Inadequacy of low-volume resuscitation with hemoglobin-based oxygen carrier hemoglobin glutamer-200 (bovine) in canine hypovolemia

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Stroma-free hemoglobin-based oxygen carriers (HBOC) have been developed to overcome problems associated with transfusion of allogeneic blood. We have studied the efficacy of the first licensed veterinary blood substitute, hemoglobin glutamer-200 bovine (Oxyglobin®; Biopure, Cambridge, MA, USA, Hb-200), in a canine model of acute hypovolemia and examined whether clinically commonly used criteria are adequate to guide fluid resuscitation with this product. Twelve anesthetized dogs were instrumented for measurements of physiological variables including hemodynamic, oxygenation, and blood gas and acid–base parameters. Dogs were bled to a mean arterial pressure (MAP) of 50 mmHg for 1 h followed by resuscitation with either shed blood (controls) or Hb-200 until heart rate (HR), MAP and central venous pressure (CVP) returned to baseline. Recordings were repeated immediately and 3 h after termination of fluid resuscitation. Hemorrhage (average 32 mL/kg) caused significant decreases in total hemoglobin (Hb), mean pulmonary arterial pressure (PAP), cardiac output (CO) and oxygen delivery (DO_2I), increases in HR and systemic vascular resistance (SVRI), and lactic acidosis. In controls, only re-transfusion of all shed blood returned HR, MAP and CVP to prehemorrhage values, whereas in other dogs this endpoint was reached with infusion of 10 mL/kg Hb-200. Unlike blood transfusion, Hb-200 infusion failed to return CI and DO_2I to baseline and to increase arterial oxygen content (C_aO_2) and total Hb; SVRI further increased. Thus, commonly used criteria (HR, MAP, CVP) to guide transfusion therapy in patients posthemorrhage prove insufficient when HBOCs with pronounced vasoconstrictive action are used and lead to inadequate volume repletion.

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INTRODUCTION

Transfusion of allogeneic blood has long been the mainstay in the treatment of acute blood loss and anemia despite serious concerns associated with its use, such as transmittable diseases, immunologic incompatibility, transportation and storage difficulties, short shelf-life, and supply shortage (Klein, 1994; Wallace *et al.*, 1998). Allogeneic and xenogeneic, stroma-free, ultra-purified hemoglobin (Hb) blood substitutes have now been developed to overcome these problems (Rabinivici *et al.*, 1994; Hughes *et al.*, 1996; Winslow, 1999). While several studies have demonstrated the efficacy of these blood substitutes as volume expanders and oxygen carriers (Vlahakes *et al.*, 1990; Bosman *et al.*, 1992; Harringer *et al.*, 1992; Slanetz *et al.*, 1994; Schultz *et al.*, 1995; Chappell *et al.*, 1996; Frankel *et al.*, 1996; Standl *et al.*, 1996) they also revealed unwanted physiologic effects including significant vasoconstriction in both the systemic and pulmonary vasculature (see Mallik & Bodenham, 1996; Tremper, 1997). The vasoconstrictive property may lead to ischemia in susceptible organs by limiting oxygen delivery (Aranow *et al.*, 1996; Ulatowski *et al.*, 1996; De Figueiredo *et al.*, 1997; Krieter *et al.*, 1997; Kasper *et al.*, 1998; Noone *et al.*, 1998).

Hemoglobin glutamer-200 bovine (Hb-200; Oxyglobin®, Biopure, Cambridge, MA, USA) is an ultrapure solution of highly

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polymerized bovine Hb (Rentko, 1992; Light *et al.*, 1998), and is approved for treatment of anemia in dogs only. Like other Hb-based oxygen carriers (HBOC), highly polymerized bovine Hb has been associated with increases in vascular resistance when administered in both dogs (Hughes *et al.*, 1996; Horn *et al.*, 1997; Standl *et al.*, 1997; Muir *et al.* 2000) and humans (Krieter *et al.*, 1997; Brauer *et al.*, 1998; Kasper *et al.*, 1998). Unfortunately, the composition and, hence, physico-chemical properties differ substantially among various polymerized bovine HBOC solutions tested in previous experimental studies (Vlahakes *et al.*, 1990; Bosman *et al.*, 1992; Harringer *et al.*, 1992; Slanetz *et al.*, 1994; Botzlar *et al.*, 1996; Krieter *et al.*, 1997; Standl *et al.*, 1997), probably reflecting the manufacturer's progress in developing the final product. Independent clinical efficacy studies of Hb-200 in the treatment of hemorrhagic shock are still missing. Therefore, the present study was designed to evaluate the first marketed veterinary HBOC Hb-200 in a canine model of acute hypovolemia and to examine the hypothesis that clinically most commonly used criteria, i.e. heart rate (HR) and mean arterial pressure (MAP) and central venous pressure (CVP), are unreliable predictors of adequacy of volume repletion when using Hb-200 in place of whole blood for fluid resuscitation.

MATERIALS AND METHODS

Animals

Twelve healthy, adult, mongrel dogs (30.8 ± 1.4 kg; seven female, five male) with no clinically noticeable heart disease were studied after approval by the Campus Animal Care and Use Committee of the University of California and in compliance with the Guide for the Care of Laboratory Animals (National Institutes of Health publication 86–23, revised 1985). The animals were fasted over night but had free access to water up to 2 h prior to anesthesia.

Animal preparation and instrumentation

The animals were premedicated with oxymorphone (0.02 mg/kg i.m.) and atropine (0.02 mg/kg i.m.) followed by percutaneous catheterization of the cephalic vein for continuous infusion of lactated Ringer's solution at a rate of 10 mL/kg/h throughout the preparation and instrumentation period and administration of drugs. Dogs were then anesthetized with propofol (2–4 mg/kg i.v.) and diazepam (0.5 mg/kg i.v.), intubated and attached to a small animal anesthesia machine (Frazer Harlake, Orchard Park, NY, USA) operating with an Isotec^{TM} vaporizer (Ohmeda, Milwaukee, WI, USA). During animal preparation and instrumentation, anesthesia was maintained with isoflurane in 100% oxygen (end-tidal concentration of isoflurane (ISO_{ET}) 0.8–1.2%) and an infusion of fentanyl at a rate of 0.7 μg/kg/min that followed an initial bolus of fentanyl (10 μg/kg i.v.). The animals were mechanically ventilated with an anesthesia ventilator (Model 2000; Hallowell EMC, Pietsfield, MA, USA), using tidal volumes (V_T) of 12–15 mL/kg and a respiratory rate (RR) of 9–11 breaths/min. Ventilation was subsequently adjusted to ensure an arterial partial pressure of CO_2 (P_aCO_2) in the range of 35–45 mmHg (4.6–6.0 kPa). Tidal volume (V_T) and peak airway pressures were measured using a respirometer (Mark 14 Wright; Bird Corporation, Palm Springs, CA, USA) and a pressure gauge attached to the anesthesia machine, respectively. The RR, end-tidal partial pressure of CO_2 ($P_{ET}CO_2$) and ISO_{ET}, as well as inspired oxygen concentration (F_iO_2) were continuously monitored using a Datex 254 airway gas monitor (Datex, Helsinki, Finland).

