of the NE transporter.<sup>1,2)</sup> In protracted ischemia,

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intraneuronal acidosis is caused by the failure of the H<sup>+</sup>-ATP pump as a result of ischemia activation of the Na<sup>+</sup>-H<sup>+</sup> exchanger. This leads to an accumulation of intracellular Na<sup>+</sup>. This phenomenon, combined with increased axoplasmic NE that causes a reversal of the NE transporter, was in an outward direction, eliciting a carrier-mediated NE release.<sup>2)</sup> This release strongly associates with the duration of reperfusion arrhythmias.<sup>1–3)</sup> Therefore many investigators have thought NE is to be closely related to and a crucial factor of reperfusion arrhythmia in experimental and clinical settings.<sup>2,4)</sup>

Liposomal hemoglobin (LHb) manufactured by Terumo is a polyethylene glycol (PEG)-modified liposomal human hemoglobin (Hb) solution. Unlike other Hb

solutions, LHB transfusion shows no vasoconstriction. The efficacy and safety of LHB have been previously demonstrated in hemorrhage, endotoxin shock, and cerebral ischemic models in terms of systemic and microvascular responses.<sup>5-8)</sup>

Under conditions of myocardial ischemia reperfusion, it has been reported that the use of synthetic-modified Hb-oxygen affinity improved the recovery of stunned myocardium in dogs.<sup>9)</sup> More recently, Asano and colleagues demonstrated that LHB manufactured by Terumo has a potential therapeutic value in myocardial microcirculatory failure.<sup>10)</sup> However, there are no studies about the effects of this product on reperfusion arrhythmias.

The present study was designed to determine whether the three different timings of the administration of LHB ( $P_{50}=40-45$  mmHg) could change the onset and duration of reperfusion arrhythmias with or without the modulation of NE release.

## Material and Methods

### Animals

Twenty-four male Hartley guinea pigs (Sankyo Co., Ltd., Tokyo, Japan) weighing 400 to 500 g were randomly divided into four groups ( $n=6$  in each group); Krebs-Henseleit solution (KHS) (Control) being supplemented or not with LHB as follows: pre-ischemia (PRE), reperfusion (REP), or PRE+REP groups. All animals have received humane care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86-23, revised 1985).

### Hemoglobin-based solution

Pegylated LHB was prepared at the Terumo Research Development Center (Terumo, Tokyo, Japan). A stroma-free hemoglobin solution obtained from outdated human red blood cell (RBC) was ultramixed with polyethylene glycol (PEG) to form hydrated 200 nm diameter liposomes and diluted with 0.9% NaCl to Hb concentration 6 g/dL. The oxygen transport ability of this artificial blood substitute is 4.5 mL/dL. This is the same as fresh human blood. The oxygen affinity of this product was as follows:  $P_{50}=40-45$  mmHg, final conc. 1 mg/mL.

### Langendorff perfusion experiments

Isolated guinea pig hearts were the basis of our previous study.<sup>3)</sup> In brief, the animals were killed by cervical dis-

location under anesthesia with CO<sub>2</sub> vapor. The hearts were rapidly perfused at a constant aortic pressure of 30 mmHg in a constant pressure Langendorff model with a Krebs-Henseleit solution (KHS) containing (in mM) NaCl, 118.2; NaHCO<sub>3</sub>, 25; KH<sub>2</sub>PO<sub>4</sub>, 1.0; KCl, 4.83; MgSO<sub>4</sub>, 2.37; CaCl<sub>2</sub>, 2.5; and glucose, 11.1. KHS was saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub> at 37°C. Electrocardiograms were continuously recorded with surface electrodes placed on the right atrium and the left ventricle to measure heart rate (HR) and to document any arrhythmias. Cardiac surface temperature was measured by attaching a thermoprobe (PTI-200; Unique Medical Co., Tokyo, Japan). The left ventricular-developed pressure (LVDP) was determined via the left ventricle using a fluid-filled latex balloon connected to a transducer (Baxter, McGaw Park, IL) that was recorded on a polygraph (model DS-1060; Fukuda Denshi Co., Tokyo, Japan). The volume of the balloon was initially adjusted to achieve a left ventricle end-diastolic pressure of 10 mm Hg, which was maintained throughout the experiment.

