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THE ROLE OF NITRIC OXIDE SCAVENGING IN HEMOGLOBIN-BASED OXYGEN CARRIER INDUCED HYPERTENSION: SYSTEMIC AND MICROVASCULAR EFFECTS

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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August 2014

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Abbreviations: HBOC = Hemoglobin-based oxygen carrier; HSA = human serum albumin; NO = nitric oxide; MAP = mean arterial pressure; HR = heart rate; SV = stroke volume; CI = cardiac index; CO = cardiac output; BSA = body surface area; ET = endothelin; IV = intravenous; IP = intraperitoneal; RBC = red blood cell; NOS = nitric oxide synthase; SEM = standard error of the mean, SpO_2 = oxygen saturation; SVR = systemic vascular resistance

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Abstract

THE ROLE OF NITRIC OXIDE SCAVENGING IN HEMOGLOBIN-BASED OXYGEN CARRIER INDUCED HYPERTENSION: SYSTEMIC AND MICROVASCULAR EFFECTS

By Alan R. Ottarson, MS

A thesis submitted in partial fulfilment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Director: Roland N. Pittman, PhD, Professor, Department of Physiology & Biophysics

The purpose of this study was to identify the effects of a hemoglobin-based oxygen carrier, HBOC-201, on the cardiovascular system. Systemic cardiovascular parameters of mean arterial pressure (MAP), pulse pressure, heart rate, and oxygen saturation, as well as vascular resistance, were examined. A murine model of the cardiovascular system and microvasculature was employed. Sprague-Dawley rats (male; 230-530g; N = 13) were anaesthetised and surgically prepared for intravital microscopy of the spinotrapezius muscle. Increasing doses of HBOC-201 (2 mg/kg, 22 mg/kg, 230 mg/kg, and 780 mg/kg) and an iso-oncotic volume control were administered to assess for a dose-response relationship. MAP displayed a significant increase from baseline for both treatment groups, with no significant difference between the two. Arteriolar diameter displayed no changes from baseline, or between treatment groups or across doses. Based on these results, the noted changes in MAP were due to hypervolemia, and not a property of HBOC-201, itself.

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Introduction

Trauma and associated hemorrhage often result in the development of hypovolemic shock and subsequent tissue and cell death due to ischemia. Many patients suffering from traumatic injury often require massive blood product transfusion in order to maintain blood pressure, tissue oxygenation, and normal coagulation status(Hess, Holcomb, & Hoyt, 2006). This massive transfusion is often defined as 10 or more units of packed red blood cells (PRBCs) in a 24-hour period, worsening the prognosis for these patients and increasing mortality. Currently, blood product transfusion is the standard of care for hemorrhaging trauma patients, along with crystalloid fluid administration to restore volume quickly(Como, Dutton, Scalea, Edelman, & Hess, 2004; Davis, Johannigman, & Pritts, 2012; Geeraedts, Kaasjager, van Vugt, & Frolke, 2009; Harris, Thomas, & Brohi, 2012; Holcomb et al., 2008). Unfortunately, many areas of the world do not have ready access to blood products, and those that do face the challenge of finding enough donors to meet the demand. Without an adequate and consistent source of human blood products, the goal of maintaining tissue perfusion may often be unobtainable.

Efficient transport of respiratory gases is imperative for the maintenance of tissue perfusion. As stated earlier, large-volume crystalloid fluid administration is used to restore volume quickly. However, it comes at a price. Without administration of additional oxygen-carrying red blood cells (RBCs), the patient will develop a condition termed isovolemic anemia. This occurs in the presence of adequate blood volume, but low or inadequate hemoglobin

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concentration(Gutierrez, Reines, & Wulf-Gutierrez, 2004). This condition has been documented as normal for certain patient populations (e.g.: renal failure), and benign in healthy volunteers, but does come with certain physiological characteristics. In a study of healthy volunteers subjected to acute isovolemic hemodilution to a hemoglobin level of 5 g/dL (normal range = 12-17 g/dL), subjects demonstrated significant increases in heart rate, stroke volume, and cardiac index ($CI = \frac{CO}{BSA} = \frac{SV*HR}{BSA}$), though hypoxia was absent(Weiskopf et al., 1998). While not detrimental to healthy individuals, these physiological changes almost certainly impact the outcome of a patient experiencing hemorrhagic shock.

The problem of supply versus demand has plagued blood product therapy since its beginning, and with growing populations, has only gotten worse. Many research groups have attempted to solve this issue by developing blood product replacements such as artificial oxygen carriers, plasma and platelets. Many of these products have extended shelf lives, wide environmental ranges, and can be mass-produced(Greenburg & Kim, 2004). These attributes make blood product replacements a desirable area of focus, though many issues have been found with those currently in development. These side effects, some of which will be discussed in detail later, serve as a roadblock for the industry, preventing many products from ever reaching the market. It is only through understanding the mechanisms behind such effects that safe and reliable replacements can be created. The main area of interest for this project involves artificial red blood cell replacements, otherwise known as hemoglobin-based oxygen carriers (HBOCs).

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Hemoglobin-based oxygen carriers are natural or synthetic red blood cell substitutes that were originally designed to enhance the transport of respiratory gases in patients suffering from hemorrhagic shock or general hypovolemia. Ideally, these solutions are shelf-stable and readily exchange oxygen with the surrounding tissue, acting as a suitable transfusion in lieu of whole blood or packed red blood cells(Hughes et al., 1996). Their designs are generally proprietary, but often include some form of crosslinking or polymerization that results in differing sizes of “particles” depending on the manufacturer (Greenburg & Kim, 2004; Sakai et al., 2000; Silverman, Weiskopf, & Planning Committee and the Speakers, 2009; Tsai et al., 2006).

