Cardioprotective Effect of a Hemoglobin-Based Oxygen Carrier on Cold Ischemia/Reperfusion Injury

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Key Words

Hemoglobin-based oxygen carrier • Ischemia/reperfusion injury • Inflammation • Toll-like receptors

Abstract

Objectives: The etiology of myocardial ischemia/reperfusion (I/R) injury is multifactorial, but activation of the innate immune system and the resulting inflammatory response are important components of I/R injury. The aim of this study was to investigate the protective effect of a hemoglobin-based oxygen carrier (HBOC) on cold I/R heart and to explore the underlying mechanisms. **Methods:** Isolated Sprague-Dawley rat hearts were perfused in the Langendorff mode. After 30 min of basal perfusion, rat hearts were arrested with histidine-tryptophan-ketoglutarate solution (HTKs) with or without an HBOC and hypothermically stored (4°C) for 9 or 14 h, followed by 2 h of reperfusion. **Results:** Compared with HTKs alone, the HBOC in HTKs greatly improved heart contraction and decreased infarct size, necrosis and apoptosis, which was related to the reduced expression of Toll-like receptor 2 (TLR 2), TLR 4, TNF- α , IL-1 β and nuclear factor- κ B (NF- κ B) activation. **Conclusions:** Our results demonstrated that the HBOC protected isolated rat

heart from cold I/R injury and this protection was associated with attenuation of the expression of the TLR 2 and TLR 4/NF- κ B signaling pathway, which may down-regulate the inflammatory response.

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Introduction

Ischemia/reperfusion (I/R) injury may lead to myocardial contractile dysfunction, cardiac arrhythmias, myocardial infarction and cardiomyocyte apoptosis [1, 2]. Extensive studies have been performed to identify therapeutic strategies that might protect the heart from I/R injury, but most of these have not been applied to patients in the clinical setting due to the lack of a significant effect [3, 4]. The pathogenesis of I/R injury is complex and reperfusion-induced inflammatory responses play a critical role in mediating myocardial I/R injury. This injury releases cytokines and other inflammatory mediators, initiating a vicious circle of inflammation that exacerbates myocardial damage. Thus, inhibition of the inflammatory response seems a rational way to ameliorate cardiac I/R injury [5, 6].

Fig. 1. Schematic representation of the experiment. Immediately after removal from the chest cavity, rat hearts were perfused with KHB for 30 min at 37°C as baseline and then arrested with HTKs, with or without PolyPHb, and placed in hypothermic storage (4°C) for 9 h (HTK₉ and HBOC₉) or 14 h (HTK₁₄ and HBOC₁₄). Hearts were then reperused with KHB for 2 h. The sham control group was subjected to 150 min of perfusion without I/R injury.

Toll-like receptors (TLRs) are a family of molecules that play a critical role in innate immunity. TLRs are evolutionarily conserved transmembrane receptors that serve as pattern recognition receptors, recognizing conserved microbial motifs in molecules. Activation of TLRs by any of these molecules results in an inflammatory response, alerting the host to the presence of a microbial invasion and initiating an immune response. Recent observations demonstrate that some TLR family members, such as TLR 2 and TLR 4, also become activated by endogenous molecules released from damaged or ischemic tissues. TLR signals finally activate the transcription factor nuclear factor- κ B (NF- κ B) and permit the activation of proinflammatory cytokine genes, increasing the expression of cytokines and inflammatory mediators to exacerbate organ damage. The TLR/NF- κ B signaling pathway plays an important role in initiating the inflammatory response in I/R [7–9].

Hemoglobin-based oxygen carriers (HBOCs) were initially developed as a blood substitute. Glutaraldehyde-polymerized human placenta hemoglobin (PolyPHb) is a particularly promising HBOC because it allows the transport of more oxygen (O₂) to hypoxic tissues because of its higher affinity for O₂, lower viscosity and smaller mean

diameter than human red blood cells [10, 11]. Previous studies of HBOCs, including research from our group, demonstrated the potential protective effect of HBOC on the heart against I/R injury, and that the underlying molecular mechanisms quench myocardial oxidative stress and restore the nitroso-redox balance [12, 13]. However, the protective effect of HBOC on the inflammatory response has not been reported. Thus, this study was designed to investigate the effect of PolyPHb on the TLR/NF- κ B signaling pathway in isolated rat heart during cold I/R injury.

Materials and Methods

All experiments were approved by the Institutional Animal Care and Use Committee of Sun Yat-Sen University and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

PolyPHb Preparation

PolyPHb was prepared as described previously [12, 13]. Briefly, hemoglobin from fresh human placenta blood (donated by Tianjin Union Stem Cell and Genetic Engineering Ltd., Tianjin, China) was purified and virally inactivated by heat treatment. Intra- and inter-molecular cross-linking were performed using pyridoxal phosphate and glutaraldehyde, respectively. Ultrafiltration and molecular sieve chromatography were performed to harvest the PolyPHb in the molecular weight range of 64–600 kDa. The P₅₀ value of PolyPHb was significantly lower than that of fresh human blood (5–9 vs. 26 mm Hg). Prior to use, the PolyPHb was mixed with histidine-tryptophan-ketoglutarate solution (HTKs) to a final concentration of 0.3 g Hb/dl, as a cardioplegic solution, and then equilibrated with 100% O₂ gas at 4°C for 5 min.

