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A polymerized bovine hemoglobin oxygen carrier preserves regional myocardial function and reduces infarct size after acute myocardial ischemia

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George, Isaac, Geng-Hua Yi, Allison R. Schulman, Brad T. Morrow, Yanping Cheng, Anguo Gu, Geping Zhang, Mehmet C. Oz, Daniel Burkhoff, and Jie Wang. A polymerized bovine hemoglobin oxygen carrier preserves regional myocardial function and reduces infarct size after acute myocardial ischemia. *Am J Physiol Heart Circ Physiol* 291: H1126–H1137, 2006. First published April 14, 2006; doi:10.1152/ajpheart.00076.2006.—The purpose of this study was to test if HBOC-201, a hemoglobin-based oxygen-carrying solution, can decrease infarct size (or Inf) during acute, severe myocardial ischemia and reperfusion. To test the impact of HBOC-201 on infarct size, ischemia was produced in 18 dogs by coronary stenosis to achieve 80–95% flow reduction for 195 min along with pacing 10% above the spontaneous heart rate, followed by 180 min of reperfusion. Animals were randomized to intravenous infusion of HBOC-201 (1 g/kg) ($n = 6$), normal saline (NS) ($n = 6$), or phenylephrine (Phe) ($n = 6$, as a control for the increased blood pressure seen with HBOC-201), given 15 min after the start of ischemia. Amount of infarct was quantified as the ratio between area at risk (AAR) and Inf after Evans blue and 2,3,5-triphenyltetrazolium chloride staining. Hearts were divided into five layers from base (*layer A*) to apex (*layer E*) and photographed for digital image analysis of AAR and Inf. Regional myocardial function (RMF) was also measured after 60 min of ischemia and 15 min of reperfusion. Inf/AAR was significantly reduced after HBOC-201 therapy (4.4 ± 2.2%) vs. NS (26.0 ± 3.6%) and Phe (25.7 ± 4.1%) (both, $P < 0.05$). RMF after reperfusion was restored to 92% of baseline with HBOC-201 compared with 11% of baseline after NS ($P < 0.05$) and 49% after Phe (P = not significant). HBOC-201 administration after induction of severe myocardial ischemia by acute coronary stenosis reduces infarct size and improves myocardial viability.

coronary; blood; nitric oxide inhibitor

OVER 600,000 people die each year in the United States from complications of ischemic heart disease, including acute myocardial infarction (AMI), congestive heart failure (CHF), and fatal arrhythmias (22), and its treatment continues to challenge the medical community. With an estimated 1.1 million new cases of AMI each year, the burden on the health care system and society is enormous (2). The need for improved myocardial protection after acute ischemia is paramount. Despite a vast array of therapies that reportedly attenuate ischemia-reperfusion injury, clinical application of these therapies has been meager and disappointing (22). The trend for earlier percutaneous revascularization (PCI) has helped to improve outcomes (11). However, the problems of contractile dysfunction during acute ischemia, expansion of infarct size (or Inf), and reperfusion injury have largely remained unaddressed and are more complex than simply restoring flow. In addition, the logistical requirements for early PCI, such as trained interventional cardiologists, specialized equipment, and transport time to treatment, limit its use as an immediate treatment for patients with AMI. Thus a noninvasive cardioprotective strategy that can be applied in a prehospital setting is an attractive therapeutic goal.

HBOC-201 (Hemopure; Biopure, Cambridge, MA) has been used as a hemoglobin (Hb)-based oxygen carrier solution in humans under investigational status. Manufactured from bovine whole blood filtration and ion-exchange high-pressure liquid chromatography and devoid of plasma, red blood cell (RBC) stroma, and endotoxins, HBOC-201 is prepared in a balanced electrolyte solution and lacks an immunological response when given to animals and humans (14, 23). Hemodynamically, increases in mean arterial pressure (MAP) and systemic vascular resistance are seen after HBOC-201 administration (18), likely through nitric oxide (NO) inhibition (12, 15). Oxygen delivery to tissue has been shown to be faster and three times more efficient than human Hb (20, 26). These properties, along with a lower viscosity and smaller mean diameter than a human RBC, suggest that blood substitutes may be an effective therapy in acute myocardial ischemia. Data from a canine model with HBOC-201 given 30 min before myocardial ischemia showed significant protection against reperfusion injury (4). However, pretreatment for myocardial infarction is rare, given the acuity and rapid progression of ischemia with little forewarning. In addition, prior animal experiments employing complete coronary occlusion with HBOC-201 treatment have failed to show any effect on regional myocardial function or infarct size (J. Wang, unpublished observations), suggesting HBOC-201 is dependent on vessel patency for benefit to be obtained.

In the present experiment, models of ischemia with treatment after the onset of ischemia were used to produce myocardial dysfunction and myocardial infarction through severe coronary stenosis, thus allowing minimal coronary perfusion. The primary purpose of this study was to test if HBOC-201 can improve regional myocardial function (RMF) and decrease infarct size when administered after induction of acute myo-

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cardial ischemia and reperfusion in a canine model in this setting. In addition, the effect of HBOC-201 on infarct size when administered at a reduced dose and a delayed infusion time point was evaluated.

MATERIALS AND METHODS

Studies were performed in compliance with the *Guide for the Care and Use of Laboratory Animals*, prepared by the Institute of Laboratory Animal Resources, National Research Council, and published by the National Academy Press (Revised 1996). This study was approved by the Institutional Animal Care and Use Committee of Columbia University.

Experimental Design

Two distinct canine models were employed in this project. The first model (regional myocardial ischemia model, $n = 12$) was designed to measure RMF in the territory of ischemia after acute coronary stenosis and subsequent HBOC-201 administration. This model was designed to imitate acute myocardial ischemia due to severe coronary artery stenosis or partial coronary embolism. Briefly, ischemia was produced by left anterior descending coronary artery (LAD) stenosis to 80–95% flow reduction from baseline, followed by HBOC-201 infusion 15 min after the onset of ischemia. Ischemia was continued for a total of 60 min, followed by 15 min of reperfusion. Colored microspheres were used to quantify endocardial and epicardial perfusion.

The second model (myocardial infarct model, $n = 35$) was designed to produce a significant infarction after coronary stenosis and evaluate if HBOC-201 infusion affects myocardial infarct size. Infarcts were created by LAD stenosis to 80–95% flow reduction from baseline with concurrent cardiac pacing at a rate of 10% above spontaneous heart rate to mimic additional myocardial stress for 195 min, followed by 180 min of reperfusion. The effect of HBOC-201 on infarct size at different doses and delayed administration was tested by using this model. HBOC-201 was administered intravenously 15 min (1 and 0.5 g/kg) and 60 min (1 g/kg) after the onset of ischemia. Myocardial staining for area at risk (AAR, intracoronary Evans blue) and Inf [2,3,5-triphenyltetrazolium chloride (TTC)] was performed at the conclusion of reperfusion. Systemic hemodynamics and coronary blood flow were monitored throughout the experiment.

Both RMF and infarct experiments were performed at physiological arterial oxygen tension (Pa_{O_2}, 90–120 mmHg, 30% inspired O_2) to eliminate any possible bias of hyperoxygenation effects in HBOC-201-treated animals, as well as imitate clinical use in humans at room air at time of administration. Animals treated with normal saline (NS), phenylephrine (Phe), and N^G-nitro-L-arginine methyl ester (L-NAME) served as experimental controls. Because it is known that HBOC-201 produces an increase in MAP (18) in addition to providing greater O_2 delivery, Phe was included to investigate the confounding vasopressor actions of HBOC-201 as a possible mechanism of recovery. Likewise, HBOC-201 has been shown to reduce NO activity in vitro after septic shock (15). L-NAME, a NO synthase inhibitor, also served as another control group to examine the possible hemodynamic effects of HBOC-201 via NO inhibitory pathways (7, 15). The number of animals studied in each group is detailed below.