In order to understand how HBOCs affect blood pressure, it is important to understand the mechanisms by which blood pressure is normally regulated. There are many systems and pathways that interact with each other and keep each other in check to achieve homeostasis. Of particular interest in this investigation, nitric oxide (NO) is a prime example of a regulatory molecule that produces vasodilation. Likewise, endogenous catecholamines produce vasoconstriction, which leads to an increase in blood pressure. It is this constant balancing act between hypotensive and hypertensive mechanisms that goes awry in the presence of an outside force, such as disease, trauma, or pharmacotherapeutics.

Nitric oxide (NO) is an important gaseous, free radical molecule that is involved in many processes as a messenger(Lowenstein, Dinerman, & Snyder, 1994). NO is synthesised endogenously by nitric oxide synthase (NOS), which is

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found in the endothelium (eNOS), neuronal system (nNOS), and as an immune system inducible isoform (iNOS). Each of these is a known constituent of their respective tissues, and has the ability to catalyze a number of reactions that generate NO from its precursor, L-arginine(Knowles & Moncada, 1994). Of particular importance to the topic at hand is eNOS, which as stated before, is found in vascular endothelial cells.

The synthesis of NO in the vascular system helps to regulate blood pressure by direct stimulation of vascular smooth muscle adjacent to the endothelium(Rees, Palmer, & Moncada, 1989; Umans & Levi, 1995). NO has an affinity for the iron molecules of endogenous heme groups that is much higher than that of oxygen and respiratory gases. This affinity leads to NO binding of heme on guanylate cyclase, activating the enzyme that leads to production of cyclic guanosine monophosphate (cGMP) and subsequent muscle relaxation(Ignarro, 1991; Lowenstein et al., 1994). Though direct muscle relaxation is a significant mechanism in the role of NO in vascular tone, it is not the only one.

Nitric oxide acts centrally on the sympathetic nervous system, as well as peripherally by upregulation of endothelin (ET) receptors. Direct injection of NO into the central nervous system has demonstrated a small, but significant vasoconstriction response that can be terminated by transection of the spinal cord (Togashi et al., 1992). Similarly, NO is thought to play a role in regulation of the potent vasoactive peptide, ET. This 21-amino acid polypeptide binds to several receptors, and derives its vasoconstrictor actions from the ET-A receptor

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subtype. Binding of endothelin to ET-A directly stimulates smooth muscle contraction, resulting in an increase in blood pressure (Redmond, Cahill, Hodges, Zhang, & Sitzmann, 1996; Rubanyi & Polokoff, 1994).

Cell free hemoglobin has the ability to scavenge nitric oxide from the blood and chemically alter their forms (methemoglobin and nitrate [NO_3^-], respectively) (Doherty et al., 1998; Huang et al., 2005; Olson et al., 2004; Smani, Fifre, Labrude, Vigneron, & Faivre, 2007; Tsai et al., 2006; Yu et al., 2010). This scavenging and subsequent loss of the vasoregulatory agent, NO, leads to marked increases in blood pressure, one of the side effects that has kept HBOCs from being introduced to the market(Alayash, 1999; Angele, Schneider, & Chaudry, 2008; Fitzpatrick, Savage, Kerby, Clouse, & Kashyap, 2004; Olson et al., 2004; Tsai et al., 2006; Yu et al., 2010). In the case of major traumatic hemorrhage, it has been found prudent to titrate volume replacements to a systolic blood pressure of 90-100 mmHg in order to decrease instances of bleeding while maintaining perfusion. Systolic blood pressures of greater than 100 mmHg are detrimental to hemostasis, and do not enhance vital perfusion already taking place. Hence, HBOC-induced blood pressure changes currently make this an unsuitable treatment for this patient population (Davis et al., 2012; Harris et al., 2012; Hess et al., 2006; Moore, Johnson, Cheng, Masuno, & Banerjee, 2005).

Given the different sizes and compositions of each HBOC, it is currently unknown as to the full extent of these differences in terms of physiological effects and pharmacokinetics. In 2000, Sakai *et al* demonstrated that HBOC particle size

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affected arterial constriction and hypertension, while a study by Tsai *et al* (2006) showed inconsistency in hypertensive effects based on HBOC molecular configuration (Sakai et al., 2000; Tsai et al., 2006). Therefore, it is not unreasonable to conclude that size affects the distribution of HBOCs, possibly affecting their mechanism of action.

The specific HBOC of interest in this study is a bovine hemoglobin glutamer-250 known as HBOC-201. It is manufactured by the Biopure Corporation, now known as OPK Biotech, and consists of particles that weigh, on average, 250 kDA, or approximately four times the molecular weight of a standard endogenous hemoglobin tetramer. Its oxygen affinity (P_{50}) is approximately 36 mmHg, while that of endogenous RBC hemoglobin is 13 mmHg(Dube, Vranckx, & Greenburg, 2008; Standl et al., 2003). Oxygen affinity has a negative correlation with percent saturation. Therefore, this cross-linked bovine hemoglobin has a much lower affinity for oxygen than its endogenous counterpart.

It is our hypothesis that administration of a HBOC will directly affect blood pressure, vascular resistance, and other cardiovascular parameters. Specifically, we predict there will be an increase in mean arterial pressure (MAP), systemic vascular resistance (decreased arteriolar diameter), and possibly heart rate. We also hypothesize that oxygen saturation will decrease, as evidenced by past studies on pulse oximetry and HBOC administration (Hughes et al., 1996). These parameters will display responses directly proportional to the dose of HBOC administered, producing a standard dose-response curve.