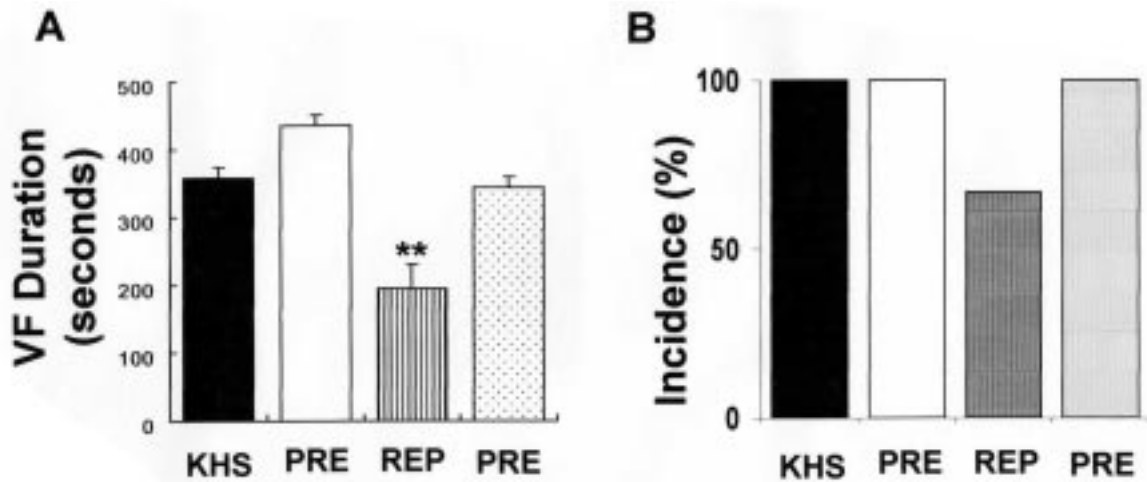
Initial background (pre-ischemic period, 30 min) measurements were obtained after 30 min of stabilization, and coronary effluent was collected into sample tubes every 5 min for 30 min. Normothermic global ischemia for 30 min was induced by the stop of coronary perfusion. This was followed by a 30-min reperfusion period. In the first 10 min of reperfusion, sample tubes were replaced every 2 min; they were replaced every 5 min during the last 35 min. To evaluate the effects of the administered method, we added LHB (1 mg/mL in KHS with 10 mg/mL bovine serum albumin; BSA) during PRE, REP, or PRE+REP. At the end of each experiment, the hearts were weighed as wet weight. Coronary flow rate (CFR) was calculated as mL/min/g.

### Evaluation of ventricular fibrillation (VF)

Reperfusion arrhythmia was analyzed from continuously recorded electrocardiogram tracings according to guidelines defined by the Lambeth Conventions.<sup>11)</sup> Although ventricular premature contraction and ventricular tachycardia were rare and inconsistent, VF is the most common and persistent type of reperfusion arrhythmia. Thus only VF was taken as an index of reperfusion arrhythmias.

### Norepinephrine (NE) assay

The amount of NE was assayed in the coronary effluent by high-performance liquid chromatography coupled with an electrochemical detector (Eicom, Kyoto, Japan) and software for analysis (AD Instruments Co., Tokyo, Japan).



**Fig. 1.** The duration of ventricular fibrillation (VF, **A**) and the incidence of VF during 45 min of reperfusion (**B**) followed by 30 min of stop flow in low affinity Lhb (1 mg/mL) during the pre-ischemic period, reperfusion period, or both periods.

Each bar represents mean  $\pm$  SEM ( $n=6$  in each group).  $**P<0.05$ , significantly different from the pre-ischemic period by one-way analysis of variance with Bonferroni's  $t$ -test used for post hoc analysis.

These procedures have been previously described.<sup>3)</sup> The values were expressed in picomoles per gram of wet heart weight.