Experimental Protocol

In total, 75 adult Sprague-Dawley rats, weighing 280–350 g, were injected intravenously with heparin (50 IU) and anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). The hearts were rapidly excised, mounted on a Langendorff apparatus (Radnoti, Monrovia, Calif., USA) and perfused with Krebs-Henseleit buffer (KHB) at constant pressure (100 cm H₂O). The KHB was equilibrated with 95 and 5% CO₂ at 37°C for 30 min prior to use. After 30 min of basal perfusion, the hearts were randomly divided into 5 groups, arrested with HTKs with or without PolyPHb and stored in 0.9% sodium chloride under hypothermic storage (4°C) for 9 h [HTKs group (HTK₉) and HBOC group (HBOC₉)] or for 14 h [HTKs group (HTK₁₄) and HBOC group (HBOC₁₄)]. The hearts were then reperused with KHB for 2 h. Rat hearts without arrest were used as the sham control and subjected to 150 min of perfusion with KHB. The experimental scheme is illustrated in figure 1.

Cardiac Function Measurement

A water-filled latex balloon was immediately inserted into the left ventricle (LV) via the mitral valve after the heart was mount-

ed on the Langendorff apparatus. The balloon was attached to a pressure sensor (model SP844; MEMSCAP, Durham, N.C., USA) connected to a PowerLab data-acquisition system (AD Instruments Pty., Bella Vista, N.S.W., Australia). Measured parameters included left ventricular developed pressure (LVDP), left ventricular end-diastolic pressure (LVEDP), maximum LVDP increase (+dp/dt), and decrease rate (−dp/dt). Coronary flow (CF) rate was calculated from the coronary effluent, sampling time and cardiac wet weight.

Oxygen Releasing Capacity Determination

The O₂ releasing capacity of PolyPHb was calculated from the O₂ partial pressure (pO₂) and O₂ saturation (sO₂) (i-STAT portable blood gas analyzer; i-STAT Corp, Windsor, N.J., USA) of the cardioplegic solutions (0.3 g Hb/dl PolyPHb in HTKs or HTKs alone) and the corresponding effluents collected during arrest, using the following formula [12]:

$$\text{O}_2 \text{ releasing capacity} = [(p\text{O}_2 \times s\text{O}_2)_{\text{cardioplegic solution}} - (p\text{O}_2 \times s\text{O}_2)_{\text{effluent}}] / \text{cardiac wet weight.}$$

To assess the levels of anaerobic glycolysis and O₂ supply during ischemia, cardiac lactic acid release was also measured from the effluents collected during the first 5 min of reperfusion (i-STAT portable blood gas analyzer).

Quantitative Real-Time PCR Analysis of mRNA Expression

Rat myocardium samples were homogenized in the TRIZOL reagent (Invitrogen, Carlsbad, Calif., USA). Total RNA concentration and purity were determined by spectrophotometric absorbance measurement (260 and 280 nm). Isolated RNA (2 µg) was converted into cDNA using a high-capacity cDNA synthesis kit (TaKaRa, Japan). Real-time PCR (RT-PCR) was performed with an ABI Prism 7,500 sequence detection system (PE Applied Biosystems). The SYBR green RT-PCR master mix kit (TaKaRa, Japan) was used in subsequent PCR assays according to the manufacturer's protocol. The RT conditions were 42°C for 15 min followed by 95°C for 2 min; PCR conditions included predenaturing at 95°C for 10 s, followed by 40 cycles of 95°C for 5 s, of 60°C for 30 s and 72°C for 1 min. In this experiment, 18S rRNA and GAPDH were used as housekeeping genes. Levels of TLR 2, TLR 4, TNF-α and IL-1β mRNA were calculated based on the 2^{−ΔΔCt} method of comparative quantification [14, 15]. Primers were as follows:

TLR 2; sense primer 5'-ACGCAGTGAGTGGTGCAAGTAT-3'; antisense primer 5'-CTTCTCAATGGGTTCAGCAA-3'.

TLR 4; sense primer 5'-TGGTGTAGCCATTGCTGCCAACA-3'; antisense primer 5'-AGCGGCTACTCAGAACTGCCA-3'.

TNF-α; sense primer 5'-GAAACACACGAGACGCTGAA-3'; antisense primer 5'-CCACTCAGGCATCGACATTC-3'.

IL-1β; sense primer 5'-TCACAGCAGCATCTCGACA-3'; antisense primer 5'-GGAGAGCTTTTCAGCTCACAT-3'.

18srRNA; sense primer 5'-CCTGGATACCGCAGCTAGGA-3'; antisense primer 5'-GCGGCGCAATACGAATGCCCC-3'.

GAPDH; sense primer 5'-GACAACCTTTGGCTCGTGGA-3'; antisense primer 5'-ATGCAGGGGTTCTGG-3'.