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Methods

Subjects and Instrumentation

A murine model was employed for this investigation, with male rats (spp. *Rattus norvegicus*; Sprague-Dawley) weighing 200-500 g being sedated via initial intraperitoneal (IP) injection of ketamine (75 mg/kg) and acepromazine (2.5 mg/kg). After successful sedation, the femoral triangles, abdomen, and ventral neck were shaved and cleaned with ethyl alcohol. The subjects were placed on a normothermic (37˚C), heated conduction pad for facilitation of electrocautery. A patent airway was established via surgical tracheotomy and maintained for the duration of the experiment.

After assurance of adequate ventilation, the right or left femoral vein was surgically isolated and cannulated for access to the central venous circulation and administration of additional intravenous (IV) anesthesia. Alfaxan (alfaxalone acetate; Jurox, Inc., Kansas City, MO) was administered at a rate of 0.1 mg/kg/min to maintain general anaesthesia. A toe pinch was performed at 15-minute intervals to assess for the effectiveness of anesthesia, and levels were adjusted appropriately. The right or left femoral artery was then surgically isolated and cannulated for hemodynamic monitoring, including mean arterial pressure (MAP), pulse pressure, and heart rate (HR). A second central venous line was inserted into the right or left internal jugular vein as a route for administration of test solutions.

Surgical exteriorization and preparation of a unilateral section of spinotrapezius muscle was performed in order to isolate a segment of skeletal

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muscle vasculature. (Gray, 1973) The isolated muscle was reflected and displayed over a heated (37˚C) Lexan polycarbonate (SABIC, Riyadh, Saudi Arabia) platform for viewing by intravital microscopy. The muscle was kept moist by the addition of normal saline and a Krehalon covering, which was also used to prevent atmospheric interference. At the conclusion of each experiment, the rats were euthanized by IV administration of 0.4 mL/kg Euthasol (pentobarbital 390 mg/mL, phenytoin 50 mg/mL; Delmarva Laboratories, Midlothian, VA). All procedures and treatments were approved as part of this protocol by the VCU Institutional Animal Care and Use Committee.

Physiological Monitoring

Arterial blood pressure and heart rate were continuously measured after initial surgical instrumentation by a pressure transducer attached to the IV cannula in the carotid artery. Data for hemodynamic status were recorded using integrated software (BIOPAC Systems, Inc., Goleta, CA). Body temperature was continuously monitored by use of a rectal thermometer, and non-invasive pulse oximetry (Nonin PulseSense™, Nonin Medical, Inc., Plymouth, MN) provided data on SpO_2 and HR.

Treatment

Rats were divided into two study groups: HBOC-201 (Hemopure® bovine hemoglobin glutamer-250, Biopure® Corporation, Cambridge, MA) IV administration and Human Serum Albumin (Albumin, Human 25%, ZLB Behring

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AG, Berne Switzerland) volume control IV administration. A top-load infusion was utilized for this study. As described in previous studies, a dose-response effect was sought by sequential administration of escalating doses of HBOC-201 and HSA control. The stock solution of 25% HSA was diluted to an iso-oncotic concentration of 5.9% (Mongan et al., 2009). Doses for HBOC-201 IV administration included Dose 1, 2 mg/kg; Dose 2, 22 mg/kg; Dose 3, 230 mg/kg; and Dose 4, 780 mg/kg. HSA control doses were administered to correspond with infused volumes of HBOC-201, and included Dose 1, 0.4 mL/kg; Dose 2, 0.4 mL/kg; Dose 3, 3.8 mL/kg; and Dose 4, 13.1 mL/kg. Due to the inherent and relatively low blood volume of rats, the volumes of administered doses had to be adjusted accordingly by concentration to maintain a reasonable infusion volume. Therefore, the highest dose (13.1 mL/kg) was reduced to 6.55 mL/kg, and an undiluted stock solution of HBOC 201 was used. The subsequent control was also lowered to 6.55 mL/kg.

Rats were allowed an equilibration period of approximately 15 minutes prior to obtaining the baseline measurements and administering the first infusion. Each dose was administered at a rate of 1 mL/min, and a 10-minute interval between doses was observed.

Microscopy

In vivo microcirculatory studies were performed by intravital microscopy (Figure 1) of the skeletal muscle vasculature using an Axioimager A1m microscope (Carl Zeiss Microscopy, LLC, Oberkochen, Germany). Each rat was