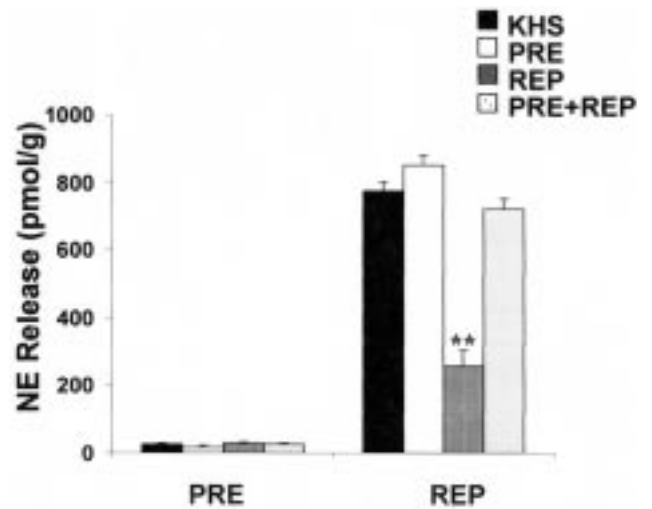
**Statistical analysis**

Experimental values were expressed as mean  $\pm$  standard error (SEM) for the 6 hearts in each group. A comparison of more than two groups was performed by one-way analysis of variance (ANOVA) with the Bonferroni  $t$ -test used for post hoc analysis. A value of  $P < 0.05$  was considered statistically significant.

**Results**

As shown in Fig. 1, A and B, the timing of administration in the reperfused period significantly shortened the duration of VF (KHS:  $358.7 \pm 14.1$  s; PRE:  $436.0 \pm 14.0$  s; REP:  $143.5 \pm 46.0$  s; PRE+REP:  $345.2 \pm 15.3$  s), but did not markedly change the incidence of VF (KHS: 100%; PRE: 100%; REP: 67%, PRE+REP: 100%).

In the amount of NE release during the 30 min pre-ischemic period, there was no significant difference among the three groups (Fig. 2). The cumulative NE release during the 30 min of reperfusion evoked by 30 min normothermic global ischemia rose about 8–46 times compared to those of the pre-ischemic periods (Fig. 2). Similar to the results of the duration of VF, Lhb given during the reperfused period significantly attenuated NE



**Fig. 2.** Norepinephrine (NE) release during pre-ischemic and reperfused period in all experimental groups.

Each bar represents mean  $\pm$  SEM ( $n=6$  in each group).  $**P<0.05$ , significantly different from the pre-ischemic period by one-way analysis of variance with Bonferroni's  $t$ -test used for post hoc analysis.

release (KHS:  $776 \pm 25.3$  pmol/g; PRE:  $852 \pm 29.5$  pmol/g; REP:  $258 \pm 46$  pmol/g; PRE+REP:  $720.8 \pm 2.5$  pmol/g) in 30 min of reperfusion (Fig. 2).

There was no difference among the three groups in HR, LVDP, and CFR during reperfusion followed by 30

**Table 1. Heart rate (HR), left ventricular-developed pressure (LVDP), and coronary flow rate (CFR) during 45-min reperfusion followed by 30-min stop in the presence of LHB (1 mg/mL). Each value represents mean  $\pm$  SEM ( $n=6$  in each group). \*\* $P<0.05$ , significantly different from KHS by one-way analysis of variance with Bonferroni's  $t$ -test used for post hoc analysis.**

		KHS	PRE	REP	PRE+REP
HR (beats/min)	PRE	238.2 $\pm$ 3.2	235.3 $\pm$ 8.5	242.7 $\pm$ 7.6	222.1 $\pm$ 8.8
	REP 0	Vf	Vf	Vf	Vf
	REP 10	204.4 $\pm$ 23.9	258.0 $\pm$ 11.5	227.0 $\pm$ 6.2	218.6 $\pm$ 11.1
	REP 45	206.1 $\pm$ 12.1	217.3 $\pm$ 2.5	208.0 $\pm$ 6.5	207.3 $\pm$ 12.0
	PRE	43.4 $\pm$ 4	34.1 $\pm$ 2.6	36.1 $\pm$ 1.6	26.6 $\pm$ 3.6
LVDP (mmHg)	REP 0	—	—	—	—
	REP 10	20.0 $\pm$ 3.3	15.0 $\pm$ 1.0	24.5 $\pm$ 4.2	10.7 $\pm$ 2.2
	REP 45	22.7 $\pm$ 3.3	18.0 $\pm$ 4.3	25.2 $\pm$ 3.9	16.5 $\pm$ 5.3
CFR (mL/min/g)	PRE	5.4 $\pm$ 0.3	5 $\pm$ 1.3	8.5 $\pm$ 0.6	7.0 $\pm$ 1.5
	REP 0	4.2 $\pm$ 0.6	6.8 $\pm$ 1.1	9.2 $\pm$ 1.0	6.3 $\pm$ 1.5
	REP 10	3.9 $\pm$ 0.5	5.7 $\pm$ 0.8	5.3 $\pm$ 0.9	5.3 $\pm$ 0.8
	REP 45	3.7 $\pm$ 0.5	4.5 $\pm$ 0.3	4.7 $\pm$ 0.7	5.0 $\pm$ 0.8

min of ischemia (Table 1).