Western Blot Analysis of Protein Levels

Western blotting was performed with monoclonal antibodies against TLR 2 and TLR 4 (anti-TLR 2, Epitomics, USA; anti-TLR 4, Cell Signaling Technology, USA; diluted 1:1,000). LV tissue was

homogenized and the protein concentration of its supernatant was determined by the bicinchoninic acid method (Pierce, Rockford, Ill., USA). Protein (50 µg) was separated by 8% SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The blot was blocked in PBS containing 0.1% Tween-20 and 5% skimmed milk at 37°C for 1 h. The membrane was then incubated in primary antibody at 4°C overnight, followed by an HRP-conjugated secondary antibody. The results were scanned and analyzed using the Scion Image software (Scion, Frederick, Md., USA). Protein levels of TLR 2 and TLR 4 were corrected by β-actin and the results were shown by relative optical density.

Electrophoretic Mobility Shift Assay of NF-κB Activity

NF-κB activity in rat cardiomyocytes was determined using an electrophoretic mobility shift assay. Nuclear extracts were prepared using the NE-PER kit (Pierce) following the manufacturer's protocol. Equal amounts of nuclear extract protein (3 µg) were incubated with 10× binding buffer, 1 µg/µl poly(dI-dC), and 400-fmol biotin-labeled double-stranded NF-κB-binding consensus oligonucleotides 5'-AGTTGAGGGGACTTTCCCAGGC-3' (total volume 15 µl) using a LightShift Chemiluminescent electrophoretic mobility shift assay kit (Viagene Biotech). Specificity of binding was ascertained by competition with a 50-fold excess of unlabeled consensus oligonucleotides. The film was analyzed using Labworks 4.6.

Determination of Myocardial Apoptosis and Necrosis

Myocardial apoptosis was detected using the terminal deoxynucleotidyl nick-end labeling (TUNEL) assay (Promega, USA). Myocardial nuclei were stained with DAPI (Sigma). TUNEL-positive cardiomyocytes were evaluated under single blinding fashion by a pathologist. The percentage of TUNEL-positive cells was determined by dividing the number of positive-stained nuclei by the total number of nuclei of the cells [16]. Myocardial necrosis was estimated by the release of creatine kinase-MB (CK-MB) and lactate dehydrogenase using an autoanalyzer (model AU5400; Olympus Diagnostics, Melville, N.Y., USA). The LV tissue was stained with hematoxylin and eosin and then assessed in a blinded fashion by a pathologist for the following histological characteristics: acute myocardial necrosis, interstitial edema and cellular swelling.

Estimation of Myocardial Infarct Size

The size of the myocardial infarction in the 5 groups was estimated by 2,3,5-triphenyltetrazolium chloride (TTC) staining. Briefly, after reperfusion, the hearts were weighed and cut into 2-mm-thick slices vertical to the atrioventricular groove. The slices were stained by incubation in 1% TTC solution in phosphate buffer (0.1 M) at 37°C for 10 min and then fixed in 4% paraformaldehyde solution. Infarct size (unstained by TTC) was measured by planimetry, using the Image Pro Plus 5.0 software (Media Cybernetics, Silver Spring, Md., USA), and expressed as a percentage of the total heart.

Oxidative Stress in LV Tissue

Maleic dialdehyde (MDA) content and superoxide dismutase (SOD) activity in LV tissue were used as markers of oxidative stress. The MDA content of heart tissue homogenates was measured with a commercially available assay kit (Nanjing Jiancheng). MDA production was read from a standard curve and corrected for tissue protein content (nmol/mg protein). Bradford's method

Fig. 2. a O₂-releasing capacity of PolyPHb during cardiac arrest (n = 15). **b** Cardiac lactic acid release during the first 5 min of reperfusion. Values are presented as means $\pm$ SEM. * p < 0.05 vs. the HTK₉ group; # p < 0.05 vs. the HTK₁₄ group.

Table 1. Main parameters of PolyPHb in HTKs

Parameter	Results or range
Placenta hemoglobin, g/l	5
Methemoglobin, %	<3
P ₅₀ , mm Hg ^a	5–9
Colloid osmotic pressure, mm Hg	20–25
Osmolality, mosm/kg	290–310
Molecular mass, kDa	64–600
Unpolymerized hemoglobin, %	<3

^a P₅₀, the pO₂ level at which hemoglobin is half-saturated with O₂.

was used to assess protein concentration. The total SOD activity in LV tissue was measured with a commercially available assay kit (Nanjing Jiancheng).

Statistical Analysis

All values are presented as mean $\pm$ SEM. The results of LVDP, LVEDP, $\pm$ dp/dt and recovery of CF were analyzed by 2-factor ANOVA with repeated measures and the use of a post hoc t test with a Bonferroni correction for multiple comparisons. Other data in this study were subjected to 1-way ANOVA followed by a Bonferroni correction for a post hoc t test; p values <0.05 were considered to indicate statistical significance.