The animals were instrumented in dorsal recumbency for continuous recording of the electrocardiogram (ECG; Monitor Model 78353 B, Hewlett Packard, Andover, MA, USA) and arterial oxygen saturation (SpO_2; Model N-180 pulse oximeter, Nellcor Inc., Hayward, CA, USA). Both femoral arteries were percutaneously catheterized for measurement of systemic arterial blood pressures (ABP) and collection of arterial blood. The lateral saphenous vein was cannulated using a large bore catheter to facilitate rapid blood removal during hemorrhage. An 8-Fr balloon-tipped flow-directed thermodilution pulmonary arterial catheter (OptiQ^{TM}, Abbott Laboratories, Chicago, IL, USA) was also inserted via the jugular vein and floated into the pulmonary artery under direct pressure monitoring for measurements of pulmonary arterial pressure (PAP), pulmonary artery occlusion pressure (POP), central venous pressure (CVP), core body temperature and cardiac output (CO). This and one of the femoral arterial catheters were connected to membrane transducers (Model 1290 A, Hewlett Packard, Waltham, MA, USA). Prior to each experiment, all pressure transducers were calibrated against a mercury manometer and against atmospheric pressure using the mid-thoracic inlet of the supine dog as zero level. Systolic, diastolic and mean ABP, PAP, POP, CVP were recorded with a multichannel, amplified, strip chart recording system (7754B System, Hewlett-Packard, Palo Alto, CA). The pulmonary arterial catheter was connected to a CO computer (Critical Care Systems QVUE, Oximetrix 3, Abbott Laboratories, Chicago, IL, USA) for continuous CO monitoring. The CO was also assessed by thermodilution in triplicate using 10 mL of saline at room temperature. Body temperature was maintained between 37 and 39 °C by means of a heating pad and circulating warm air blanket (Bair Hugger^{R} Model 505, Augustine Medical Inc., Eden, MN, USA) placed underneath and on top of the animal, respectively.

Dogs have the capacity to sequester substantial amounts of red blood cells in their spleen and may release those cells into the circulation during acute hypovolemia. As this response has been observed in two previous canine hemorrhagic shock studies with HBOCs (Bosman *et al.*, 1992; Harringer *et al.*, 1992), all animals underwent surgical splenectomy immediately after placement of the thermodilution catheter.

Measurements

The following parameters were measured throughout the experiment: V_T, peak airway pressures, RR, $P_{ET}CO_2$, ISO_{ET}, F_iO_2, HR, SpO_2, SAP, DAP, and MAP, mean PAP, POP (at end-

expiration), CVP, and CO. Arterial and mixed venous blood samples were collected intermittently from the femoral artery and right atrium, respectively. Immediately after collection, blood samples were air sealed and stored on ice. Subsequently, hematocrit (Htc) and total solid content (TS) were measured by means of centrifugation of capillary tubes and refractometry, respectively; arterial oxygen saturation (S_aO_2), total (Hb_{total}) and plasma Hb (Hb_{Plasma}; after centrifugation) as well as methemoglobin (Met-Hb) were measured in these samples using a Nova co-oximeter (Nova Biomedical, Waltham, MA, USA), and arterial and mixed venous oxygen content (C_aO_2; C_vO_2) was directly determined in duplicate using an oxygen-specific electrode ($LEXO_2CON$-K, Hospex Fiberoptics, Chestnut Hill, MA, USA). Arterial lactate ($Lactate_a$) concentrations were determined in duplicate by means of a Sport Lactate Analyzer (Model 1500; YSI Inc., Yellowsprings, OH, USA), and arterial pH (pH_a) and partial pressures of oxygen (P_aO_2) and CO_2 (P_aCO_2) were analysed with a blood gas analyzer (Model 170, Corning Medical, Medfield, MA, USA). Blood gas values were corrected for the body temperature of the animals at the time of sampling. Arterial base excess (SBE_a) was calculated by the blood gas analyser. All laboratory analysers used were recently validated for use with Hb-200 (Jahr *et al.*, 2000a,b, 2001).

Cardiac index (CI), stroke volume index (SVI), systemic (SVRI) and pulmonary vascular resistance indexes (PVRI) were calculated using standard formulae, which were previously reported (Driessen *et al.*, 1999). Cellular (red blood cell) Hb (Hb_{RBC}) was calculated as the difference of Hb_{total} and Hb_{Plasma}. Systemic oxygen delivery index (DO_2I) was calculated as $C_aO_2 \times CI$, and systemic oxygen consumption index (VO_2I) as $(C_aO_2 - C_vO_2) \times CI$.