These two models were designed to represent a broad range of presentation times for a patient suffering a myocardial infarction: *1*) an early presentation that with proper treatment can salvage ischemic myocardium to improve contractile function, and *2*) a late presentation where infarction reduction rather than acute function is optimized to improve long-term outcomes.

Surgical Preparation

On the day of each surgery, a mongrel dog (22–25 kg, male and female) (total $n = 47$) was induced with thiopental sodium (15–20 mg/kg iv; Abbott Laboratories, North Chicago, IL). The animal was then intubated and maintained under general anesthesia using isoflurane at 1.5–2.5% on mechanical ventilation. Lactated Ringer (12 ml/kg) (Baxter Healthcare, Deerfield, IL) was administered intravenously over 15 min to replace insensible losses. A maintenance solution of Lactated Ringer and lidocaine (2%, 140 mg per 500 ml) (Abbott Laboratories) was given at 0.1 $ml \cdot kg^{-1} \cdot min^{-1}$ throughout the remainder of the experiment to reduce the potential for ventricular arrhythmia during cardiac manipulation. Millar Mikro-Tip pressure catheters (SPC-360S, Houston, TX) were placed in the left ventricle (LV) and aorta, respectively, via carotid artery cannulation. A left thoracotomy was performed between the fourth and fifth intercostal space to expose the heart. A thin Silastic catheter was placed in the left atria for colored microsphere administration to quantify myocardial perfusion. The LAD was isolated, and a custom-designed coronary constrictor (producing adjustable coronary stenosis) (28) was placed around the LAD, immediately distal to the bifurcation of the left main coronary artery. Ultrasonic flow probes (model 25618, Transonic Systems, Ithaca, NY) were placed around the LAD (distal to the constrictor) and left circumflex coronary arteries (LCX), respectively. Pacing wires were placed on the anterior and free wall of the LV. In addition, two sonomicrometric crystals were placed in the midwall of the LV in the territory of LAD ischemia and connected to a sonomicrometer system (Sonometrics, model C1096, London, Canada). These crystals were used to quantify regional function on the basis of LV pressure-segment length relationships. All visible collaterals from the LCX and right coronary artery (including right posterior descending artery) were ligated for each animal (on average, 6 collaterals per animal). After completion of instrumentation, inhaled oxygen content was reduced by adding compressed nitrogen and measuring arterial blood gases (Radiometer Copenhagen, ABL 700 series, Copenhagen, Denmark) until physiological arterial partial pressures (Pa_{O_2}, 90–120 mmHg at 30% O_2, Pa_{CO_2} 36–40 mmHg) were reached.

Regional Myocardial Ischemia Model

Ischemia via LAD stenosis was achieved in 12 animals through progressive constrictor tightening until flow reduction to 80–95% of baseline was reached (28). Impairment of function was confirmed by real-time LV pressure vs. segment length loops, as measured by sonomicrometric crystals, before proceeding. Once a stable level of ischemia was maintained for 15 min, each animal was randomized to HBOC-201 (1 g/kg) ($n = 5$), 0.9% NS (7 ml/kg) ($n = 4$) (Baxter Healthcare, Deerfield, IL), or Phe (0.02 mg/ml drops) ($n = 3$) (American Regent Laboratory, Shirley, NY) infused intravenously at 0.1 $ml \cdot kg^{-1} \cdot min^{-1}$ for 2 min, then 0.3 $ml \cdot kg^{-1} \cdot min^{-1}$ for the remainder of infusion. The average HBOC-201 infusion time was ~23 min. Phe was titrated until a MAP equal to that of HBOC-201-treated animals was reached and maintained throughout the ischemic period. Ischemia was maintained for a total of 60 min, followed by release of the coronary stenosis. Hemodynamics were monitored for 15 min after reperfusion, and animals were then killed.

Assessment of regional myocardial function. Myocardial segment length was recorded by sonomicrometric crystals in the regional myocardial ischemia model at 248 Hz using Sonosoft Software (Sonometrics) for off-line analysis. RMF was quantified by regional stroke work, determined from the area inside the LV pressure vs. segment length loop, divided by end-diastolic segment length (PSLA/EDSL), as previously described (27). Measurements were taken at baseline, at 15 min of ischemia (before HBOC-201 or control treatment), at the end of 60 min ischemia (after infusion of HBOC-201 or control treatment), and after 15 min of reperfusion. Calculations were performed by using custom software (Labview, National Instruments, Austin, TX).

Assessment of myocardial perfusion. Colored microspheres (Dye-Trak, Triton Technology, San Diego, CA) were infused into the left atrium through the left atria catheter (2 ml, 6×10^6 spheres) in the regional myocardial ischemia model at baseline, 15 min of ischemia,

the end of 60 min of ischemia, and after 15 min of reperfusion. A blood sample representing a reference for data analysis was obtained before each microsphere injection by constant-rate blood withdrawal (7 ml/min for 2 min) from the aorta using a syringe pump. To assess myocardial perfusion, whole heart perfusion maps were created on the basis of colored microsphere data, as previously described (24). At the conclusion of each experiment, the heart was cut into 5 levels (labeled *A* through *E*, with base as *A* and apex as *E*), and each level was then cut into pieces, resulting in a total of 36 transmural segments (see Fig. 3*D*). Blood flow in each level was calculated from the colored microsphere data according to standard techniques (24). Normalized myocardial perfusion, expressed as percentage of baseline flow, was then plotted as a function of transmural segment number, 1 through 36. Regional myocardial perfusion of the ischemic zone (LAD territory) was expressed in milliliters per minute per gram. Hb and RBC levels were measured from the blood samples taken at baseline, 15 min of ischemia, after 60 min of ischemia, and after 15 min of reperfusion.

Myocardial Infarct Model

Ischemia was similarly created in 35 animals at 30% O_2 by LAD stenosis to 80–95% flow reduction. After 15 min of ischemia and confirmation of functional impairment by LV loops, each animal was randomized to HBOC-201 (0.5 g/kg) ($n = 6$), HBOC-201 (1 g/kg) ($n = 6$), NS (7 ml/kg) ($n = 6$), Phe (0.02 mg/ml drops) ($n = 6$), or L-NAME (30 mg/kg) (Sigma-Aldrich, St. Louis, MO) ($n = 6$), which was infused intravenously at 0.1 $ml \cdot kg^{-1} \cdot min^{-1}$ for 2 min, then at 0.3 $ml \cdot kg^{-1} \cdot min^{-1}$ for the remainder of infusion. The average HBOC-201 infusion time was ~23 min. The LV was paced at a rate 10% higher than the spontaneous heart rate (Pace Medical, Waltham, MA) to stress the myocardium, beginning 15 min after induction of ischemia; pacing was necessary to reliably create large and reproducible infarcts. Phe was titrated until a MAP equal to that achieved in the HBOC-201-treated animals was reached and maintained throughout the ischemic period. L-NAME was continuously infused during both ischemia and reperfusion. Ischemia was maintained for a total of 195 min, at which point pacing was stopped, followed by reperfusion for 180 min. Staining for AAR and Inf was performed at the conclusion of reperfusion.

To examine if delayed administration of HBOC-201 could reduce infarct size after severe ischemia, the same ischemic and cardiac pacing experimental protocol was used, and HBOC-201 (1 g/kg) ($n = 5$) was administered intravenously after 60 min of ischemia. Ischemia was maintained for a total of 195 min, after which point pacing was stopped, followed by 180 min of reperfusion. Myocardial staining for AAR and Inf was performed at the conclusion of reperfusion.