Results

Oxygen-Releasing Capacity of HBOC

Table 1 displays the main parameters of PolyPHb in HTKs and indicates a higher O₂ affinity and theoretical higher capacity to carry O₂ by PolyPHb (table 1). As shown in figure 2a, the O₂-releasing capacity of 0.3 g Hb/dl PolyPHb was nearly 3-fold higher than that of the HTKs alone (305.60 $\pm$ 5.43 vs. 109.86 $\pm$ 4.45 mm Hg/g

wet weight; p < 0.05). Consistently, cardiac lactic acid release in the HBOC groups was largely inhibited compared with the HTKs groups (HBOC₉ 12.87 $\pm$ 2.54 and HBOC₁₄ 16.48 $\pm$ 2.12 μ mol/min/g wet weight vs. HTK₉ 15.37 $\pm$ 3.35 and HTK₁₄ 24.84 $\pm$ 2.73 μ mol/min/g wet weight, p < 0.05, respectively; fig. 2b).

Cardiac LV Function

At basal perfusion, there was no significant difference in LVDP, $\pm$ dp/dt, LVEDP or CF among the 5 groups. However, during reperfusion, the LVDP was greatly improved in the HBOC groups when compared with the HTKs groups (p < 0.05; fig. 3a). The $\pm$ dp/dt was also greatly increased in the HBOC groups, compared with the HTKs groups (p < 0.05 for both; fig. 3b, c). Consistently, the LVEDP elevation after I/R injury was also significantly attenuated in the HBOC groups (vs. the HTKs groups, p < 0.05; fig. 3d). Also, the recovery of CF at 60, 90 and 120 min of reperfusion (HBOC₉ 72.74 $\pm$ 3.52% and HBOC₁₄ 50.66 $\pm$ 3.39%, p < 0.05, respectively) of PolyPHb groups was significantly higher than in the HTKs group (HTK₉ 58.84 $\pm$ 3.46% and HTK₁₄ 37.88 $\pm$ 3.82%, p < 0.05, respectively; fig. 3e). HBOC did not totally reverse the damage caused by I/R injury when compared with the sham group (p < 0.05).

TLR 2, TLR 4, TNF- α and IL-1 β mRNA Expression in Cardiomyocytes

TLR 2, TLR 4, TNF- α and IL-1 β mRNA levels of the HBOC groups were markedly lower than in the HTKs groups (fig. 4a–d; p < 0.05). The mRNA levels of TLR 2, TLR 4, TNF- α and IL-1 β were decreased to a greater extent in the HBOC₁₄ group than in the other groups (TLR 2 0.35 $\pm$ 0.89, TLR 4 0.46 $\pm$ 1.09, TNF- α 0.15 $\pm$ 0.75, IL-1 β 0.20 $\pm$ 0.78 relative units vs. other groups, p < 0.05, respectively).

Fig. 3. LVDP (**a**), $\pm dp/dt$ (**b**, **c**) and LVEDP (**d**) of the 5 groups. **e** Recovery of CF at baseline and time points of 30, 60, 90 and 120 min of reperfusion. Values are presented as means $\pm$ SEM ($n = 15$). * $p < 0.05$ vs. the HTK₉ group; # $p < 0.05$ vs. the HTK₁₄ group.

TLR 2 and TLR 4 Protein Levels in Cardiomyocytes

As shown in figure 5, the Western blotting results showed that TLR 2 and TLR 4 protein levels in HBOC groups were significantly lower than in the HTKs groups ($p < 0.05$). Protein levels in HBOC₁₄ were markedly lower than other groups (TLR 2 0.47 ± 1.12 , TLR 4 0.28 ± 1.07 vs. other groups, $p < 0.05$).

NF- κ B Activity in Cardiomyocytes

The band gray value of NF- κ B p65/p50 protein was regarded as the corresponding NF- κ B activity (fig. 6a).

The NF- κ B activities in the 5 groups were sham control 79.67 ± 1.20 , HTK₉ 84.01 ± 1.52 , HBOC₉ 69.01 ± 1.73 , HTK₁₄ 104.67 ± 1.76 , and HBOC₁₄ 91.33 ± 1.45 (the differences among the 5 groups were significant, $F = 36.291$; $p < 0.05$). NF- κ B activity was significantly lower in the HBOC groups than in the HTKs groups ($p < 0.05$; fig. 6b).

Myocardial Apoptosis and Necrosis

After hypothermic storage (4°C) for 9 or 14 h and followed by 2 h of reperfusion, myocardial apoptosis in the

Fig. 4. Expression of TLR 2, TLR 4, TNF- α , and IL-1 β mRNA levels in the different experimental groups (n = 15). Values are presented as means $\pm$ SEM. * p < 0.05 vs. the HTK₉ group; # p < 0.05 vs. the HTK₁₄ group.

Fig. 5. Western blot analysis of TLR 2 and TLR 4. Representative Western blots of these proteins are shown at the top. Values are expressed as means $\pm$ SEM. * p < 0.05 vs. the HTK₉ group; # p < 0.05 vs. HTK₁₄ group.

Fig. 6. NF- κ B p65/p50 levels in cardiomyocytes. 1 = Negative control; 2 = positive control; 3 = sham control; 4 = cold competition reaction; 5 = HTK₉ group; 6 = HBOC₉ group; 7 = HTK₁₄ group; 8 = HBOC₁₄ group. NF- κ B activity in the PolyPHb groups was significantly lower. Values are expressed as means $\pm$ SEM. * p < 0.05 vs. the HTK₉ group; # p < 0.05 vs. the HTK₁₄ group.