Experimental protocol

Following completion of the surgical procedure, the infusion of lactated Ringer's solution was discontinued, the inspired concentration of oxygen reduced to room air conditions (F_iO_2: 21 ± 1%), and anesthesia maintained with a decreased end-tidal concentration of isoflurane (ISO_{ET}: 0.7–0.8%) and a reduced infusion rate of fentanyl (0.35 μg/kg/min). After these adjustments the dogs were allowed to stabilize for 45 min (*Equilibration period*), after which all measurements (*Baseline*) were taken. Subsequently, approximately 40% of the dogs' blood volume was withdrawn simultaneously from the lateral saphenous vein and femoral artery at a rate of 60–70 mL/min until a mean ABP of about 50 mmHg was reached. Shed blood was collected in plastic containers containing 63 mL anti-coagulant citrate phosphate dextrose solution (Blood-Pack® Unit PL 146R, Baxter Healthcare Corp., Deerfield, IL 60015, USA), which were weighed before and after blood withdrawal using a portable standard scale (Model LS 2000, Ohaus, Florham Park, NJ, USA) in order to estimate total volume of shed blood. Additional small amounts of blood were withdrawn to maintain the main blood pressure at 50 mmHg for 60 min. The collection bags were stored at 25–30 °C. The CO decreased by more than 50% from baseline under these conditions. At the end of the hypovolemic period all measurements were repeated (*Posthemorrhage*) and then the dogs were randomly allocated to the control or study group. Control animals were resuscitated by transfusion of shed blood at a rate of 30 mL/kg/h while animals in the study group received Hemoglobin glutamer-200 (Hb-200; Oxyglobin®, Biopure; for specifics see Table 1) at the manufacturer's recommended rate of 10 mL/kg/h. Transfusion of shed blood or Hb-200 was discontinued once HR, mean ABP and CVP returned to baseline and stabilized. All measurements were repeated immediately (*Resuscitation I*) and 3 h (*Resuscitation II*) after fluid resuscitation had been terminated. The animals remained anesthetized throughout the procedure and were killed following the last measurements with an overdose of potassium chloride without regaining consciousness.

Statistical analysis

Results are given as arithmetic mean ± SEM. Statistical evaluation of data within each group (i.e. test for differences between time points) included an analysis of variance (ANOVA) for repeated measures. When ANOVA indicated significant differences, statistical testing was followed by comparisons between baseline and time points using a Student's *t*-test for independent samples and post hoc Bonferroni correction. Statistical evaluation of data between both groups was performed using an ANOVA for repeated measures followed by a Student's *t*-test. $P < 0.05$ was taken to be statistically significant.

RESULTS

All hemodynamic and blood parameters measured prior to hemorrhage (*Baseline*) were well within the normal range

Table 1. Specifications of hemoglobin glutamer-200 bovine (Oxyglobin®, Hb-200)

Polymerized bovine Hb	13 g/dL
NaCl	113 mM
KCl	4 mM
$CaCl\text{-}2H_2O$	1.4 mM
NaOH	10 mM
Na-lactate	27 mM
N-acetyl-L-cysteine	200 mg/dL
Osmolality	300 mOsm/kg
pH	7.8
COP*	42 torr
P_{50}†	34 torr
Unpolymerized Hb	<5%
Molecular weight of 50% of Hb polymers	65–130 kDa
Molecular weight of 10% of Hb polymers	>500 kDa
Free-glutaraldehyde	<3.5 μg/mL
Endotoxin	<0.05 EU/mL

*Measured with Colloid Oncometer 4420 (Wescor, Logan, UT, USA). *Source*: Rentko, 1992. †The P_{50} represents the oxygen tension at which the polymerized bovine Hb molecule is half saturated with oxygen.

previously reported for dogs (Muggenburg & Mauderly, 1974; Haskins *et al.*, 1985; Haskins & Patz, 1990) without any statistically significant differences in any of these variables between both animal groups (see Tables 2–4 and Figs 1–4). Following the initial equilibration phase, in all dogs there were no significant changes in body temperature, RR, airflow, V_T, F_iO_2, ISO_{ET}, $P_{ET}CO_2$, P_aO_2, and P_aCO_2 over time. There was also no statistically significant difference in these parameters between both animal groups at any time.

The total amount of blood removed during hemorrhage averaged 31.6 ± 4.1 and 31.5 ± 4.1 mL/kg in the control and Hb-200 group, respectively, corresponding to about 40% of the estimated canine blood volume (85 mL/kg body weight). As expected, arterial Htc and total Hb as well as C_aO_2 decreased by 15–18% in both groups (Fig. 4 and Tables 2 & 4). The animals in both groups responded hemodynamically to the acute blood loss in a similar manner (Figs 1–3; Table 3). MAP, MPAP, CI, SVI, and DO_2I decreased on average by 44, 23, 55, 75, and 65%, respectively. During hypovolemia the pulse pressure amplitude also decreased, as is evident from SAP and DAP recordings (Table 3). The HR and SVRI increased by an average of 87 and 38%, respectively (Figs 2 & 3; Table 2). The POP and CVP decreased immediately posthemorrhage (not shown), however, at the end of the hypovolemic period, when posthemorrhage values were recorded, these effects were not statistically significant anymore (Table 3), probably because of progressively increasing peripheral vasomotor tone. Oxygen content of mixed venous blood decreased rapidly following hemorrhage as a result of increased tissue oxygen extraction, reaching a nadir of about 44% of baseline at the end of the hypovolemic phase (Table 4). There was no difference between groups in this hypovolemia-related compensatory response, as there was also no difference

Table 2. Effects of hemorrhage and subsequent resuscitation with autologous blood (control) or hemoglobin glutamer-200 bovine (Hb-200) on arterial blood composition

Variable	Resuscitation group	Baseline	Post hemorrhage	Resuscitation I	Resuscitation II
Htc (%)	Control	44 ± 2	36 ± 2**	41 ± 2*	44 ± 2
	Hb-200	44 ± 2	37 ± 1**	29 ± 1**##	29 ± 1**##
Hb_{Plasma}	Control	0 ± 0	0 ± 0	0 ± 0	0 ± 0
(g/dL)	Hb-200	0 ± 0	0 ± 0	2.9 ± 0.1**##	2.5 ± 0.1**##
Met-Hb	Control	0.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0
(%)	Hb-200	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	0.4 ± 0.1
TS (g/dL)	Control	7.2 ± 0.2	5.9 ± 0.3**	6.5 ± 0.2**	6.9 ± 0.2
	Hb-200	7.7 ± 0.3	6.3 ± 0.2**	7.9 ± 0.1#	7.1 ± 0.3

Variables determined were hematocrit (Htc), free plasma Hb (Hb_{Plasma}), percent met-Hb (Met-Hb) of total Hb, total solids (TS). Values are mean ± SEM of six dogs in each group. Significant differences from baseline values: $^*P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

Table 3. Effects of hemorrhage and subsequent resuscitation with autologous blood (control) or hemoglobin glutamer-200 bovine (Hb-200) on physiological parameters