Assessment of AAR and Inf. At the conclusion of reperfusion period in the myocardial infarct model, 20,000 U of heparin (Baxter Healthcare) was given intravenously. The heart was then excised and perfused with 1 liter of warm (37°C) 0.9% NS down the aortic root to clear any remaining blood and thrombus. One liter of 0.24% Evans blue (Sigma) was then infused at 100 mmHg after complete LAD ligation at the site of the original stenosis. After Evans blue administration, a small Silastic catheter was inserted down the LAD. One liter of warm 1% TTC (Sigma) was delivered at 100 mmHg via this catheter. The heart was then sliced into five layers equal in length after TTC staining, from base (*A*) to apex (*E*). This protocol stains all perfused areas dark blue. In ischemic zones, infarcted tissue appears as a pale, whitish color, and viable tissue stains a reddish-pink color. Digital images of each layer were recorded and analyzed using digital image software (ImagePro v. 4.5.1.22, Media Cybernetics, Silver Spring, MD). AAR was defined as the area of the outlined portions of LV unstained by Evans blue (ischemic area), while Inf was defined as infarcted area (appearing white) within the AAR. Total AAR, total LV, and total Inf were expressed as the sum of *layers A–E* of each area per layer multiplied by the weight per layer, respectively, as previously described (4). This portion of the analysis was performed by an investigator blinded to the treatment group.

Hemodynamic Measurements

The Millar pressure catheters were calibrated before each experiment ex vivo using Sonosoft software. LV pressure, aortic pressure, LAD blood flow, and LCX blood flow were continuously recorded at 248 Hz on an eight-channel thermal writing chart recorder (30-V8808–10, Gould Electronics, Chandler, AZ), and periods of interest were digitized [Gateway 2000 486 computer (San Diego, CA), equipped with a National Instruments analog-to-digital conversion system] for offline analysis. Mean values of aortic pressure were all determined online by use of 3-Hz averaging filters (DA26, Medtron Engineering, Olivinhein, CA). To evaluate the effects of HBOC-201 on the coronary microvasculature, an indirect measure of coronary flow reserve (CFR) was determined by the ratio of LAD hyperemic flow after stenosis release to baseline LAD flow, and minimum coronary resistance (MCR) was calculated as the ratio of baseline MAP to LAD hyperemic flow after stenosis release. This definition of hyperemic flow, rather than administration of adenosine, which was not feasible in this study, was representative of the maximal vasodilatory properties of the coronary vessel due to the buildup of endogenous vasodilatory agents during ischemia.

Statistics

All data are expressed as means ± SE. Differences within treatment groups were evaluated by using paired *t*-testing, while two-tailed independent *t*-testing with Levene's test for equality of variances was used to evaluate differences among treatment groups. For multiple treatment groups, data were analyzed by using one-way ANOVA with Bonferroni post hoc analysis. All statistics were analyzed by using SPSS software (v. 11.5, Chicago, IL).

RESULTS

Regional Ischemia Model

Systemic hemodynamics. A number of consistent hemodynamic changes were observed with the administration of HBOC-201, similar to previous reports (4, 18), as shown in Table 1. A significant increase in MAP was seen immediately after infusion of HBOC-201 at 30% O_2. This increase in afterload was maintained throughout the duration of the experiment as expected because the reported half-life of HBOC-201 is 23 h (17). Control animals receiving NS had a much smaller increase in MAP after infusion and reperfusion ($P < 0.05$ vs. HBOC-201). Control animals receiving Phe experienced an equivalent increase in MAP and a higher LV end-diastolic pressure (LVEDP) ($P < 0.05$). The first derivative of LV pressure with respect to time (LV dP/d*t*) was significantly improved after HBOC-201 treatment vs. NS ($P < 0.05$). LAD flow was equally reduced by 85–95% across treatment and control groups after 15 min of stable ischemia (but before treatment). LAD flow improved dramatically after infusion in the HBOC-201 group (7.6 ± 1.8 to 16.4 ± 2.4 ml/min) vs. the NS (2.4 ± 0.6 to 1.8 ± 0.4 ml/min) and Phe (5.7 ± 0.7 to 5.3 ± 3.2 ml/min) groups (both, $P < 0.05$). Mean LAD resistance after infusion and before stenosis release was calculated as MAP/mean LAD flow to examine if HBOC-201 peripheral vasopressor properties adversely affected coronary vasculature. LAD resistance was significantly lower with HBOC-201 throughout the ischemic period vs. NS ($P < 0.05$) and after 30 min vs. Phe ($P < 0.05$) (see Fig. 1*A*).

Table 1. *Regional myocardial ischemia model: hemodynamics at 30% O_2*

	Baseline	Preinfusion/15 min Ischemia	Postinfusion/End of Ischemia	15 min Reperfusion
MAP, mmHg				
NS	75.5±3.6	69.7±1.8	79.4±2.7	60.0±9.7
Phe	69.1±7.4	71.8±4.3	94.6±10.8	90.4±10.2
HBOC-201	87.2±5.5	76.2±6.3	89.7±7.8[d]	90.4±7.6[a,d]
LVSP, mmHg				
NS	100.5±3.4	85.5±7.1	100.5±5.3	78.0±11.6
Phe	99.3±3.3	95.3±6.4	123.3±6.6	117.3±14.3
HBOC-201	101.6±2.8	91.2±5.9	105.6±7.5[d]	104.8±6.0
LVEDP, mmHg				
NS	13.0±1.7	25.0±5.3	30.0±7.5	21.5±6.1
Phe	10.7±0.7	18.7±1.8	35.3±5.3[d]	22.0±9.5
HBOC-201	6.8±1.4[a,b]	14.8±2.7[c]	13.6±4.0[b]	11.2±3.4[d]
LV dP/d*t*, mmHg/s				
NS	1,538±159	1,296±145	1,304±135	803±165[c,d]
Phe	1,281±82	1,310±155	1,262±147	1,280±70
HBOC-201	1,486±110	1,225±43	1,361±78	1,277±91[a]
LAD flow, ml/min				
NS	50.0±15.4	2.4±0.6[c]	1.8±0.4	35.0±10.3
Phe	48.3±11.7	2.7±0.7	5.3±3.2	52.0±13.6
HBOC-201	47.6±16.9	7.6±1.8[a]	16.4±2.4[a,b]	45.2±12.0[d]

All data expressed as means ± SE; $n = 4$ for normal saline (NS), $n = 3$ for phenylephrine (phe), and $n = 5$ for Hb-based oxygen-carrier solution HBOC-201. MAP, mean arterial pressure; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; LAD, left anterior descending coronary artery; LV dP/d*t,* 1st derivative of left ventricular pressure with respect to time. [a]$P < 0.05$ vs. NS, [b]$P < 0.05$ vs. Phe, [c]$P < 0.05$ vs. baseline, [d]$P < 0.05$ vs. preinfusion/15 min ischemia, [e]$P < 0.05$ vs. postinfusion/end of ischemia.

CFR was determined by the ratio of LAD hyperemic flow after stenosis release to baseline LAD flow (see Table 2), and MCR was calculated as the ratio of baseline MAP to LAD hyperemic flow after stenosis release. No significant differences in CFR or MCR were seen among groups. No adverse hemodynamic effects, reactions, or anaphylaxis were seen in any treated animals after HBOC-201 administration.

Regional myocardial function. Sample LV pressure-segment length loops at baseline, 15 min of ischemia (preinfusion), end of ischemia (postinfusion), and after 15 min of reperfusion are shown in Fig. 2, *A–C*. Coronary stenosis produced equal reductions in RMF, as measured by PSLA/EDSL, in both treatment and control groups (see Fig. 2*D*). A trend toward improvement in RMF was seen during ischemia after HBOC-201 treatment compared with NS ($P = 0.08$) and compared with Phe ($P = 0.11$). These improvements are shown to be independent of MAP, as shown in Table 1. HBOC-201 provided significant protection against reperfusion injury, ultimately restoring 92% of baseline function compared with only 11% of baseline function for NS ($P < 0.05$) and 49% of baseline function for Phe ($P = 0.17$) after coronary stenosis release.