Fig. 7. TUNEL assay. DAPI-stained myocardial nuclei appear light blue, TUNEL-positive nuclei appear green (colors refer to the online version only; 5 fields for each specimen). Values are expressed as means $\pm$ SEM. * $p < 0.05$ vs. HTK₉ group; # $p < 0.05$ vs. HTK₁₄.

5 groups were: sham control 2.63 ± 0.89 , HTK₉ 16.73 ± 1.38 , HBOC₉ 11.46 ± 1.15 , HTK₁₄ 31.12 ± 1.72 , and HBOC₁₄ 22.86 ± 1.53 (the differences between the 5 groups were significant; $F = 229.709$, $p < 0.05$). The percentage of apoptotic cells was significantly lower in the HBOC groups than in the HTKs groups (fig. 7a, b; $p < 0.05$, respectively).

HBOC significantly decreased the percentage infarct size (HBOC₉ $13.81 \pm 1.27\%$ vs. HTK₉ $23.01 \pm 1.72\%$, $p < 0.05$; HBOC₁₄ $35.65 \pm 1.84\%$ vs. HTK₁₄ $60.39 \pm 2.18\%$, $p < 0.05$; fig. 8a, b). HBOC also significantly decreased the release of CK-MB and lactate dehydrogenase into the coronary effluent (vs. the HTK₉ group and HTK₁₄ group; $p < 0.05$; fig. 8c, d) and lessened the extent of cellular swelling and extracellular matrix edema (fig. 8e).

Oxidative Stress in LV Tissue

HBOC significantly increased SOD activity in LV tissue in comparison with the HTK groups (HBOC₉ 83.67 ± 1.45 and HBOC₁₄ 66.46 ± 1.52 vs. HTK₉ 71.34 ± 1.85

and HTK₁₄ 36.47 ± 1.28 U/mg protein, $p < 0.05$; fig. 9a) and decreased the formation of MDA (HBOC₉ 1.21 ± 0.58 and HBOC₁₄ 1.66 ± 0.61 vs. HTK₉ 1.55 ± 0.62 and HTK₁₄ 2.10 ± 0.96 nmol/mg protein, $p < 0.05$; fig. 9b).

Discussion

In this study, we treated isolated rat hearts with 0.3 g Hb/dl PolyPHb to investigate the potential effect of HBOC on the cold I/R heart. The results demonstrated that HBOC treatment greatly improved cardiac functional recovery compared with the HTK groups. Additionally, myocardial infarction and necrosis were significantly reduced in the HBOC groups, accompanied by significantly elevated myocardial tissue SOD activity and decreased levels of myocardial tissue MDA. To reveal the underlying mechanism, we investigated the TLR/NF- κ B signaling pathway expression in these hearts. We found that HBOC could reduce the mRNA and protein levels of TLR 2 and TLR 4 as well as NF- κ B activation. The mRNA

Fig. 8. PolyPHb limits the myocardial infarct size and reduces cardiac enzyme release. **a** Representative mid-myocardial cross sections of TTC-stained hearts for the 5 groups. Red-stained areas indicate viable tissue and nonstained pale areas indicate infarct tissue. **b** Myocardial infarct size of hearts in the 5 groups. **c, d** Total CK-MB and lactate dehydrogenase (LDH) release in

coronary effluent after 2 h of reperfusion. Values are expressed as means $\pm$ SEM. * $p < 0.05$ vs. the HTK₉ group; # $p < 0.05$ vs. the HTK₁₄ group. **e** Representative photomicrographs of hematoxylin-and-eosin-stained left ventricular tissue sections. Original magnification $\times 400$.

levels of TNF- α and IL-1 β were consistently significantly decreased. The results indicate that the cardioprotective effect of HBOC on cold I/R injury could be via down-regulation of the TLR signaling pathway.

Semi-synthetic or synthetic preparations of hemoglobin, now termed HBOCs, have been in development as

an alternative to erythrocytes for several decades. However, nonclinical and clinical studies of HBOCs have raised questions about their safety, presenting an obstacle to their development [17–19]. Thus, in addition to improving the quality of HBOCs themselves, we have started to explore their alternative clinical uses [12, 13, 20].

Fig. 9. Oxidative stress in left ventricular tissue. Total SOD activity and MDA formation. One unit of SOD activity corresponded to a 50% reduction of absorbance at 550 nm. Values are expressed as means $\pm$ SEM. * $p < 0.05$ vs. the HTK₉ group; # $p < 0.05$ vs. the HTK₁₄ group.

PolyPHb was initially developed as an HBOC by the research group of Prof. Chengmin Yang to treat patients with tumors and hemorrhagic shock [21]. Our previous studies demonstrated that PolyPHb provided more profound protection to the I/R heart than whole blood, showing that the effect of HBOC cannot be totally attributed to its O₂-transporting capacity, because it is a multifunctional protein. Moreover, we demonstrated that the cardioprotective effect of PolyPHb was closely related to the attenuation of nitric oxide-mediated myocardial apoptosis and restoration of the nitroso-redox balance in the myocardium [12, 13]. Animal studies of HBOCs have indicated their potential cardioprotective effects in I/R hearts [22–24]. In our experiments, we tested our hypothesis that the TLR 2 and TLR 4/NF- κ B signaling pathway played an important role in the cardioprotective effects of PolyPHb. The results confirmed the hypothesis and demonstrated the regulatory effect of HBOC on the expression of the TLR 2 and TLR 4/NF- κ B signaling pathway for the first time.