Variable	Resuscitation group	Baseline	Post hemorrhage	Resuscitation I	Resuscitation II
SAP (mmHg)	Control	133 ± 7	83 ± 7**	122 ± 3	151 ± 7
	Hb-200	125 ± 6	74 ± 7**	138 ± 7#	130 ± 19
DAP (mmHg)	Control	85 ± 7	38 ± 3**	63 ± 3**	77 ± 3
	Hb-200	76 ± 6	34 ± 2**	71 ± 3#	62 ± 12
MPAP (mmHg)	Control	12 ± 1	9 ± 2*	17 ± 3*	15 ± 2
	Hb-200	10 ± 1	8 ± 2**	10 ± 1#	9 ± 2#
POP (mmHg)	Control	5 ± 1	4 ± 1	6 ± 1	7 ± 1
	Hb-200	3 ± 1	2 ± 1	3 ± 1	3 ± 2
CVP (mmHg)	Control	4 ± 1	3 ± 2	6 ± 2	6 ± 1
	Hb-200	3 ± 1	0 ± 1	4 ± 2	4 ± 1
SVI (mL/m^2/beat)	Control	31 ± 3	8 ± 2**	30 ± 2	41 ± 4
	Hb-200	30 ± 3	7 ± 1**	19 ± 3*##	17 ± 4*##
DO_2I (mL/m^2/beat)	Control	673 ± 80	244 ± 40**	733 ± 58	724 ± 31
	Hb-200	606 ± 42	208 ± 30**	381 ± 15**##	299 ± 43**##
VO_2I (mL/m^2/beat)	Control	113 ± 25	141 ± 33	136 ± 21	101 ± 26
	Hb-200	138 ± 14	121 ± 15	167 ± 15	135 ± 29

Variables determined were systolic (SAP) and diastolic arterial blood pressures (DAP), mean pulmonary arterial pressure (MPAP), pulmonary occlusion pressure (POP), central venous pressure (CVP), stroke volume index (SVI), oxygen delivery index (DO_2I), and oxygen consumption index (VO_2I). Values are mean ± SEM of six dogs in each group. Significant differences from baseline values: $^*P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

between groups regarding the metabolic acidosis, which developed during the sustained hypovolemic phase. Arterial pH, SBE and lactate concentrations all indicated a similar metabolic derangement in both groups (Table 4).

Following resuscitation, significant group differences in hemodynamics and blood oxygen contents were observed (Figs 2 & 3; Tables 3 & 4). In controls, transfusion of all shed blood (average 32 mL/kg) was required to return HR, MAP, and CVP to baseline, but in the study group infusion of only 10 mL/kg of Hb-200 was required to reach the same resuscitation end-point (Figs 1 & 2; Table 3). The return of HR, MAP, and CVP to or above baseline values occurred somewhat slower in the control than Hb-200 group (see also Fig. 1). In control animals all other measured hemodynamic and oxygenation parameters, including CI, C_aO_2, C_vO_2 and DO_2I returned to or above baseline (but still within normal limits) following blood transfusion. In the Hb-200 group CI, SVI, C_aO_2, C_vO_2 and DO_2I remained significantly below, and SVRI significantly above, baseline and control values throughout the entire observation period (Figs 2 & 3; Tables 3 & 4). In fact, CI, C_aO_2, and DO_2I did not recover at any time to more than 76, 83, and 63% of baseline, respectively, and SVRI continued to rise to 154% of baseline following resuscitation. In both groups, VO_2I returned to baseline concentrations, but was somewhat increased in the Hb-200 group during the early postresuscitation phase (Table 3).

Table 4. Effects of hemorrhage and subsequent resuscitation with autologous blood (control) or hemoglobin glutamer-200 bovine (Hb-200) on blood oxygenation, gas and acid–base parameters

Variable	Resuscitation group	Baseline	Post hemorrhage	Resuscitation I	Resuscitation II
C_aO_2 (mL/dL)	Control	19.2 ± 0.6	16.5 ± 1.2*	18.7 ± 0.8	19.0 ± 0.6
	Hb-200	20.3 ± 0.5	16.1 ± 0.8**	16.8 ± 0.6*#	16.9 ± 0.8*#
C_vO_2 (mL/dL)	Control	16.0 ± 0.7	7.4 ± 2.1**	15.2 ± 0.9	16.4 ± 0.9
	Hb-200	15.6 ± 0.7	6.6 ± 1.0**	9.4 ± 0.6**##	9.6 ± 1.4**##
S_aO_2 (%)	Control	97.9 ± 0.6	96.5 ± 1.1	97.7 ± 0.5	97.5 ± 0.6
	Hb-200	95.9 ± 0.5	95.2 ± 0.5	96.5 ± 0.5	96.7 ± 0.5
P_aO_2	Control	112 ± 11	110 ± 10	118 ± 12	112 ± 13
(mmHg [kPa])		[14.9 ± 1.4]	[14.6 ± 1.3]	[15.7 ± 0.3]	[15.0 ± 1.7]
	Hb-200	95.1 ± 5.3	86.2 ± 4.6	95.6 ± 6.1	93.5 ± 4.7
		[12.7 ± 0.7]	[11.5 ± 0.6]	[12.7 ± 0.8]	[12.5 ± 0.6]
P_aCO_2	Control	37.4 ± 1.8	39.9 ± 2.2	41.1 ± 2.3	35.6 ± 1.9
(mmHg [kPa])		[5.0 ± 0.2]	[5.3 ± 0.3]	[5.5 ± 0.3]	[4.7 ± 0.3]
	Hb-200	35.4 ± 1.1	39.9 ± 1.3	39.7 ± 1.1	35.2 ± 2.0
		[4.7 ± 0.1]	[5.3 ± 0.2]	[5.3 ± 0.1]	[4.7 ± 0.3]
pH_a	Control	7.37 ± 0.01	7.25 ± 0.01**	7.27 ± 0.02**	7.40 ± 0.03
	Hb-200	7.38 ± 0.01	7.28 ± 0.01**	7.33 ± 0.01**	7.37 ± 0.06
SBE_a (mmol/L)	Control	−2.6 ± 0.7	−9.2 ± 0.9**	−7.4 ± 1.1**	−1.8 ± 1.2
	Hb-200	−2.6 ± 0.6	−7.4 ± 0.6**	−4.4 ± 0.7*	−4.3 ± 2.4
$Lactate_a$ (mmol/L)	Control	1.48 ± 0.22	3.59 ± 0.41**	2.15 ± 0.15*	1.54 ± 0.25
	Hb-200	1.20 ± 0.22	2.60 ± 0.32**	2.09 ± 0.10*	2.10 ± 1.33