Myocardial perfusion. Myocardial perfusion was separated into endocardial and epicardial flow patterns on the basis of the location of the transmural segment from the ventricular wall (see Fig. 3). Figure 3 shows sample endocardial microsphere-derived myocardial blood flow maps from one animal. Regional endocardial perfusion, in the territory of LAD ischemia, was significantly higher at the end of ischemia with HBOC-201 treatment compared with NS ($P < 0.05$) (Fig. 4). No significant changes were seen during reperfusion. Epicardial perfusion was not affected by HBOC-201 administration (data and microsphere-derived flow maps not shown). In addition, the volume of HBOC-201 administered produced no significant changes in plasma RBC or Hb levels throughout the experiment (data not shown).

Myocardial Infarct Model

Systemic hemodynamics. Hemodynamics in the infarct model demonstrated similar trends to those seen in the ischemia model (see Table 3). It is notable that increases in MAP after HBOC-201 infusion at the dose of 1 g/kg were sustained throughout the course of ischemia. Despite an equivalent MAP to HBOC-201 (1 g/kg), Phe- and L-NAME-treated animals did not experience an equivalent increase in LAD flow. LAD flow was uniformly reduced at the start of ischemia (15 min ischemia) in both control and treatment groups [% of baseline flow reduction: 89% (NS), 92% (Phe), 94% (L-NAME), 85% (HBOC-201; 0.5 g/kg), 89% (HBOC-201; 1 g/kg), 90% (HBOC-201; delayed)]. LAD flow was significantly increased after HBOC-201 infusion vs. all control groups, as well as the reduced dose HBOC-201 (all, $P < 0.05$). Mean coronary (LAD) resistance was lowered in HBOC-201-treated animals after 60 min of ischemia (postinfusion) in contrast to animals treated with NS who had large increases in coronary resistance that likely limited coronary perfusion (see Fig. 1*B*).

Infarct reduction. Sample digital images of infarct sizes from HBOC-201-treated and control hearts are shown in Fig. 5. Total AAR/LV was not significantly different among groups, as shown in Fig. 6*A*. However, HBOC-201 (1 g/kg) administration after the onset of ischemia significantly reduced total infarct size per AAR (4.4 ± 2.2%) compared with NS (26.0 ± 3.6) and Phe (25.7 ± 4.1) (see Fig. 6*B*) (both $P < 0.05$). Trends toward infarct reduction (AAR/Inf) after HBOC-201 (0.5 g/kg) and HBOC-201 (delayed) treatment were also seen compared with NS and Phe therapy but did not reach statistical significance (0.5 g/kg: $P = 0.06$, $P = 0.07$ vs. NS, Phe; delayed: $P = 0.11$, $P = 0.13$ vs. NS, Phe). No significant differences were seen between HBOC-201 and L-NAME therapy. Infarct size expressed as a percentage of the left ventricle (Inf/LV) demonstrated significant reductions with HBOC-201 (1 g/kg) vs. NS and Phe (both, $P < 0.05$) (Fig. 6*C*).

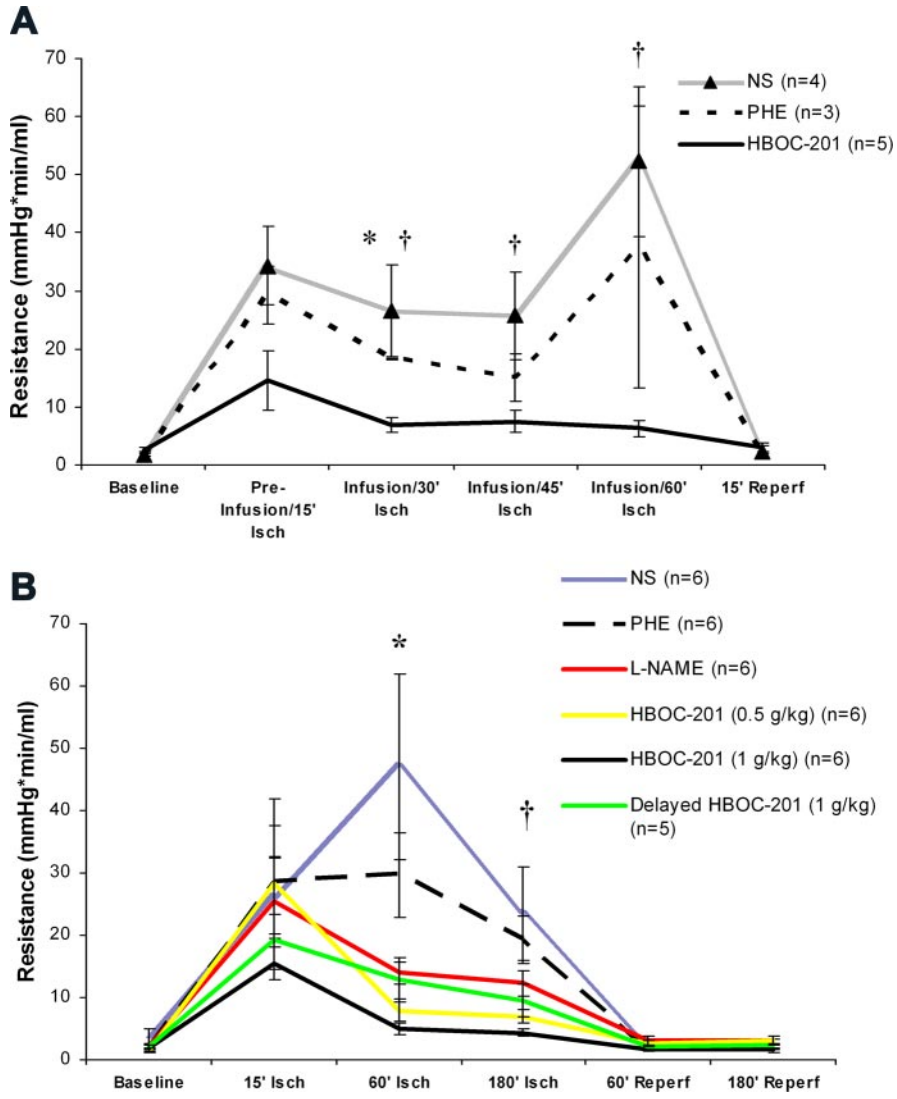


Fig. 1. Mean left anterior descending coronary artery (LAD) resistance in the myocardial ischemia model (*A*) and myocardial infarct model (*B*). In the myocardial ischemia model, LAD resistance was significantly lower in HBOC-201 therapy after 30 min vs. phenylephrine (Phe) (*$P < 0.05$) and throughout infusion/ischemia (Isch) and reperfusion (Reperf) vs. normal saline (NS) (†$P < 0.05$). In the myocardial infarct model, there were no differences in coronary resistance at baseline; after 60 and 180 min of ischemia (both postinfusion), LAD resistance was significantly lower after HBOC-201 treatment compared with NS [*$P < 0.05$: HBOC-201 (0.5 g/kg), HBOC-201 (1 g/kg) vs. NS; †$P < 0.05$, HBOC-201 (1 g/kg) vs. NS]. L-NAME, N^G-nitro-L-arginine methyl ester; HBOC-201, hemoglobin-based oxygen-carrying solution.

When infarct size (Inf/AAR) was examined per layer (as depicted in Fig. 7), a significant reduction in *layer D*, the region most vulnerable to LAD ischemia, after HBOC-201 therapy was also apparent. Specifically, Inf/AAR was 6.3 ± 4.4% in HBOC-201 (1 g/kg) compared with NS (40.2 ± 4.6%) and Phe (43.8 ± 9.0%) ($P < 0.01$ vs. NS, Phe). HBOC-201 at the reduced dose (0.5 g/kg) also significantly decreased infarct sizes compared with Phe and NS (both $P < 0.05$). Infarct reduction after HBOC-201 treatment can still be seen when infused 60 min after the onset of ischemia (delayed) vs. NS and Phe (both $P < 0.05$). Large infarcts were not produced in any group in *layers A* or *B*. Infarcts were present in all animals at the apex, largely due to the extensive ligation of collaterals, including the right posterior descending coronary artery.