## Discussion

This study demonstrated that LHB administered in reperfusion significantly inhibited the duration of VF and NE release, but not post-ischemic cardiac function (coronary flow rate, heart rate, and LVDP) and the incidence of VF.

The administration of LHB in reperfusion markedly inhibited NE release in the present study. However, the modes of NE release were divided into exocytotic release, and carrier-mediated release linked to Na<sup>+</sup>/H<sup>+</sup> exchanger.<sup>1,2)</sup> To confirm the mode of NE release in the present study, we used the Na<sup>+</sup>/H<sup>+</sup> exchanger inhibitor (5-[N-ethyl-N-isopropyl]-amiloride, 10  $\mu$ M) and the NE transporter inhibitor (desipramine hydrochloride, 10 nM). This inhibitor (desipramine hydrochloride; DMI, 10 nM)<sup>1)</sup> significantly inhibited NE release in 30-min reperfusion subjected to 30-min ischemia and completely abolished reperfusion arrhythmias (data not shown). Also, the inhibition of the Na<sup>+</sup>/H<sup>+</sup> exchanger suppressed NE release and abolished reperfusion arrhythmias (data not shown). These suggest that the carrier-mediated NE release linked to the Na<sup>+</sup>/H<sup>+</sup> exchanger in this experimental model concurs with the previous studies.<sup>1,3)</sup> Taken together, LHB most likely inhibited the carrier-mediated NE release linked to the Na<sup>+</sup>/H<sup>+</sup> exchanger in the present study.

It is well known that the amount of NE release in reperfusion has a strong correlation with the deterioration of reperfusion arrhythmias.<sup>1-3)</sup> However, Hb is also

well known as one of the strong inducible factors in reperfusion arrhythmias. Hb can readily generate or interact with free radicals,<sup>12)</sup> leading to induced reperfusion arrhythmias.<sup>13)</sup>

To address whether NE release is one of the important inducible factors on reperfusion arrhythmias in our experimental model, we used a nonselective  $\beta$ -adrenoceptor antagonist, *dl*-propranolol hydrochloride (1  $\mu$ M).<sup>14)</sup> Propranolol did not affect an NE release in reperfusion, but it completely inhibited the incidence and the duration of VF in reperfusion (data not shown). Thus it was suggested that NE is one of the important inducible factors of reperfusion arrhythmias in this experimental model. Collectively, although there are many mediators like free radicals and calcium overload, LHB at least modulates NE release associated with reperfusion arrhythmias in this study.

Myocardial post-ischemic cardiac dysfunction is the conversion of normal or injured cells to more severely injured cells during the restoration of blood flow. This resultant damage is mediated by molecular O<sub>2</sub>.<sup>15)</sup> However, many reports showed that the pretreatment of Hb-based artificial oxygen transporters improved postcardiac dysfunction.<sup>16,17)</sup> Nevertheless, there was no significant difference in post-ischemic cardiac dysfunction (HR, CFR, and LVDP) in the three different administered methods.

The LHB used in the present study was manufactured differently from the other hemoglobin-based oxygen carriers (HBOC), like an HBOC-201. Thus although LHB in the present study is a capsulated HBOC, HBOC-201 is a

noncapsulated HBOC. Also, the discrepancies between our study and John et al. include (1) human hemoglobin vs. bovine hemoglobin and (2) modified PEG vs. glutaraldehyde-polymerized.<sup>16</sup> Also, the duration of the global ischemia might be too short in the present study.

In the present study, LHB administered during the reperfused period may inhibit NE release, and this may lead to the prevention of reperfusion arrhythmias. However, our findings in this study could not completely exclude the LHB itself to reperfusion arrhythmias generation by an action on cardiomyocytes and also could not exclude the effect on free radicals and calcium overloads. Therefore, we soon need further experiments to clarify the inhibitory mechanism of low affinity LHB.