Variables determined were arterial and mixed venous oxygen content (C_aO_2, C_vO_2), arterial oxygen saturation (S_aO_2), partial pressures of arterial oxygen (P_aO_2) and CO_2 (P_aCO_2), arterial pH (pH_a), standard base excess (SBE_a), and lactate ($Lactate_a$). Values are mean ± SEM of six dogs in each group. Significant differences from baseline values: $^*P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

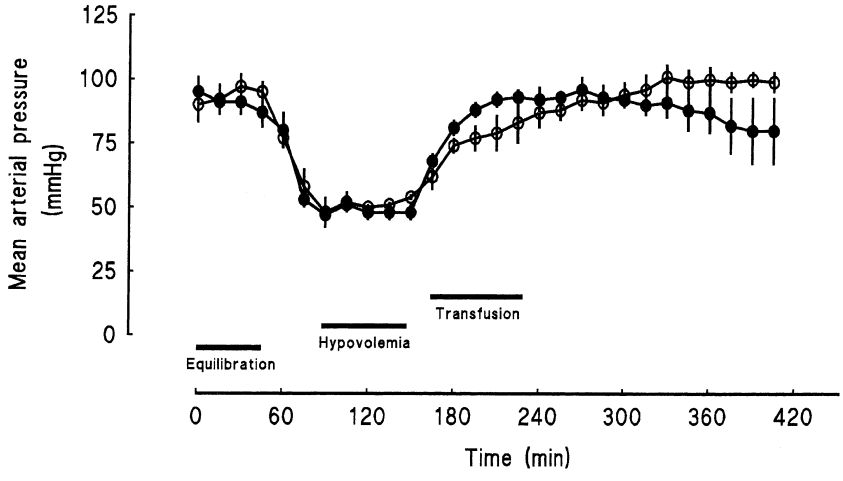

Fig. 1. The MAP in anesthetized dogs during equilibration, hypovolemia, and following transfusion of autologous blood (○, control) or hemoglobin glutamer-200 bovine (●, Hb-200) illustrating the time course of the experiment. After equilibration, dogs were bled about 40% of their blood volume and remained hypovolemic for 60 min. Thereafter, either shed blood (32 mL/kg) or Hb-200 (10 mL/kg) was transfused. Values are mean ± SEM of six dogs in each group.

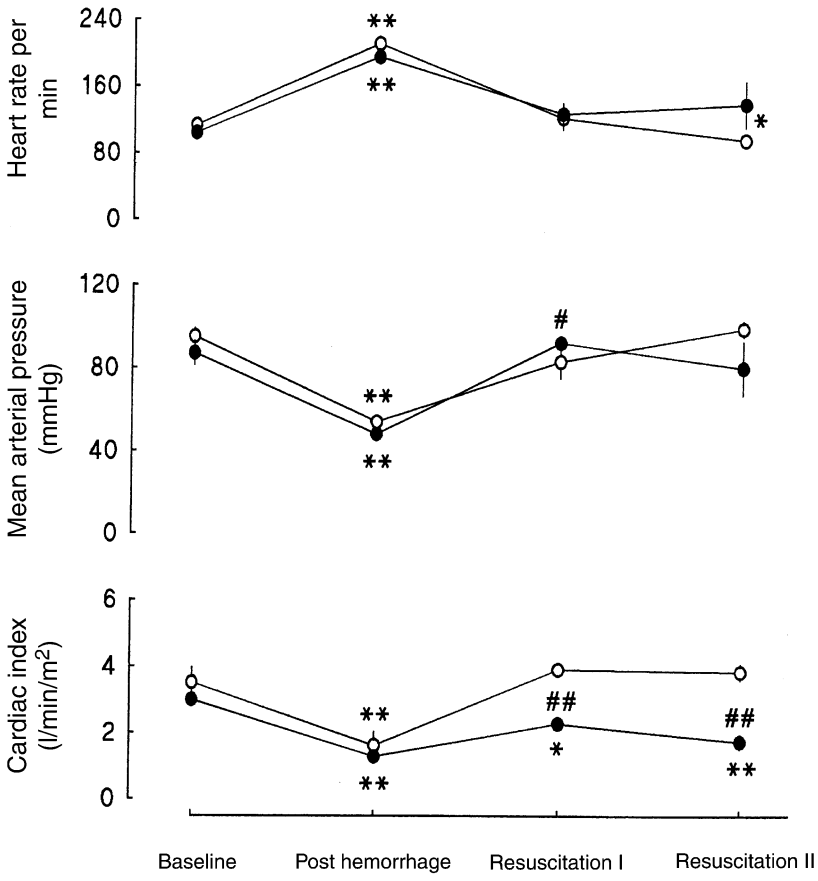

Fig. 2. The HR, MAP and CI in anesthetized dogs at baseline (end of equilibration), 60 min posthemorrhage (end of hypovolemia), and immediately (Resuscitation I) and 3 h (Resuscitation II) following transfusion of autologous blood (○, control) or hemoglobin glutamer-200 bovine (●, Hb-200), respectively. Values are mean ± SEM of 6 dogs in each group. Significant differences from baseline values: $^{*}P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