Table 2. *Regional myocardial ischemia model: CFR and MCR*

	CFR, %	MCR, mmHg·min·ml^{-1}
NS	1.75±0.69	1.38±0.30
Phe	2.10±0.22	0.77±0.19
HBOC-201	2.33±0.79	1.38±0.44

All data expressed as means ± SE. CFR, coronary flow reserve; MCR, minimum coronary resistance.

DISCUSSION

Conventional treatments (PCI, coronary artery bypass grafting) for myocardial ischemia have focused on restoring flow in an occluded or stenotic coronary vessel. However, few options exist, except for anti-platelet therapy, in treating active ischemia before arriving in the cardiac catheterization laboratory, which introduces a significant time delay, during which significant myocardial stunning and/or necrosis occurs (3). In the present study, regional contractile function was shown to be restored during ischemia, and reperfusion injury was reduced by administration of HBOC-201, a polymerized bovine Hb oxygen-carrier solution (12, 15). Most importantly, infarction size was reduced after prolonged ischemia after HBOC-201 therapy. As a result, HBOC-201 shows potential as an adjunctive therapy for providing myocardial protection during acute myocardial ischemia caused by coronary stenosis.

Several possible mechanisms exist to account for the beneficial effects of HBOC-201 on ischemic myocardium. First, in

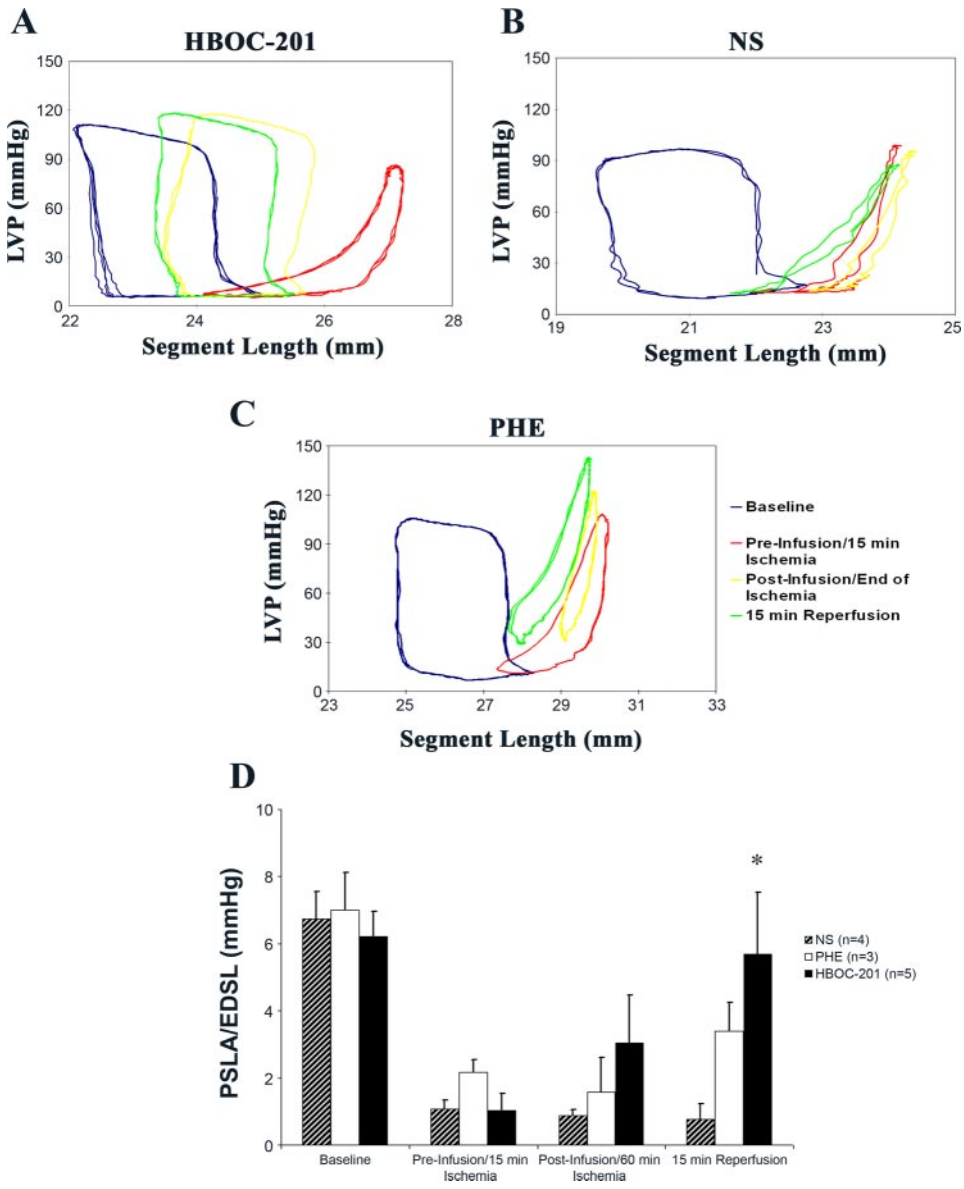


Fig. 2. Regional myocardial ischemia model: sample left ventricular pressure (LVP)-segment loops from HBOC-201 (*A*), NS (*B*), and Phe-treated (*C*) animals. *D*: regional myocardial function (RMF) at 30% O_2. Animals receiving only NS showed progressive deterioration in myocardial function; a trend toward higher RMF during ischemia was seen after HBOC-201 compared with both NS ($P = 0.08$) and Phe ($P = 0.11$). RMF in animals treated with HBOC-201 also improved after reperfusion vs. NS (*$P < 0.05$ vs. NS), and a trend toward higher RMF vs. Phe was evident ($P = 0.117$). PSLA/EDSL, area inside the LVP vs. segment length loop divided by end-diastolic segment length.

the present study, several consistent hemodynamic changes occurred with HBOC-201 administration that may improve RMF and reduce infarct size. MAP and LAD flow increased significantly after HBOC-201 infusion, thus increasing myocardial tissue perfusion through conduit vessels, despite an initial fixed coronary stenosis. Although HBOC-201 requires either vessel patency or adequate collateral circulation to derive maximal benefit, stenoses as great as 96% flow reduction in this study were effectively treated without invasive intervention; preservation of endothelial function through prevention of zero-pressure flow phenomenon, a critical perfusion threshold whereby coronary flow goes to zero despite a lack of complete stenosis, may be involved (16). Strict ligation of all collaterals in this study additionally may underestimate clinical results in humans as well, in which collaterals can limit infarction size and preserve long-term myocardial viability (1, 6, 13, 25). The inability of HBOC-201 to reach affected target myocardium in a model of complete coronary ligation may explain the lack of improvement in regional function or infarct size reduction (J. Wang, unpublished data), similar to historical treatments failing to help during ischemia. It is believed that HBOC-201 modulates vascular smooth muscle by decreasing free NO, resulting in vasoconstriction (12, 15); the current data support this hypothesis, as MAP and afterload increased predictably in all treated animals. Increases in MAP associated with HBOC-201 administration did not impair coronary flow or adversely affect cardiac performance. In addition, there was no evidence of significant coronary vasoconstriction in treated animals. In contrast, a significant increase in coronary resistance greatly impaired coronary flow, as shown in NS- and Phe-treated animals. L-NAME control animals also demonstrated a trend toward higher coronary resistance compared with HBOC-201.