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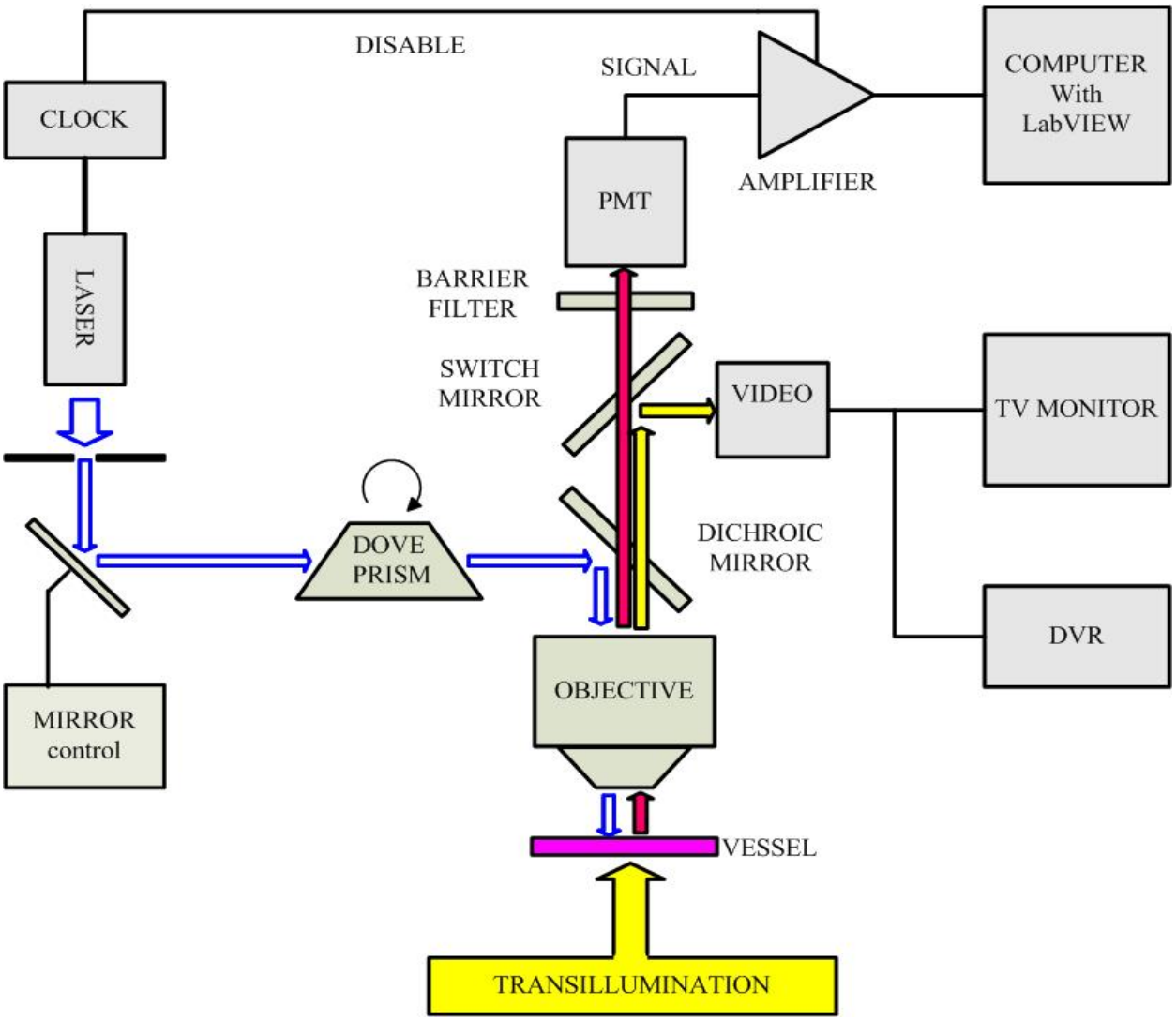

Figure 1. Intravital microscope diagram. Note: PMT was not used in this series of experiments, but remained as a part of the microscope setup. (Credit: Aleksander Golub)

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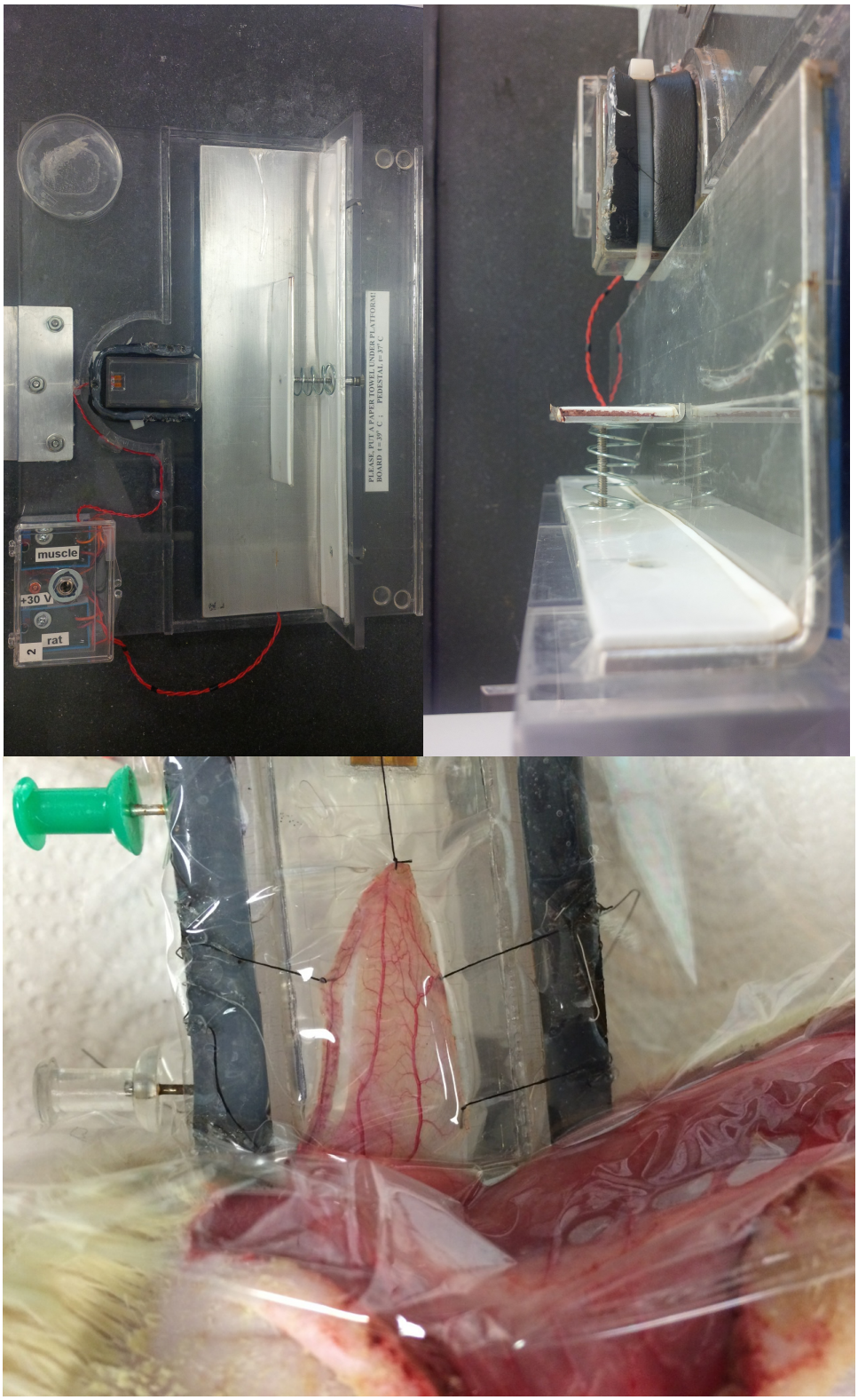