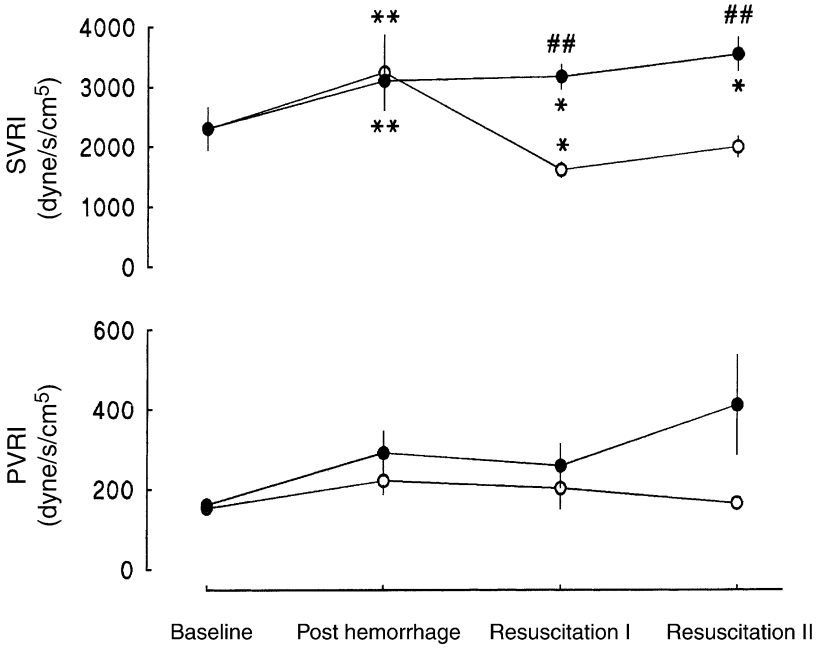

Fig. 3. The SVRI and PVRI in anesthetized dogs at *baseline* (end of equilibration), 60 min posthemorrhage (end of hypovolemia), and immediately (Resuscitation I) and 3 h (Resuscitation II) following transfusion of autologous blood (○, control) or hemoglobin glutamer-200 bovine (●, Hb-200), respectively. Values are mean ± SEM of six dogs in each group. Significant differences from baseline values: $^{*}P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

As expected, total Hb (i.e. Hb_{RBC}) returned to baseline in control animals after resuscitation with autologous blood. In contrast, total Hb did not increase in dogs receiving Hb-200 but remained at the low posthemorrhage concentration despite a significant increase in free plasma Hb, amounting to about 25% of total Hb (Fig. 4; Table 2). The Htc and Hb_{RBC} continued to decrease during the infusion of Hb-200. The Met-Hb concentrations were not affected in either group throughout the experi-

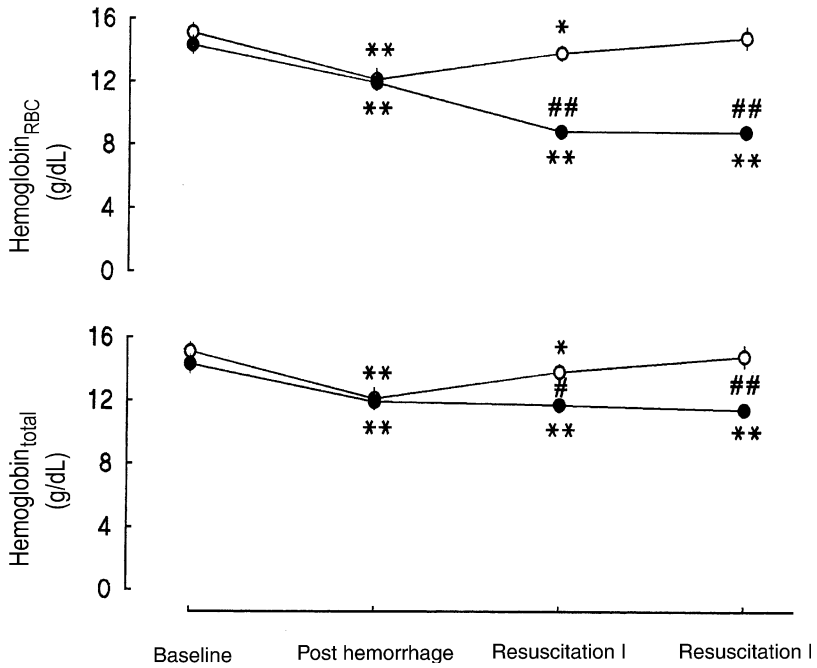

Fig. 4. Total and red blood cell Hb in anesthetized dogs at baseline (end of equilibration), 60 min posthemorrhage (end of hypovolemia), and immediately (Resuscitation I) and 3 h (Resuscitation II) following transfusion of autologous blood (○, control) or hemoglobin glutamer-200 bovine (●, Hb-200), respectively. Values are mean ± SEM of six dogs in each group. Significant differences from baseline values: $^{*}P < 0.05$, $^{**}P < 0.01$. Significant differences between control and Hb-200 group: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$.

ment (Table 2). Numerous yellow-red, ecchymosis-like discolorations were detected in nonpigmented areas of skin, particularly along the ventral abdominal wall, by the end of the experiment in dogs receiving Hb-200. The manufacturer described these signs as transient clinical phenomenon associated with Hb-200 administration, and are probably related to extravasation of Hb-200 into interstitial and lymph fluids, which are red-coloured after HBOC infusion in sheep (Dr R. Gunther, personal communication).

In comparison with controls, animals in the Hb-200 group had a significantly lower C_vO_2, which did not return to baseline tension at any time postresuscitation, but remained at stable tension about 40% below baseline (Table 4), indicating continued increased tissue oxygen extraction. Nevertheless, acid–base parameters returned in both treatment groups to or at least close to baseline values following resuscitation with no significant differences in effects on acid–base status between both groups (Table 4).

DISCUSSION

The development of Hb-based blood substitutes has added a new potential treatment option for resuscitation of patients suffering from severe hemorrhage. One chief advantage of highly purified stroma-free HBOCs is their complete or almost complete lack of anti-genicity (Hughes *et al.*, 1996; Winslow, 1999), thus offering, particularly in emergency situations, the potential of immediate HBOC administration to hypovolemic patients without the requirement of time-consuming blood typing and cross matching. In most clinical instances, volume replacement in the acute hypovolemic patient is guided by measurements of HR, ABP, and CVP (Sturm & Wisner, 1985) as more detailed hemodynamic data are rarely available at the time of resuscitation. In order to evaluate the efficacy of Hb-200 under as close as possible clinical conditions, we have also chosen in the present study a return of these clinically most commonly used parameters to baseline as the endpoint of volume resuscitation, followed by a 3-h period for continued cardiovascular monitoring. Hb-200 was administered to the animals at the rate recommended by the manufacturer warning of excessive circulatory overload in case of excessively rapid fluid administration (>10 mL/kg/h, corresponding to 1.3 g/kg/h of bovine Hb).