Second, tissue oxygen delivery and unloading properties of HBOC-201 may also account for improvements in RMF and infarct size reduction, as well. HBOC-201 has been shown previously to more than double the oxygen extraction ratio in canines after hemodilution, which is due to a lower oxygen

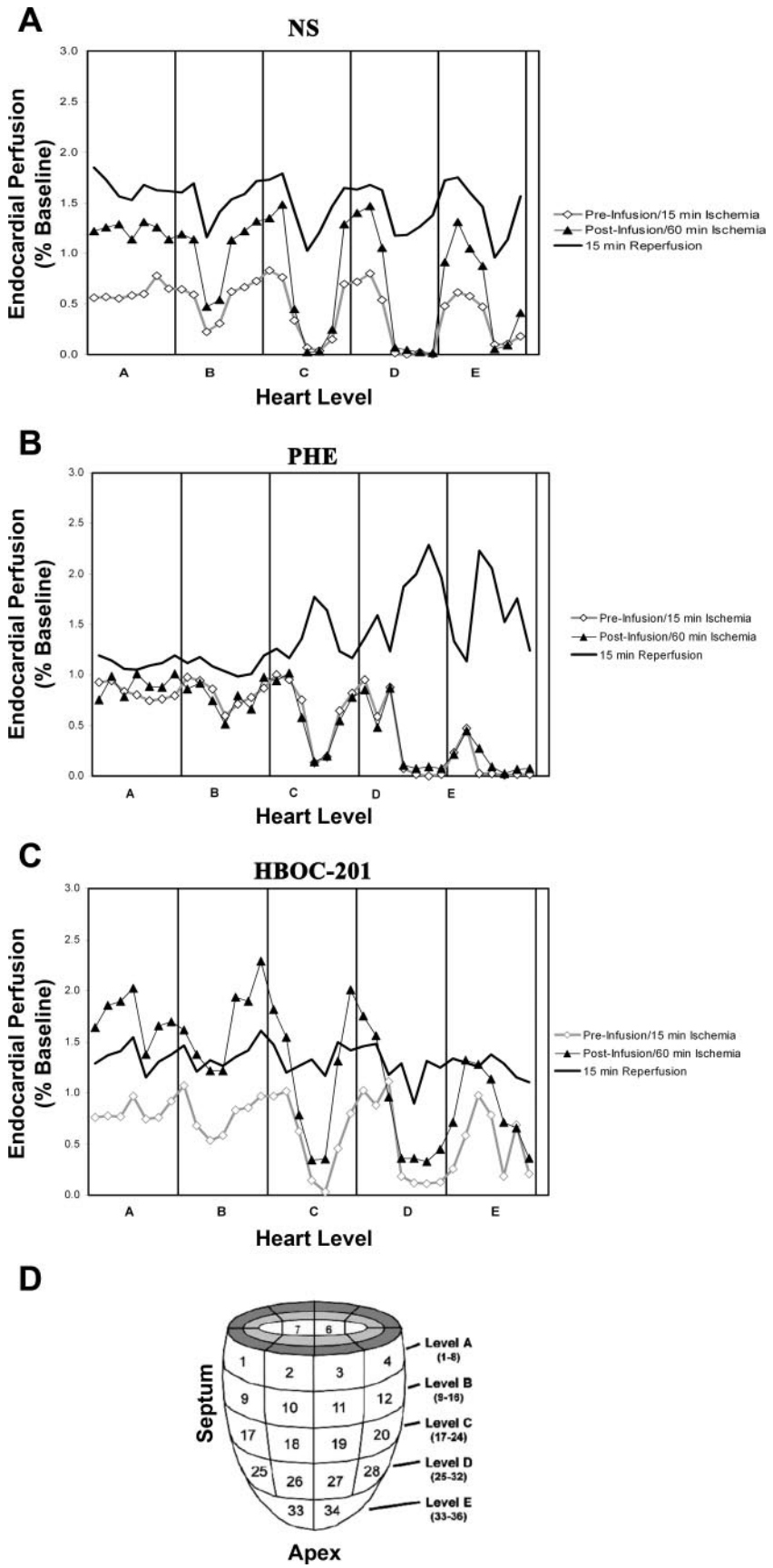


Fig. 3. Regional myocardial ischemia model: sample endocardial perfusion flow maps from one animal after NS (*A*), Phe (*B*), and HBOC-201 (*C*), as measured by colored microsphere analysis. Representative diagram (*D*) outlines the endocardial and epicardial sectioning performed at each heart level.

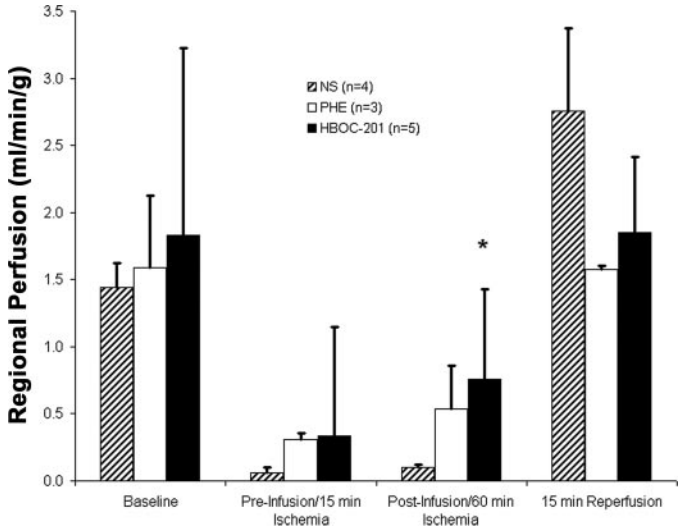


Fig. 4. Regional myocardial ischemia model: regional endocardial perfusion by colored microsphere analysis. Treatment with HBOC-201 significantly increased endocardial perfusion after ischemia (*$P < 0.05$ vs. NS).

affinity to allow greater oxygen unloading compared with human or canine RBCs (26). Tissue oxygen delivery has also been demonstrated to be at least 20% higher and at a faster rate than human Hb (26). In a swine model of hemorrhage by McNeil et al. (20), HBOC-201 reversed anaerobic metabolism and normalized arterial lactate and base excess. Improved RMF during ischemia and infarct size reduction cannot be accounted for only by increased coronary flow, as seen in the selected treated and NS, Phe, and L-NAME groups that had matched LAD flow but improved cardiac performance only after HBOC-201 treatment. This suggests that HBOC-201 rescued myocardium in part by delivering more oxygen to ischemic tissue. This hypothesis is supported by the dose-dependent effects of HBOC-201 on myocardial infarct size, in which HBOC-201 at higher doses with greater total oxygen-carrying capacity reduced infarct size greater than the lower dose of HBOC-201. The inherent oxygen binding and unloading characteristics of HBOC-201 are an important feature lacking in L-NAME and other NO inhibitors.

Finally, HBOC-201 provides protection against reperfusion injury, consistent with a prior study by Caswell et al. (4). Treated animals showed trends to less severe declines in LV dP/d*t* and LVEDP; despite long reperfusion times, RMF displayed significant improvements after reperfusion, and myo-