As discussed previously, the etiology of myocardial I/R injury is multifactorial, but activation of the innate immune system and the resulting inflammatory response are important components of I/R injury. TLRs are a family of proteins that are mammalian homologs of the *Drosophila* Toll, a protein that functions in development and immunity [25]. TLRs are ubiquitously expressed pattern-recognition receptors central to the inflammatory response in a broad variety of species. The TLR/NF- κ B signaling pathway has been demonstrated to bridge innate immunity and myocardial I/R injury [26]. TLRs serve as pattern recognition receptors by sensing the presence of conserved microbial motifs and alerting the host to the presence of microbial invaders. Previous studies had also found that TLR 2, TLR 4 and TLR 9 can sense the presence of a number of endogenous molecules termed ‘dan-

ger-associated molecular patterns’ (DAMPs), which include heat-shock proteins, high-mobility-group box 1, and breakdown products of fibronectin, heparin sulfate and hyaluronic acid [27]. TLR signals can activate NF- κ B and NF- κ B then translocates to the nucleus to induce proinflammatory and proapoptotic gene expression promoting cell dysfunction and death [28]. That is to say, the inhibitory effect of HBOC on the mRNA levels of TNF- α , IL-1 β and NF- κ B activation is likely to be associated with decreased TLR 2 and TLR 4 expression, thereby reducing the inflammatory response and myocardial injury during cold I/R.

Another important finding of this study is the relationship between oxidative stress and expression in the TLR 2 and TLR 4/NF- κ B signaling pathway. We found that the HBOC attenuates the fall in SOD and the rise in MDA, respectively. Consistently, the TLR 2 and TLR 4/NF- κ B signaling pathway expression were significantly inhibited compared with the HTKs groups ($p < 0.05$); the reasons for these results are not yet understood. Injury caused by oxidative stress occurs in many clinical scenarios involving ischemia and reperfusion, such as organ transplantation, hemorrhagic shock (HS), myocardial infarction, and cerebral vascular accidents. Molecules released in oxidative stress, such as various reactive oxygen species (ROS) and nitric oxide, are found in response to microbial invasion during the neutrophil and macrophage respiratory burst. Significantly, ROS function not only in their classic role of causing injury by disruption of cell membranes, DNA-strand breaks and enzyme inactivation, but also as precise signaling molecules [29, 30]. The mechanisms by which redox stress can activate an inflammatory response have not been fully determined. However, there is accumulating evidence that pattern recognition receptors of the innate immune system, such as TLRs, may be involved in mediating the response seen

in experimental I/R [31, 32]. The TLRs involved in alerting the innate immune system to danger seem to be activated by DAMPs and the mobilization of these DAMPs may represent a link between oxidative stress and the inflammatory response. DAMPs can be released as a consequence of hypoxia and I/R- or hemorrhagic shock-induced injury [33–35], and various types of ROS can contribute to DAMP activation, release and even redox modification [36, 37]. Activation of the immune system as a result of disturbances in the redox state of cells seems to contribute to tissue and organ damage in these conditions. However, the mechanism by which TLRs mediate the production of ROS during redox stress is uncertain, but could involve the membrane-associated enzyme complex NADPH oxidase [38]. There is also evidence suggesting that interplay between ROS derived from NADPH could modulate the TLRs canonical pathway, initiated by myeloid differentiation factor 88 (MyD88) and calcium-related signaling events [39]. Our study provides a key link connecting the inflammatory response, oxidative stress, innate immunity and hypoxia. PolyPHb-inhibited TLR 2 and TLR 4/NF- κ B signaling pathway expression could be via the amelioration of hypoxia and the attenuation of oxidative stress. Understanding the mechanisms of this key interaction may have clinical implications. During ischemic insult, the interaction between oxidative stress and TLR activation forms a paracrine loop, amplifying inflammation, leading to end-organ injury [40, 41].

There are several limitations to our study that need consideration. First, because inhibitors of TLR 2 and TLR 4 were not used, we could not exclude the possibility that other mechanisms, such as HBOC, could change the level of other endogenous molecule expression that regulates TLR 2 and/or TLR 4 expression. Second, we demonstrated the dose-related cardioprotective effect of PolyPHb in a previous study [12] and found that an infusion of 0.3 g Hb/dl PolyPHb provided greater myocardial protection during I/R than other doses. In this study, we did not research the relationship between different doses of PolyPHb and TLR 2 and TLR 4/NF- κ B signaling pathway expression. Further studies are needed to explore this.