The results of the present study indicate that animals in the study group, when compared with controls, were inadequately resuscitated from hypovolemia despite a rapid return of HR, MAP, and CVP to normal or baseline after infusion of 10 mL/kg of Hb-200. The persistence of a significantly diminished CO in the Hb-200 group throughout the postresuscitation period was a remarkable finding. Both cardiac and noncardiac factors may have been involved in mechanisms underlying the consistently lower CI observed in this group: (a) Reduced right or left ventricular filling pressures, i.e. ventricular preload might have resulted in a dimished stroke volume. The total resuscitation volume that was infused in animals of the Hb-200 group was roughly one third of the blood volume re-transfused in control animals. However, postresuscitation measurements of CVP and POP did not differ from baseline values either, indicating that low CO resuscitation did not primarily result from low filling pressures. Furthermore, the colloid oncotic pressure (COP) of Hb-200 we measured was 42 torr (Dr F.-R. Curry, personal communication), significantly higher than that of whole blood (22 torr), as reported by Rentko (1992) prior to FDA approval. The measured COP exceeds that of other commonly used colloids such as albumin 5% (24 torr) or hetastarch 6% (32 torr), making mobilization of interstitial fluids following Hb-200 administration

very likely, given that the total Hb concentration in this group did not increase after infusion of Hb-200. This supports the idea that volume under-loading was not a chief mechanism responsible for the low CO observed; (b) impaired myocardial contractility as a result of decreased coronary arterial blood flow or direct negative inotropic effects by Hb-200 might be another explanation, although there is no evidence reported indicating any impairment of myocardial function by highly purified HBOCs. Polymerized bovine Hb has been administered directly into the coronary circulation of dogs and found not to have any vasoconstrictive effect, instead improving myocardial oxygenation (Hodakowski *et al*., 1992); (c) increased total peripheral vascular resistance, evidenced by sustained to progressive increase in SVRI during the postresuscitation phase. This was most likely caused by Hb-200 and negatively influenced SVI and, thus, CO by increasing afterload. Significant decreases in CO resulting from pronounced vasoconstriction were also consistently found in dogs undergoing isovolemic hemodilution with polymerized bovine Hb (Standl *et al*., 1996, 1997; Krieter *et al*., 1997).

The purpose of the present study was to investigate the efficacy of Hb-200 as a resuscitation fluid for patients suffering from severe surgical or trauma-associated hemorrhage. A canine model of hypovolemia was chosen because it is a well-characterized model (Wiggers *et al*., 1945; Haskins *et al*., 1985; Haskins & Patz, 1990; Ilkiw *et al*., 1991) and Hb-200 is approved for use in dogs only. We decided to use a hemorrhage that decreased MAP to 50 mmHg. The average blood loss amounted to about 40% of circulating blood volume in both control (autologous blood transfusion) and study group (Hb-200 infusion). This method ensures a severe, but not necessarily lethal insult, which is comparable with many clinical situations of acute blood loss.

Because of the invasive nature of a splenectomy, it was necessary to perform this study under general anesthesia, optimized to minimize potential confounding hemodynamic effects. A constant rate infusion of fentanyl as the primary agent was chosen as at the dosages used fentanyl has minimal cardiovascular depressant effects (Lemmens, 1995; Ilkiw, 1999). To compensate for potential changes in fentanyl pharmacokinetics during hypovolemia (Egan *et al*., 1999), the rate of infusion of the drug was decreased by 50% at the end of splenectomy, i.e. prior to the equilibration period. Isoflurane, which is vasoactive, was added in a low dose, corresponding to only 0.5–0.6 times the minimum alveolar concentration of isoflurane reported in dogs (≈1.32 vol.%; Steffey, 1996), to ensure unconsciousness but prevent significant hemodynamically depressant effects (Ilkiw, 1999). Although this model may not truly represent the prehospital or emergency room situation, it certainly is similar to clinical circumstances of intra-operative blood loss and resuscitation under anesthesia. Hemodynamic stability was demonstrated in both animal groups during the prehemorrhage (equilibration) period and blood volume status was similar in both groups and adequate, as CVP and POP values indicated. Moreover, the animals showed hemodynamic responses to hypovolemia compatible with those collected in awake dogs (Haskins *et al*., 1985; Haskins & Patz, 1990; Ilkiw *et al*., 1991), and there were no significant differences in expected physiological responses between groups.

Bovine polymerized Hb shares its vasoactive effect with other highly purified stroma-free Hb solutions, independent of the chemical modification technique used for tetramerization, i.e. pyridoxilation, polymerization, conjugation, inter- and intramolecular cross-linkage, or liposomal encapsulation (Mallik & Bodenham, 1996). *In-vitro* experiments provide evidence that the Hb molecule itself interferes with physiological mechanisms that control vasomotor tone (see Spahn *et al*., 1994 for a review). It is thought that scavenging of endothelium-derived relaxing factor, nitric oxide, is the chief mechanism by which free Hb elicits vasoconstriction in both arterial and venous sites, but release of endothelin–1 and interaction with adrenoceptors and inositol triphosphate pathways may also be involved in this effect. A more recent investigation by Faivre-Fiorina *et al*. (1999), in which the authors demonstrated the uptake of Hb into guinea-pig aortic endothelial cells after transfusion of stroma-free human Hb, supports the idea that cell-free Hb may in fact cross cell membranes despite the large molecular size, and inactivate nitric oxide within the endothelial cell before it can reach its target site in the vascular smooth muscle cell. The degree of membrane penetration might vary dependent on the biotechnological make-up and affect the extent to which of a specific HBOC impairs vasomotor tone. For example, Rohlfs *et al*. (1998) who examined blood pressure responses to six different HBOCs in rats following 50% isovolemic exchange transfusion, found substantial quantitative differences in the vasopressor activity among different HBOCs. Noone *et al*. (1998) described quantitative differences in mean PAP responses to two different HBOC formulations in a swine hemorrhagic shock model. Another potential mechanism that has been associated with vasoconstriction following infusion of cell-free Hb is the oxidation of oxyhemoglobin to Met-Hb, leading to the generation of toxic oxygen metabolites that serve as nitric oxide inactivators or membrane destabilizers (Rooney *et al*., 1993). However, Met-Hb concentrations in our experiments were not significantly increased after Hb-200 administration, excluding this mechanism as a cause of vasoconstriction under the present study conditions.