Table 3. *Myocardial infarct model: hemodynamics*

	Baseline	Preinfusion/15 min Ischemia	Postinfusion/End of Ischemia	180 min Reperfusion
MAP, mmHg				
NS	77.9±3.8	68.7±4.9[c]	72.2±2.9	70.5±5.6
Phe	75.3±4.5	69.4±1.6	96.3±4.5[b,f]	65.7±4.1
L-NAME	90.6±2.1[d]	77.9±4.2	93.0±4.0[b,f]	86.9±10.1
HBOC-201 (0.5 g/kg)	69.0±2.8[a]	66.7±0.9	72.7±3.1	70.7±2.3
HBOC-201 (1 g/kg)	81.9±3.6	73.8±3.2	94.2±5.0[b,f]	90.2±4.4[f]
HBOC-201 (delayed)	77.2±7.4	65.3±4.8	89.0±4.3[f]	82.4±2.8[f]
LVSP, mmHg				
NS	100.7±2.8	92.7±3.6	94.0±4.8	93.4±5.1
Phe	96.7±3.4	90.0±2.9	117.0±6.0[b,d,f]	90.0±7.6
L-NAME	105.0±3.8	95.6±3.4[e]	106.0±4.1	109.0±17.1
HBOC-201 (0.5 g/kg)	90.3±1.7	83.0±4.2[e]	86.7±2.3	92.7±3.2
HBOC-201 (1 g/kg)	105.7±3.6	95.3±3.6[e]	116.7±4.8[b,d,f]	118.0±7.2[f]
HBOC-201 (delayed)	99.6±7.8	90.0±4.0	108.8±4.8[d,f]	103.6±1.7[f]
LVEDP, mmHg				
NS	11.0±2.2	16.0±1.6[e]	16.3±2.4	16.8±4.5
Phe	13.3±2.2	17.7±1.4[e]	22.3±2.9	15.6±2.1
L-NAME	9.3±1.7	17.2±3.4[e]	19.7±2.4	21.0±6.0[f]
HBOC-201 (0.5 g/kg)	14.0±1.4	19.7±1.4[e]	15.7±2.2	15.0±1.6
HBOC-201 (1 g/kg)	14.0±3.0	19.0±1.1	16.0±1.0	15.7±1.9
HBOC-201 (delayed)	13.2±2.3	18.4±2.4	18.0±2.0	14.8±2.6
LV dP/d*t*, mmHg/s				
NS	1,365±109	1,234±118	1,022±123[f]	1,093±143
Phe	1,189±58	1,089±70	1,118±98	1,027±101
L-NAME	1,268±91	1,039±63[e]	930±59	915±146
HBOC-201 (0.5 g/kg)	1,000±81	932±115[e]	786±54	889±61
HBOC-201 (1 g/kg)	1,445±96[d]	1,333±132[d]	1,252±91[d]	1,440±113[a,d]
HBOC-201 (delayed)	1,151±144	1,099±88	1,056±48	1,065±103
LAD flow, ml/min				
NS	29.2±7.8	3.3±1.4[e]	5.0±2.4	36.4±7.1[f]
Phe	42.7±7.6	3.3±0.6[e]	6.2±1.5[f]	39.2±8.7[f]
L-NAME	44.8±4.7	2.7±0.3[e]	8.7±1.5[f]	33.5±7.1[f]
HBOC-201 (0.5 g/kg)	48.5±7.9	7.2±1.7[e]	11.2±1.5[f]	33.7±5.7[f]
HBOC-201 (1 g/kg)	64.0±18.3	7.0±2.8[e]	25.0±5.2[a,b,c,d,f]	72.3±17.0[f]
HBOC-201 (delayed)	37.4±6.0	3.8±0.6[e]	13.00±2.2	34.4±1.4[f]

All data are expressed as means ± SE; $n = 6$ for NS, $n = 6$ for Phe, $n = 6$ for N^G-nitro-L-arginine methyl ester (L-NAME), $n = 6$ for HBOC-201 (0.5 g/kg), $n = 6$ for HBOC-201 (1 g/kg), and $n = 5$ for HBOC-201 (delayed). Delayed-HBOC-201 (1 mg/kg) given 60 min after the onset of ischemia. [a]$P < 0.05$ vs. L-NAME, [b]$P < 0.05$ vs. NS. [c]$P < 0.05$ vs. Phe, [d]$P < 0.05$ vs. HBOC-201 (0.5 g/kg), [e]$P < 0.05$ vs. baseline, [f]$P < 0.05$ vs. preinfusion/15 min ischemia.

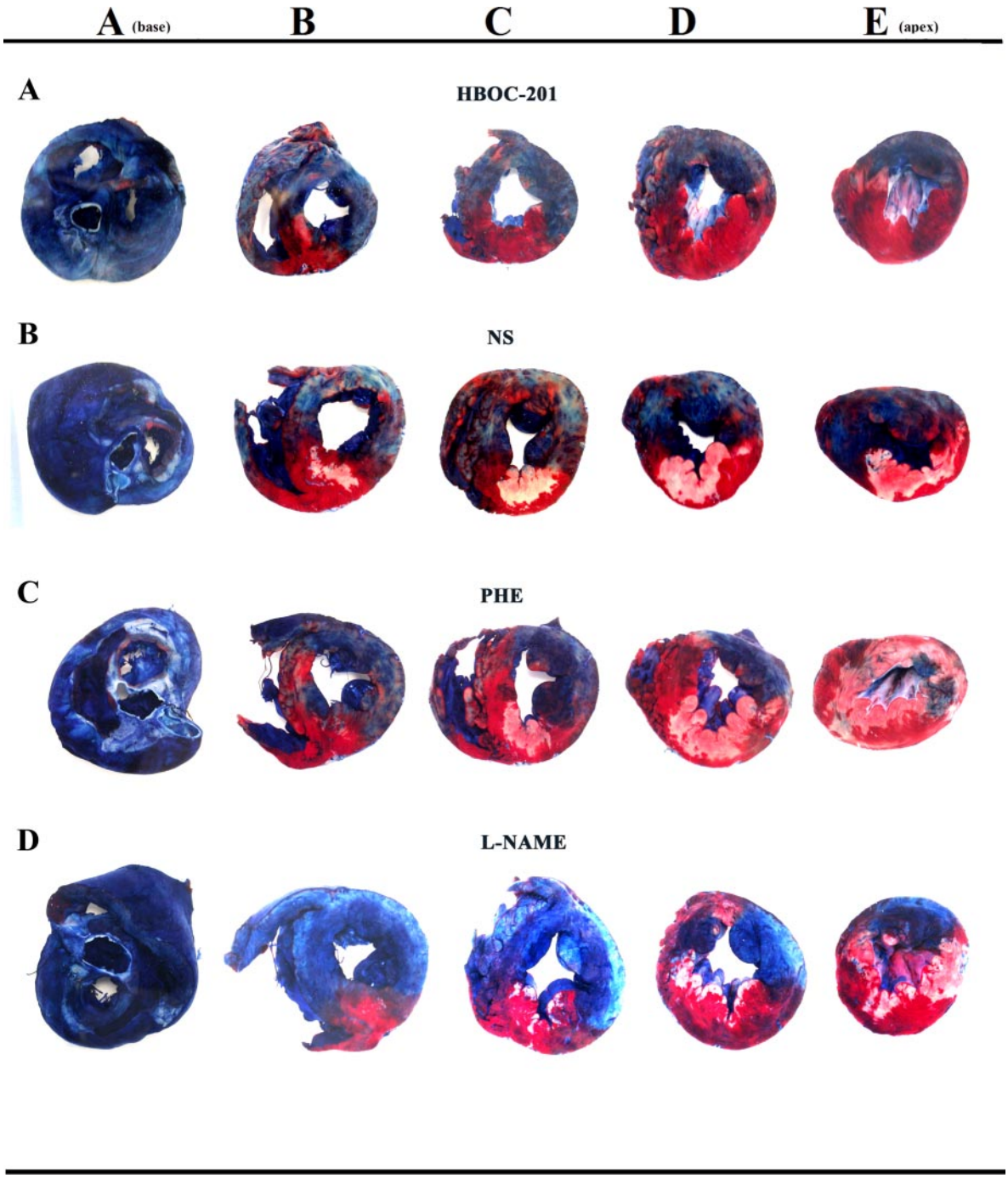
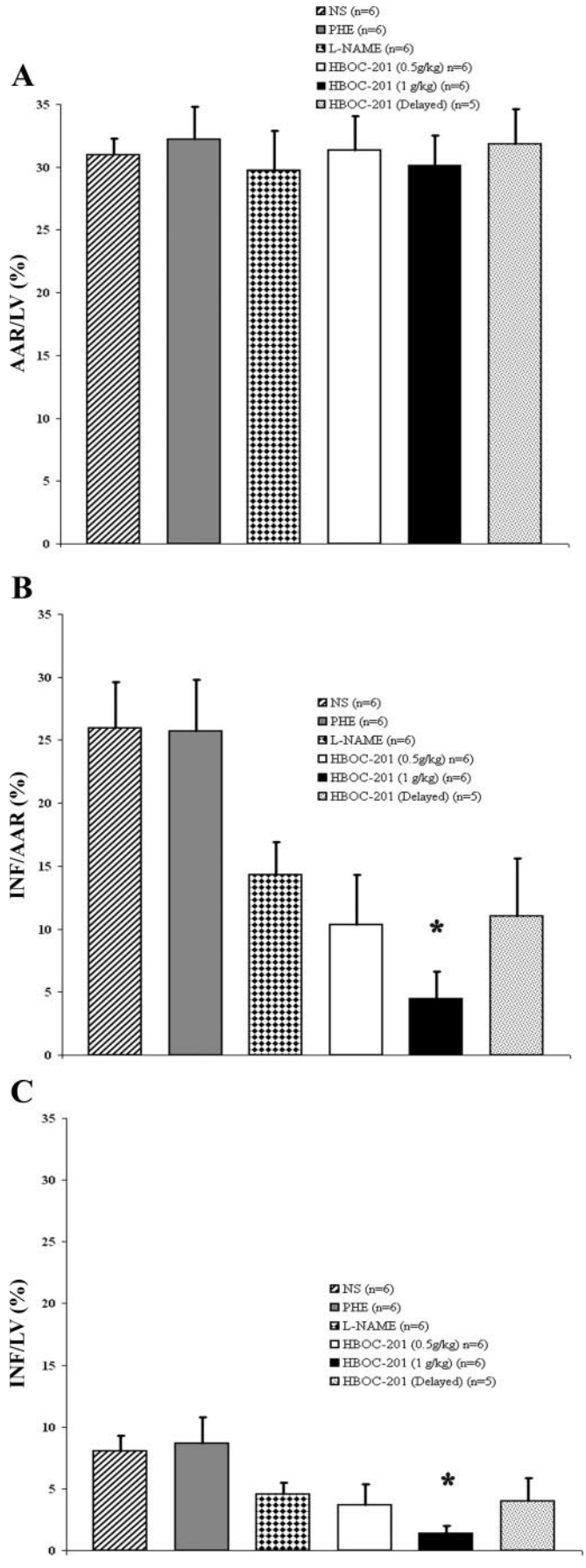