In conclusion, the results of our study demonstrated that HBOC was beneficial in the recovery of cardiac function and exerted a profound cardioprotective effect against cold I/R injury. The proposed mechanism for this protection seems to involve the downregulation of TLR 2 and TLR 4/NF- κ B signaling pathway expression. These results reveal that PolyPHb may be useful in the treatment of the myocardial I/R injury in cardiac surgery and transplantation.

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References

- 1 Yellon DM, Hausenloy DJ: Myocardial reperfusion injury. *N Engl J Med* 2007;357: 1121–1135.
- 2 Inserre J, Barrabes JA, Hernando V, Garcia Dorado D: Orphan targets for reperfusion injury. *Cardiovasc Res* 2009;83:169–178.
- 3 Murphy E, Steenbergen C: Mechanisms underlying acute protection from cardiac ischemia-reperfusion injury. *Physiol Rev* 2008; 88:581–609.
- 4 Gustafsson AB, Gottlieb RA: Heart mitochondria: gates of life and death. *Cardiovasc Res* 2008;77:334–343.
- 5 Saeed SA, Waqar MA, Zubairi AJ, et al: Myocardial ischaemia and reperfusion injury: reactive oxygen species and the role of neutrophil. *J Coll Physicians Surg Pak* 2005;15:507–514.
- 6 Parish RC, Evans JD: Inflammation in chronic heart failure. *Ann Pharmacother* 2008;42:1002–1016.
- 7 Miyake K: Innate immune sensing of pathogens and danger signals by cell surface Toll-like receptors. *Semin Immunol* 2007;19:3.
- 8 Kaczorowski DJ, Mollen KP, Edmonds R, et al: Early events in the recognition of danger signals after tissue injury. *J Leukoc Biol* 2008; 83:546.
- 9 Mollen KP, Anand RJ, Tsung A, et al: Emerging paradigm: Toll-like receptor 4-sentinel for the detection of tissue damage. *Shock* 2006;26:430.
- 10 Standl T, Freitag M, Burmeister MA, Horn EP, Wilhelm S, Am Esch JS: Hemoglobin-based oxygen carrier HBOC-201 provides higher and faster increase in oxygen tension in skeletal muscle of anemic dogs than do stored red blood cells. *J Vasc Surg* 2006;37: 859–865.
- 11 George I, Yi GH, Schulman, AR, Morrow BT, Cheng Y, Gu A, Zhang G, Oz MC, Burkhoff D, Wang JA: Polymerized bovine hemoglobin oxygen carrier preserves regional myocardial function and reduces infarct size after acute myocardial ischemia. *Am J Physiol Heart Circ Physiol* 2006;291:H1126–H1137.
- 12 Li T, Li J, Liu J, Zhang P, Wu W, Zhou R, Li G, Zhang W, Yi M, Huang H: Polymerized placenta hemoglobin attenuates ischemia/reperfusion injury and restores the nitroso-redox balance in isolated rat heart. *Free Radic Biol Med* 2009;46:397–405.
- 13 Li T, Liu J, Yang Q, Wu W, Zhang P, Yang J, Li J, Zhang W, Yang C: Polymerized placenta hemoglobin improves cardiac functional recovery and reduces infarction size of isolated rat heart. *Artif Cells Blood Substit Immobil Biotechnol* 2009;37:48–52.
- 14 Yang J, Yang J, Ding JW, Chen LH, et al: Sequential expression of TLR4 and its effects on the myocardium of rats with myocardial ischemia-reperfusion injury. *Inflammation* 2008;31:304–312.