We did not observe significant changes in the pulmonary vascular circulation following resuscitation with Hb-200, except a trend for a slight increase in pulmonary vascular resistance. Unlike increases in SVRI, a rise in pulmonary vasomotor tone seems to be a rather inconsistent and dose-independent finding with bovine HBOCs. Some studies in dogs describe evidence for increased pulmonary vascular resistance (Horn *et al*., 1997; Krieter *et al*., 1997), while others do not (Harringer *et al*., 1992; Standl *et al*., 1996, 1997). Nishikawa *et al*. (1992) analysed pressure-flow relationships in the canine pulmonary vasculature and found that the baseline vasomotor tone in pulmonary vessels is not regulated by nitric oxide in this species. Therefore, any vasoconstrictive effects of HBOCs in the canine pulmonary circulation appear to be mediated by nitric oxide-independent mechanisms, which may not always be affected by these products.

The persistence of low CO in the Hb-200 group after resuscitation contributed to the markedly lower oxygen delivery

in this group vs. the control. The product of CO and C_aO_2 is an indicator of bulk convective oxygen transport in the body (Jones, 1994). In our experiments, DO_2I rapidly returned from low posthemorrhage concentrations to baseline (increase by 200%) in control animals, but in dogs receiving Hb-200 DO_2I increased by only maximal 83%, being almost entirely related to the 78% increase in CI at the same time. In contrast, C_aO_2 differed less between the two groups after resuscitation, i.e. by only 10–11%. Nevertheless, an important finding of the present study is that the C_aO_2 after resuscitation with Hb-200 was significantly less than expected. Infusion of 10 mL/kg Hb-200 resulted in a plasma Hb concentration of 2.9 g/dL (cf. Table 2), which should have produced a 3.7-mL/dL (or 23%) increase of C_aO_2, as derived from a simplified version of the standard blood oxygen content calculation equation ($C_aO_2 = (S_aO_2) \times (1.32\ [Hb_{bov}])$, where S_aO_2 is the Hb oxygen saturation, Hb_{bov} the measured free plasma Hb concentration, and the constant 1.32 the theoretical oxygen capacity of 1 g of bovine Hb [Hüfner number; Kasper *et al.*, 1998; Jahr *et al.* 2000a). Instead, the increase in C_aO_2 amounted to only 4.3% postresuscitation. A similar discrepancy between estimated and actually observed gain in oxygen-carrying capacity has been described previously in human patients undergoing preoperative hemodilution with HBOC-201 (Hemopure®, Biopure), the proposed human oxygen carrier (Kasper *et al.*, 1998). Based on results of the direct oxygen content measurements and the lack of increase in total Hb following resuscitation, it appears then that Hb-200 indeed exerted a pronounced colloid oncotic effect that might have offset the gain in oxygen-carrying capacity by hemodilution as a result of intra-vascular fluid shifts.

Our findings of a persistently diminished CO and oxygen transport capacity following resuscitation with Hb-200 coincide with effects of diaspirin cross-linked Hb (DCLHb) studied in a swine hemorrhagic shock model (Van Iterson *et al.*, 1998). In this study, low-volume resuscitation with DCLHb (5 mL/kg), which shares important properties with Hb-200 (P_{50}: 33 torr; COP: 43 torr), also restored MAP to prehemorrhage level as a result of increased systemic vasoconstriction without improving CO or systemic oxygen delivery. However, our data contrast to some extent with results reported in two other canine studies of acute hemorrhage (Bosman *et al.*, 1992; Harringer *et al.*, 1992), in which CO of animals treated with a bovine HBOC returned to prehemorrhage level. Methodological differences between those and our study might account for the discrepancy in hemodynamic outcome observed. For example, experiments were carried out in nonsplenectomized dogs, which probably have released stored red blood cells during and after hemorrhage as Htc never declined, and HBOC solutions used, although from the same manufacturer, had different properties (Hb 11 g/dL; P_{50} 17–23 torr; COP 20 torr; Osmolality 250 mOsm/kg) than Hb-200. Moreover, in the study of Harringer *et al.* (1992) resuscitation volume (average 31 mL/kg HBOC plus 44 mL/kg saline) and data acquisition protocol (recording of baseline and shock values under isoflurane anesthesia, but postresuscitation values measured in the awake animal) were substantially different. Nevertheless, like in our experiments, in those studies C_aO_2 did not increase in either study postresuscitation but, instead, continued to decline despite the much larger volume of HBOC administered (31–32 mL/kg).

Regardless of the significantly lower oxygen transport capacity in the Hb-200 group, we did not find evidence for persistence of global tissue hypoxia in this as compared with the control group. This finding also coincides with results collected previously in the other two canine hypovolemia studies (Bosman *et al.*, 1992; Harringer *et al.*, 1992). All measured acid–base parameters in arterial blood returned in both treatment groups to or near baseline following resuscitation. In order to maintain oxygen consumption, tissues in HBOC-treated dogs markedly increased extraction of oxygen from arterial blood; this is evident from the significantly lower C_vO_2 in this group postresuscitation as compared with controls. One might therefore speculate that either blood flow at the tissue level was less compromised than increased SVRI indicated, or that, despite increased SVRI, the diffusive component of oxygen transport, i.e. the capillary oxygen transport to the cells (Jones, 1994), was improved after infusion with Hb-200. Based on *in-vitro* experiments with bovine Hb (Page *et al.*, 1998), presence of only 10% of total Hb in the plasma space would result in increased oxygen transport efficiency comparable with half of that collected with pure HBOC solution. Thus, it is conceivable that the oxygen transport efficiency and hence peripheral tissue oxygen tension was significantly augmented in dogs of the study group, in which 25% of total Hb was carried cell-free in the plasma. The enhanced tissue oxygen tension may have potentially resulted in autonomic down regulation of the cardiovascular system despite of inadequate volume repletion. Finally, there is the remote possibility that scavenging of nitric oxide by the HBOC resulted in a significant shift of the baroreceptor-cardiac reflex function causing a reduction in HR with increasing arterial pressures despite decreased circulating volume, as has been observed after nitric oxide synthase inhibition in rats (Minami *et al.*, 1995).

In summary, Hb-200 (bovine) solution, when administered to hypovolemic dogs in a resuscitation volume : blood loss ratio of 1:3, was associated with a return of HR, MAP and CVP, and acid–base status to prehemorrhage concentrations. Hb-200 did not restore CO or oxygen transport capacity, nor did it increase C_aO_2. These results emphasize (1) the importance of adequate volume repletion in hypovolemic patients and (2) the insufficiency of clinically commonly used criteria (HR, MAP, CVP) to guide transfusion therapy, particularly when HBOC with significant vasoconstrictive action are used.

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