Fig. 5. Sample infarct images. *Columns*: *layers A* (base) through *E* (apex). *Rows*: HBOC-201 (*A*; 1 g/kg) led to significant reductions in infarct size in *layers C–E* of the heart vs. NS (*B*) and Phe (*C*; $P < 0.005$ vs. NS, Phe). Treatment with L-NAME (*D*) produced infarct reduction to a lesser degree than HBOC-201. Heart is oriented with the ischemic territory facing inferiorly. Area outside the ischemic region is stained blue, infarct tissue stains white, and viable tissue within the ischemic region stains pink-red. Similar infarct reductions were seen with HBOC-201 (0.5 g/kg) and delayed treatment (after 60 min of ischemia) (1 g/kg) (not shown).

cardial infarct sizes were reduced with HBOC-201. These results may be mediated by the formation of peroxynitrite and reactive oxygen radicals, which have been strongly implicated in antioxidant injury with high NO expression and may be attenuated with HBOC-201 (10, 29).

Clinically, myocardial protection after acute ischemia is critical to improving clinical outcomes in multiple settings. Dysfunction after surgical revascularization manifests as low cardiac output requiring inotropic support in up to 30% of patients to allow weaning from cardiopulmonary bypass (19). Infarct expansion after PCI and stunning after transplantation similarly occur. Ischemia in a prehospital setting accounts for the majority of deaths after AMI, estimated at 32%, and long-term prognosis has been directly correlated with infarct size (5, 21). Both the RMF model and infarct model employ an intermediate-grade coronary stenosis to produce ischemia, as



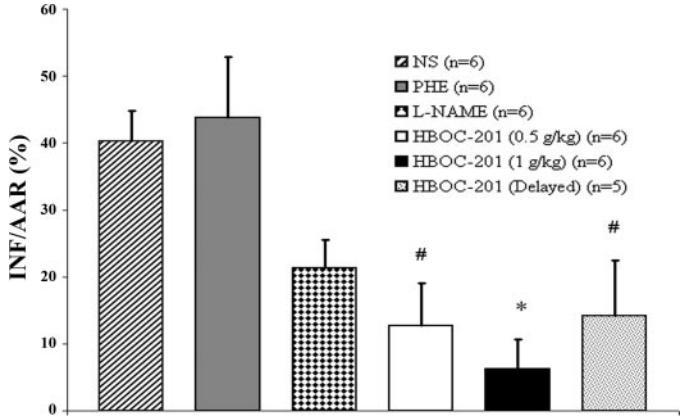


Fig. 7. Myocardial infarct model: *layer D* infarct size (expressed as Inf/AAR) after HBOC-201 infusion. Infarct sizes were reduced significantly in *layer D*, the heart layer most vulnerable to ischemia after left anterior coronary artery stenosis, after HBOC-201 (1 g/kg) administration vs. NS and Phe ($*P < 0.01$ vs. NS, Phe). Infarct sizes were also significantly reduced in *layer D* after HBOC-201 (0.5 g/kg) compared with both NS and Phe ($\#P < 0.05$ vs. NS, Phe). These results suggest a dose-dependent effect by HBOC-201 on tissue oxygenation and delivery. Finally, HBOC-201 (delayed, 1 g/kg) was administered 60 min after the onset of ischemia and produced significant reductions in infarct size in *layer D* ($\#P < 0.05$ vs. NS, Phe).

80–95% flow reduction correlates to a 56–78% reduction in LAD radius. We believe that this model is clinically relevant, as 20–70% coronary stenoses have been most commonly shown to progress to Q-wave infarction and non-Q-wave infarction (8). Acute coronary stenosis, or partial rather than complete vessel occlusion, accounts for 13% or more of all patients suffering AMI (9). This number is expected to rise with increased survival of patients with severe multivessel coronary disease and CHF and improved medical therapy. It is also important to note that HBOC-201 was given after 15 min of ischemia; there was no pretreatment of tissues to allow preconditioning or ischemic tolerance to occur. Therapy with HBOC-201 after 60 min is a foreseeable goal, especially with progressive reduction in symptom onset to presentation times, reported as under 2 h in up to 32% of patients presenting with AMI (30). Infarct reduction with HBOC-201 in this subset of patients suffering myocardial infarction with incomplete coronary occlusion, even after 60 min of ischemia, represents an important result; a method to salvage large portions of ischemic tissue at risk for necrosis could be applied to direct clinical use in a prehospital setting.

A number of limitations exist in this study. Extensive biochemical analysis of endothelial function, neurohormonal factors, and reperfusion injury were not performed to further reveal the underlying mechanisms responsible for the hemodynamic, functional, and infarct improvements. Measurement of vasoactive factors, neurohormones, and markers for ischemia-reperfusion injury may provide further valuable insight into the mechanisms of action of HBOC-201. An extensive

Fig. 6. Myocardial infarct model: total infarct size after HBOC-201 infusion. Area at risk (AAR) per left ventricle (LV) was comparable among both control and HBOC-201 treatment groups, as shown in *A*. Total infarct sizes expressed as Inf/AAR were significantly reduced after HBOC-201 (1 g/kg) compared with NS and Phe ($*P < 0.05$ vs. NS, Phe) (*B*). Infarct size expressed as Inf/LV was also reduced with HBOC-201 (1 g/kg) therapy ($*P < 0.05$ vs. NS, Phe) (*C*).

analysis of myocardial consumption, through mixed venous saturation measurement or pressure-volume loop analysis, is missing and would complement these findings. Formal calculations of oxygen delivery are missing, as well. Reperfusion was only carried out for only 15 min in the regional ischemia model, and it remains to be seen if lasting effects in a chronic model will demonstrate the same results. In the infarct model, HBOC-201 was given 60 min after the onset of ischemia; this time period should be extended to 2–3 h after the onset of ischemia to determine if late administration provides the same benefit.

In conclusion, HBOC-201 has been shown to improve RMF and reduce myocardial infarct size after acute coronary ischemia and reperfusion. Early and late administration of a bovine Hb solution after acute ischemia attenuates cardiac injury and increases tissue viability. Further study into timing of administration and specific biochemical pathways are needed to help clarify the optimal clinical use of HBOC-201 for treatment of acute myocardial ischemia.

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