- 15 Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 2006; 3:RESEARCH0034.
- 16 Caswell JE, Strange MB, Rimmer DM 3rd, Gibson MF, Cole P, Lefer DJA: Novel hemoglobin-based blood substitute protects against myocardial reperfusion injury. *Am J Physiol Heart Circ Physiol* 2005;288:H1796–H1801.
- 17 Serruys P, Vranckx P, Slagboom T, Regar E, Meliga E, de Winter R, Heyndrickx G, Schuler G, van Remortel E, Dubé GP, Symons J: Haemodynamic effects, safety, and tolerability of haemoglobin-based oxygen carrier-201 in patients undergoing PCI for CAD. *Euro Intervention* 2008;3:600–609.
- 18 Olofsson C, Nygard EB, Ponzer S, Fagrell B, Przybelski R, Keipert PE, Winslow N, Winslow RM: A randomized, single-blind, increasing dose safety trial of an oxygen-carrying plasma expander (Hemospan) administered to orthopaedic surgery patients with spinal anaesthesia. *Transfus Med* 2008;18: 28–39.
- 19 Yu B, Shahid M, Egorina EM, Sovershaev MA, et al: Endothelial dysfunction enhances vasoconstriction due to scavenging of nitric oxide by a hemoglobin-based oxygen carrier. *Anesthesiology* 2010;112:586–594.
- 20 Li T, Yu R, Zhang HH, Wang H, Liang WG, Yang XM, Yang CM: A method for purification and viral inactivation of human placenta hemoglobin. *Artif Cells Blood Substit Immobil Biotechnol* 2006;34: 175–188.
- 21 Wu W, Yang Q, Li T, Zhang P, Zhou R, Yang C: Hemoglobin-based oxygen carriers combined with anticancer drugs may enhance sensitivity of radiotherapy and chemotherapy to solid tumors. *Artif Cells Blood Substit Immobil Biotechnol* 2009;37:163–165.
- 22 You Z, Li T, Yang C, Wu H, Zeng Y: Comparison of the cardioprotective effect of deoxygenated hemoglobin-based oxygen carrier (HBOC) pretreatment and ischemia preconditioning. *Artif Cells Blood Substit Immobil Biotechnol* 2011;39:7–11.
- 23 Asanuma H, Nakai K, Sanada S, Minamino T, Takashima S, et al: S-nitrosylated and pegylated hemoglobin, a newly developed artificial oxygen carrier, exerts cardioprotection against ischemic hearts. *J Mol Cell Cardiol* 2007;42:924–930.
- 24 Burmeister MA, Rempf C, Standl TG, Rehberg S, Bartsch-Zwemke S, Krause T, Tuszyński S, Gottschalk A, Schulte am Esch J: Effects of prophylactic or therapeutic application of bovine haemoglobin HBOC-200 on ischaemia-reperfusion injury following acute coronary ligation in rats. *Br J Anaesth* 2005;95:737–745.
- 25 O'Neill LAJ: How Toll-like receptors signal: what we know and what we don't know. *Curr Opin Immunol* 2006;18:3–9.
- 26 Oyama J, Blais C Jr, Liu X, et al: Reduced myocardial ischemia-reperfusion injury in Toll-like receptor 4-deficient mice. *Circulation* 2004;109:784.
- 27 Sha Y, Zmijewski J, Xu Z, et al: HMGB1 develops enhanced proinflammatory activity by binding to cytokines. *J Immunol* 2008; 180:2531.
- 28 Liew FY, Xu D, Brint EK, O'Neill LAJ: Negative regulation of Toll-like receptor-mediated immune responses. *Nat Rev Immunol* 2005;5:446–458.
- 29 Fialkow L, Wang Y, Downey GP: Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Radic Biol Med* 2007;42:153–164.
- 30 Kim JH, Na HJ, Kim CK, Kim JY, Ha KS, Lee H, et al: The nonprovitamin A carotenoid, lutein, inhibits NF- κ B-dependent gene expression through redox-based regulation of the phosphatidylinositol 3-kinase/PTEN/Akt and NF- κ B-inducing kinase pathways: role of H₂O₂ in NF- κ B activation. *Free Radic Biol Med* 2008;45:885–896.
- 31 Arslan F, De Kleijn DPV, Timmers L, Doevendans PA, Pasterkamp G: Bridging innate immunity and myocardial ischemia/reperfusion injury: the search for therapeutic targets. *Curr Pharm Des* 2008;14:1205–1216.
- 32 Palm NW, Medzhitov R: Pattern recognition receptors and control of adaptive immunity. *Immunol Rev* 2009;227:221–233.
- 33 Zahring U, Lindner B, Inamura S, Heine H, Alexander C: TLR2 – promiscuous or specific? A critical re-evaluation of a receptor expressing apparent broad specificity. *Immunobiology* 2008;213:205–224.
- 34 Aprahamian CJ, Lorenz RG, Harmon CM, Dimmit RA: Toll-like receptor 2 is protective of ischemia-reperfusion-mediated small-bowel injury in a murine model. *Pediatr Crit Care Med* 2008;9:105–109.
- 35 Cavassani KA, Ishii M, Wen H, Schaller MA, Lincoln PM, Lukacs NW, et al: TLR3 is an endogenous sensor of tissue necrosis during acute inflammatory events. *J Exp Med* 2008; 205:2609–2621.
- 36 Khandoga AG, Khandoga A, Anders HJ, Krombach F: Postischemic vascular permeability requires both TLR-2 and TLR-4, but only TLR-2 mediates the transendothelial migration of leukocytes. *Shock* 2009;31:592–598.
- 37 Ito I, Fukazawa J, Yoshida M: Post-translational methylation of high mobility group box 1 (HMGB1) causes its cytoplasmic localization in neutrophils. *J Biol Chem* 2007;282: 16336–16344.
- 38 Lambeth JD: NOX enzymes and the biology of reactive oxygen. *Nat Rev Immunol* 2004; 4:181–189.
- 39 Kazama H, Ricci JE, Herndon JM, Hoppe G, Green DR, Ferguson TA: Induction of immunological tolerance by apoptotic cells requires caspase-dependent oxidation of high-mobility group box-1 protein. *Immunity* 2008;29:21–32.
- 40 Park HS, Jung HY, Park EY, Kim J, Lee WJ, Bae YS: Cutting edge: direct interaction of TLR4 with NAD(P)H oxidase 4 isozyme is essential for lipopolysaccharide-induced production of reactive oxygen species and activation of NF- κ B. *J Immunol* 2004; 173:3589–3593.
- 41 Clarke SJ, Khaliulin I, Das M, Parker JE, Heesom KJ, Halestrap AP: Inhibition of mitochondrial permeability transition pore opening by ischemic preconditioning is probably mediated by reduction of oxidative stress rather than mitochondrial protein phosphorylation. *Circ Res* 2008;102:1082–1090.