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## References

1. Imamura M, Lander HM, Levi R. Activation of histamine H<sub>3</sub> receptors inhibits carrier-mediated norepinephrine release during protracted myocardial ischemia. Comparison with adenosine A<sub>1</sub>-receptors and  $\alpha^2$ -adrenoceptors. *Circ Res* 1996; **78**: 475–81.
2. Schömig A. Catecholamines in myocardial ischemia. Systemic and cardiac release. *Circulation* 1990; **82** (3 Suppl): II13–22.
3. Oka J, Imamura M, Hatta E, et al. Carrier-mediated norepinephrine release and reperfusion arrhythmias induced by protracted ischemia in isolated perfused guinea pig hearts: effect of presynaptic modulation by  $\alpha_2$ -adrenoceptor in mild hypothermic ischemia. *J Pharmacol Exp Ther* 2002; **303**: 681–7.
4. Bankwala Z, Hale SL, Kloner RA.  $\alpha$ -adrenoceptor stimulation with exogenous norepinephrine or release of endogenous catecholamines mimics ischemic preconditioning. *Circulation* 1994; **90**: 1023–8.
5. Oda T, Nakajima Y, Kimura T, et al. Hemodilution with liposome-encapsulated low-oxygen-affinity hemoglobin facilitates rapid recovery from ischemic acidosis after cerebral ischemia in rats. *J Artif Organs* 2004; **7**: 101–6.
6. Sakai H, Takeoka S, Wettstein R, et al. Systemic and microvascular responses to hemorrhagic shock and resuscitation with Hb vesicles. *Am J Physiol Heart Circ Physiol* 2002; **283**: H1191–9.
7. Usuba A, Osuka F, Kimura T, et al. Liposome encapsulated hemoglobin as a resuscitation fluid for hemorrhagic shock. *Artif Organs* 1998; **22**: 116–22.
8. Whiteford M, Spirig A, Rudolph A, et al. Effect of liposome-encapsulated hemoglobin on the development of endotoxin-induced shock in the rat. *Shock* 1998; **9**: 428–33.
9. Pagel PS, Hettrick DA, Montgomery MW, et al. RSR13, a synthetic modifier of hemoglobin-oxygen affinity, enhances the recovery of stunned myocardium in anesthetized dogs. *J Pharmacol Exp Ther* 1998; **285**: 1–8.
10. Asano T, Matsumoto T, Tachibana H, et al. Myocardial microvascular perfusion after transfusion of liposome-encapsulated hemoglobin evaluated in cross-circulated rat hearts using tracer digital radiography. *J Artif Organs* 2004; **7**: 145–8.
11. Walker MJ, Curtis MJ, Hearse DJ, et al. The Lambeth conventions: guidelines for the study of arrhythmias in ischaemia, infarction, and reperfusion. *Cardiovasc Res* 1988; **22**: 447–55.
12. Winterbourn CC. Oxidative reactions of hemoglobin. *Methods Enzymol* 1990; **186**: 265–72.
13. Bernier M, Hearse DJ, Manning AS. Reperfusion-induced arrhythmias and oxygen-derived free radicals. Studies with “anti-free radical” interventions and a free radical-generating system in the isolated perfused rat heart. *Circ Res* 1986; **58**: 331–40.
14. Lewis MJ, Grey AC, Henderson AH. Inotropic  $\beta$ -blocking potency (pA<sub>2</sub>) and partial agonist activity of propranolol, practolol, sotalol and acebutolol. *Eur J Pharmacol* 1982; **86**: 71–6.
15. Vlahakes GJ, Lee R, Jacobs EE Jr, et al. Hemodynamic effects and oxygen transport properties of a new blood substitute in a model of massive blood replacement. *J Thoracic Cardiovasc Surg* 1990; **100**: 379–88.
16. Berlin G, Challoner KE, Woodson RD. Low-O<sub>2</sub> affinity erythrocytes improve performance of ischemic myocardium. *J Appl Physiol* 2002; **92**: 1267–76.
17. Caswell JE, Strange MB, Rimmer DM 3rd, et al. A novel hemoglobin-based blood substitute protects against myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 2005; **288**: H1796–801.