Figure 2. Thermostatic platform. Shown with temperature regulator and pedestal for muscle preparation. (Credit for muscle prep picture: Aleksander Golub)

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placed on its side on a thermostatic platform with a unilateral section of exteriorised and isolated spinotrapezius muscle reflected onto a pedestal (Figure 2) (Golub & Pittman, 2003). The muscle preparation was stretched and secured via silk surgical suture and covered with a polyvinylidene chloride film (Krehalon, Kureha America, Inc., New York, NY). The film was secured to the pedestal via a neoprene ring and pushpins.

A Krehalon "bag" and modified hypodermic needle were secured to the objective lens via wrapping with electrical tape. The needle served to provide an inlet by which an air compressor could keep the air between the bag and objective lens at a constant pressure (8-12 mmHg), and electrical tape prevented any unwanted air escape. A small amount of transparent lubricant was applied to the Krehalon cover of the muscle preparation to reduce friction between it and the objective lens "bag".

Randomly selected, isolated skeletal muscle arterioles with a diameter range of 30-80 μm were identified as sites for measurement during treatment, with three such sites identified for each rat. Baseline measurements were obtained after a short equilibration period, and included arteriolar diameter, MAP, pulse pressure, HR, and pulse oximetry (SpO_2). After administration of the treatment or control solutions, measurements were reobtained.

Statistical Analysis

Unless otherwise noted, all data are presented as mean ± standard error of the mean (SEM). An analysis of variance and F test were performed to detect

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differences within groups and across doses, while a Tukey HSD test was utilised to determine significant differences among specific points.

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Results

Systemic Parameters

Mean Arterial Pressure

Figures 3 and 4 display the relationship between dose and MAP for the two treatment groups (HBOC-201 and 5.9% HSA). Tables 1 and 2 display a summary of the data and statistical analysis for all parameters. Overall (Figure 5), there was a significant increase in MAP ($p < 0.05$) from baseline (102.70 ± 2.32 mmHg, N = 13) to the two highest doses (3.8 mL/kg: 124.07 ± 3.18 mmHg, N = 13; 6.55 mL/kg: 121.69 ± 2.59 mmHg, N = 13), but there was no difference between the two lowest doses or between the two highest doses (Table 2). For specific comparisons, the 3.8 mL/kg (HBOC-201: 130.84 ± 3.77 mmHg, N = 7; 5.9% HSA: 116.17 ± 3.07 mmHg, N = 6) and 6.55 mL/kg (HBOC-201: 125.87 ± 2.70 mmHg, N = 7; 5.9% HSA: 116.82 ± 3.97 mmHg, N = 6) doses of both treatment solutions were significantly different ($p < 0.05$) from the other groups, though not from each other.

Pulse Pressure

The relationship between pulse pressure and dose is displayed in Figure 6. Summary statistics for pulse pressure can be found in Tables 1 and 2. No significant differences were found overall from baseline (48.16 ± 2.76 mmHg, N = 13) or between the two treatment groups and across doses. Likewise, when considering the effects of treatment and dose together, no significant differences were found.

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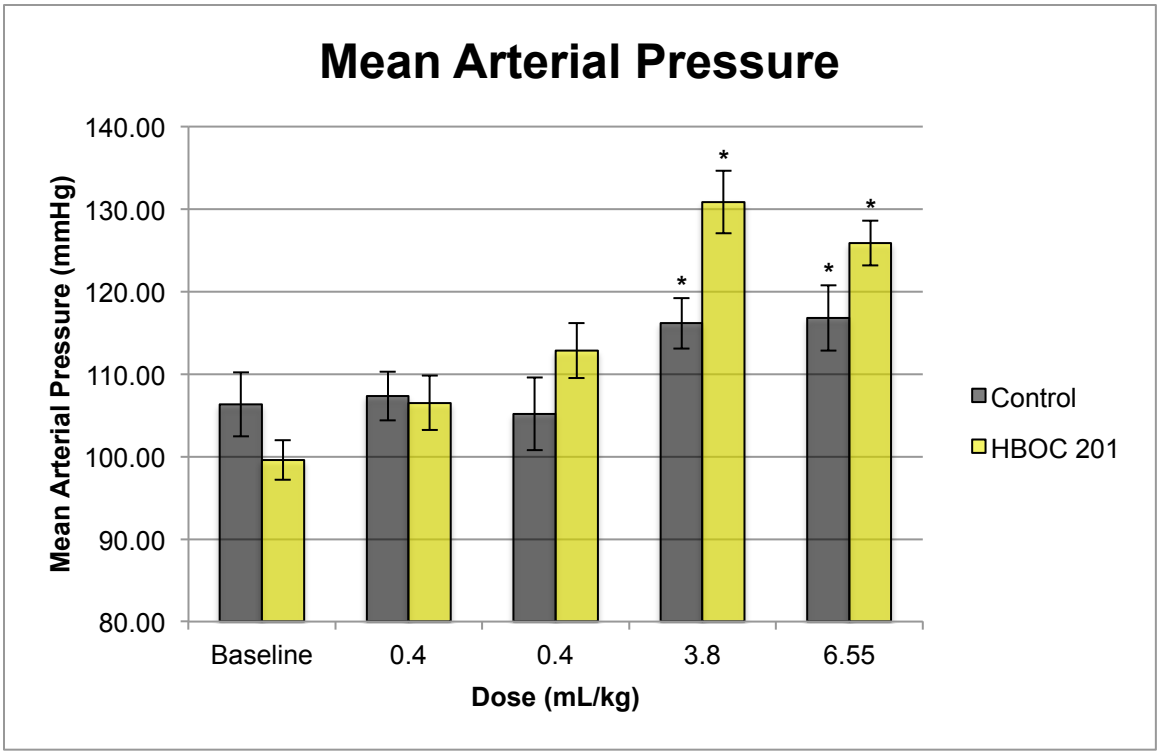

Figure 3. Dose-Response Bar Graph (MAP). HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). * denotes statistically significant difference ($p < 0.05$) from baseline.

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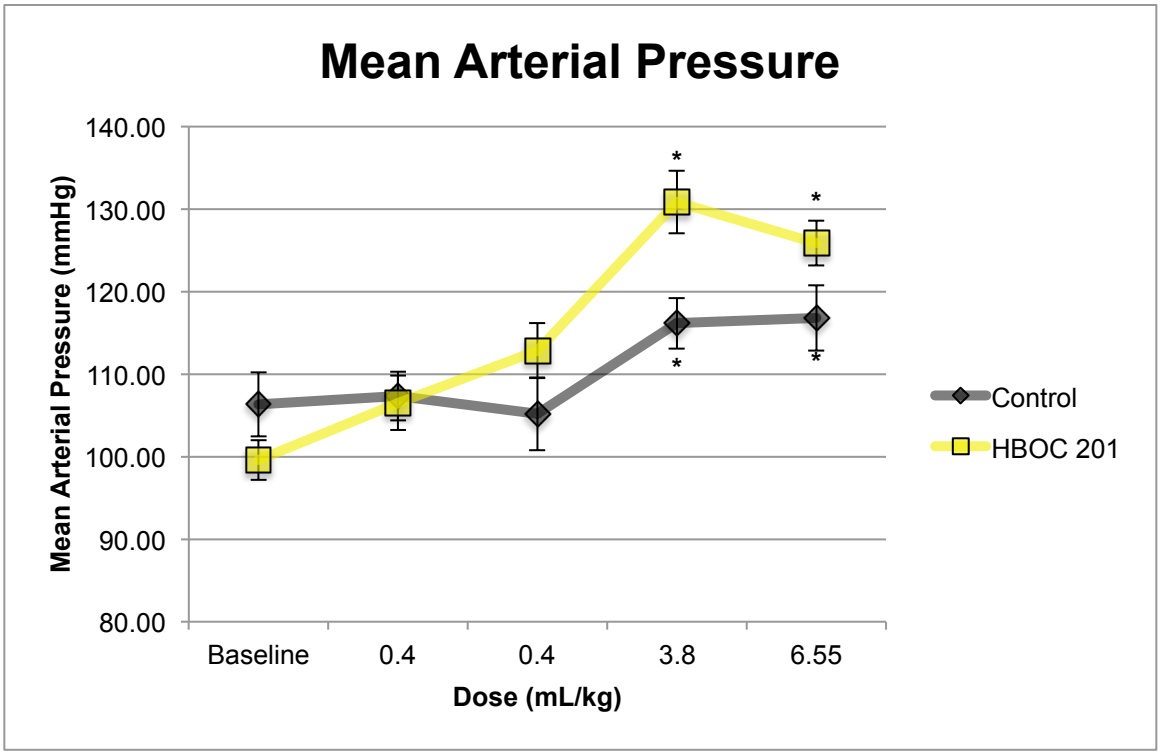

Figure 4. Dose-Response Line Graph (MAP). HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). * denotes statistically significant difference ($p < 0.05$) from baseline.

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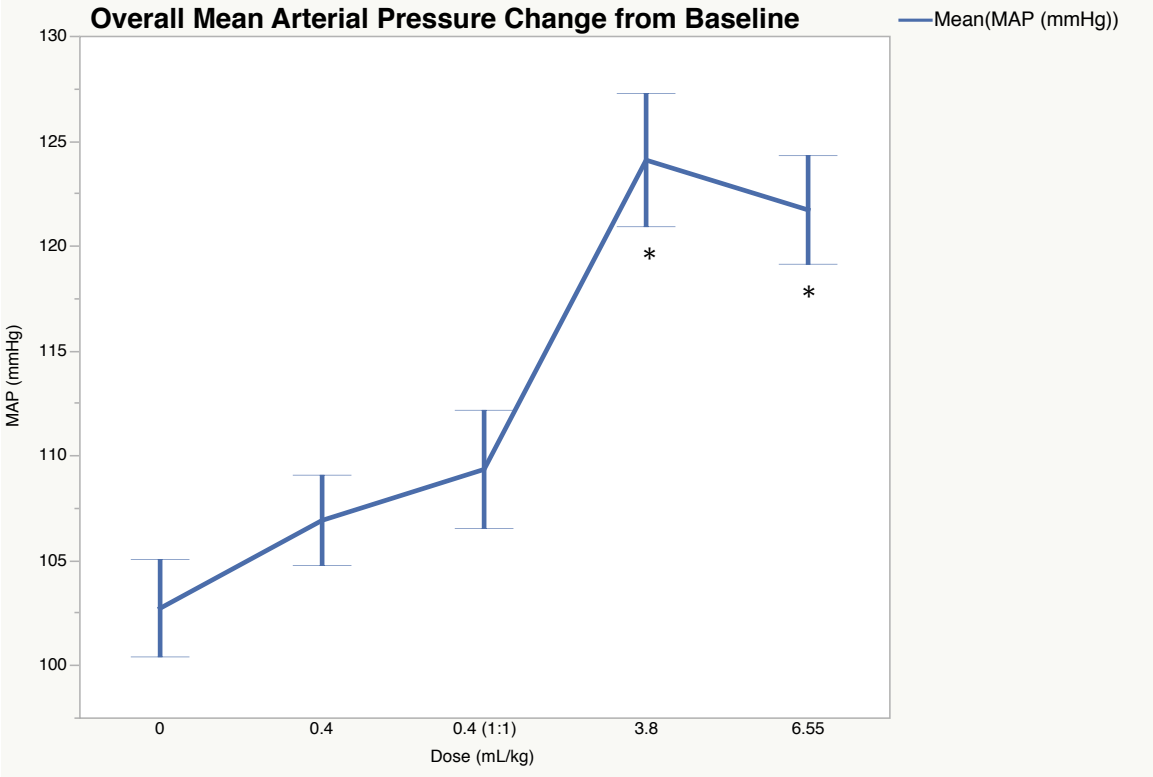

Figure 5. Combined Treatment Dose-Response (MAP). This figure represents the total mean (HBOC-201 and 5.9% HSA) change from baseline across doses. HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). * denotes statistically significant difference ($p < 0.05$) from baseline.

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			Baseline			0.4 mL/kg			0.4 mL/kg			3.8 mL/kg			6.55 mL/kg		
Parameter	**Treatment**	**N**	**Mean**	**Std Dev**	**SEM**	**Mean**	**Std Dev**	**SEM**	**Mean**	**Std Dev**	**SEM**	**Mean**	**Std Dev**	**SEM**	**Mean**	**Std Dev**	**SEM**
MAP (mmHg)	Control	6	106.33	9.50	3.88	107.33	7.26	2.96	105.20	10.82	4.42	116.17	7.53	3.07	116.82	9.72	3.97
	HBOC 201	7	99.59	6.33	2.39	106.51	8.76	3.31	112.86	8.82	3.33	130.84	9.98	3.77	125.87	7.13	2.70
Pulse Pressure (mmHg)	Control	6	49.40	13.88	5.66	50.20	16.44	6.71	52.28	14.57	5.95	56.32	17.54	7.16	57.95	19.10	7.80
	HBOC 201	7	47.10	5.88	2.22	50.04	8.25	3.12	51.17	6.66	2.52	57.79	6.61	2.50	58.29	5.42	2.05
HR (bpm)	Control	6	319.50	45.64	18.63	316.33	47.97	19.58	324.50	45.21	18.46	338.00	49.80	20.33	381.33	38.78	15.83
	HBOC 201	7	306.86	52.75	19.94	311.43	55.87	21.12	313.43	54.88	20.74	311.93	47.49	17.95	345.86	59.82	22.61
SpO2 (%)	Control	6	90.50	2.74	1.12	90.67	2.94	1.20	90.67	2.58	1.05	89.33	3.14	1.28	90.17	2.14	0.87
	HBOC 201	7	90.86	4.78	1.81	91.43	4.20	1.59	90.14	4.95	1.87	87.57	4.79	1.81	83.00	3.56	1.35

Table 1. Summary statistics for systemic parameters by treatment group. HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). SpO_2 = oxygen saturation

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		Baseline		0.4 mL/kg		0.4 mL/kg		3.8 mL/kg		6.55 mL/kg	
Parameter	**N**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**	**Mean**	**Std Dev**
MAP (mmHg)	13	102.70 ± 2.32	8.36	106.89 ± 2.16	7.78	109.32 ± 2.82	10.17	124.07 ± 3.18	11.46	121.70 ± 2.59	9.32
Pulse Pressure (mmHg)	13	48.16 ± 2.76	9.95	50.12 ± 3.36	12.11	51.68 ± 2.92	10.53	57.11 ± 3.40	12.27	58.13 ± 3.58	12.91
Heart Rate (bpm)	13	312.69 ± 13.31	47.98	313.69 ± 13.94	50.26	318.54 ± 13.56	48.89	323.96 ± 13.43	48.41	362.23 ± 14.56	52.49
SpO2 (%)	13	90.69 ± 1.06	3.82	91.08 ± 0.98	3.55	90.39 ± 1.08	3.88	88.38 ± 1.12	4.05	86.31 ± 1.30	4.70

Table 2. Summary statistics for systemic parameters not differentiated by treatment group. HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). SpO_2 = oxygen saturation

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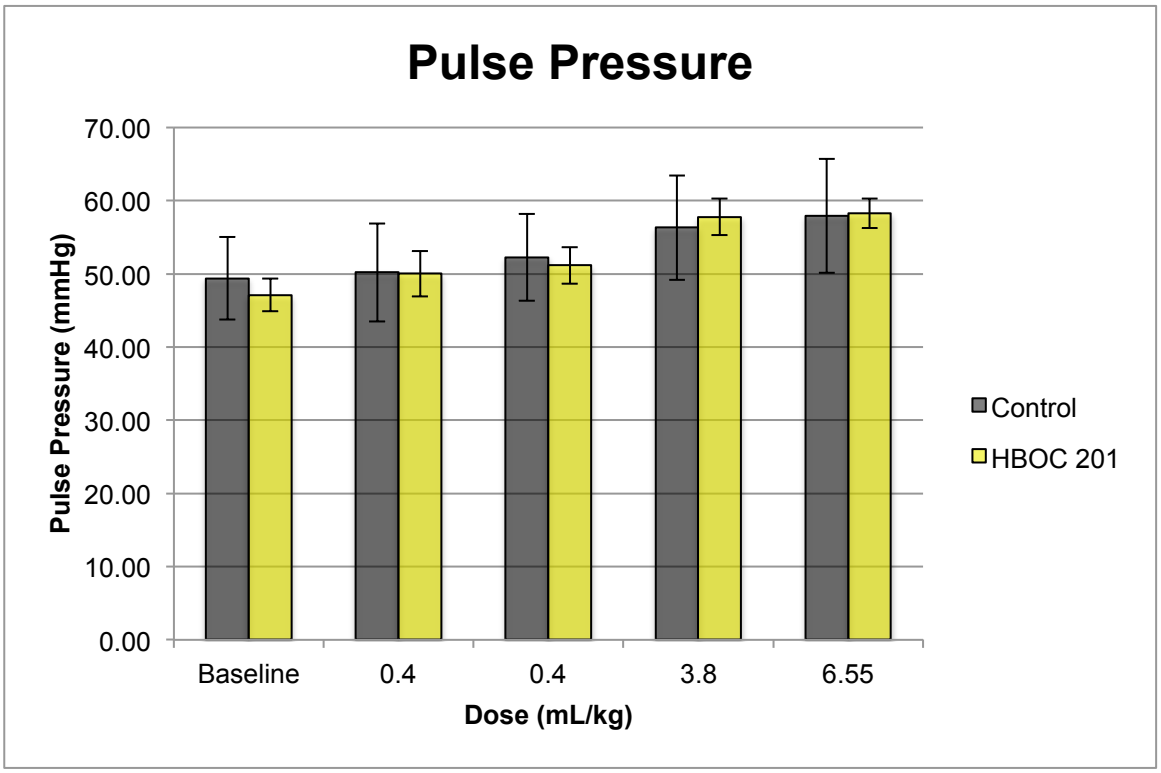

Figure 6. Dose-Response Graph (Pulse Pressure). HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). No statistically significant changes were noted in any comparisons, however, a slight upward trend can be seen in the graph.

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Heart Rate

The relationship between heart rate and dose is displayed in Figure 7. Data pertaining to heart rate can be found in Tables 1 and 2. No significant differences from the baseline (312.69 ± 13.31, N = 13) were found between the two treatment groups, or across doses. Likewise, when considering the effects of treatment and dose together, no significant differences were found.

Pulse Oximetry

The dose-response relationship between the pulse oximetry measurements and treatment is displayed in Figure 8. Tables 1 and 2 display the summary statistics for oxygen saturation (pulse oximetry). Overall, only one significant difference was noted among the doses, with the highest dose (6.55 mL/kg: 86.31 ± 1.30, N = 13, $p < 0.05$) resulting in a lower pulse oximetry reading than the other doses. Across treatment groups and doses, the 6.55 mL/kg HBOC-201 dose (83.00 ± 1.35, N = 7, $p < 0.05$) produced significantly lower pulse oximetry readings than all other doses, with the exceptions of the 3.8 mL/kg control (89.33 ± 1.28, N = 6, $p = 0.1027$) and HBOC-201 (87.57 ± 1.81, N = 7, $p = 0.4322$) doses. No other significant differences were observed.

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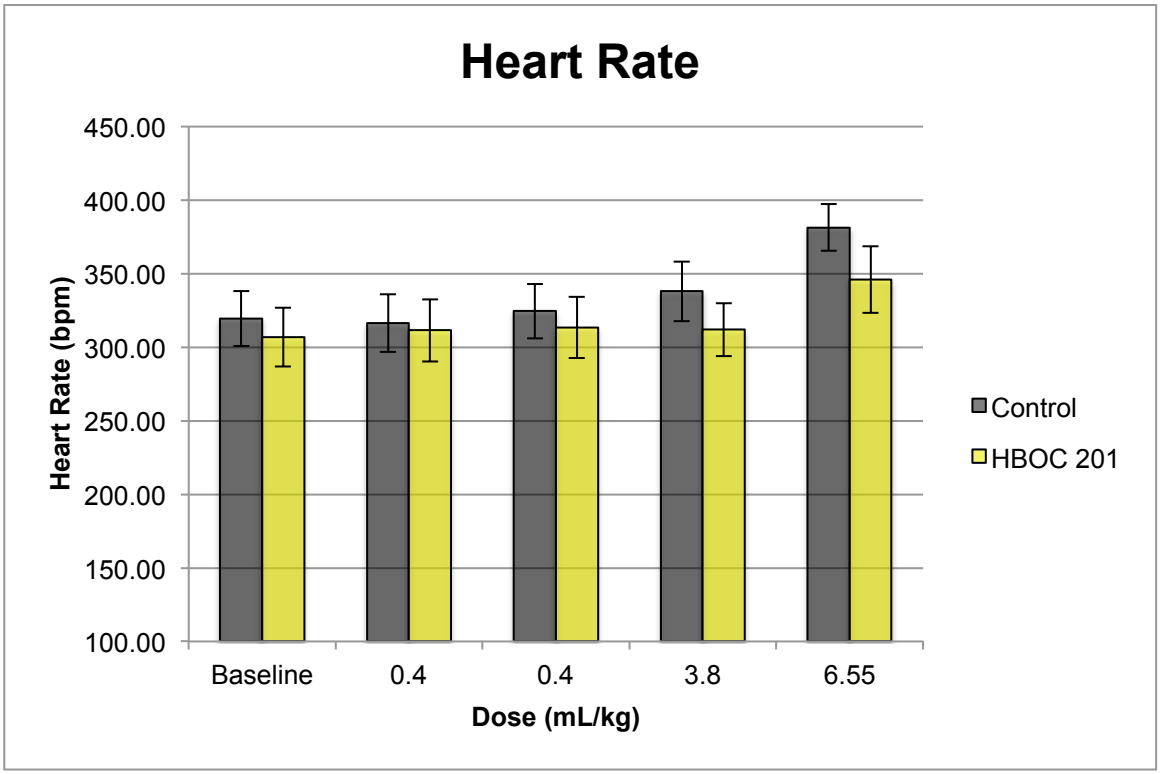

Figure 7. Dose-Response Graph (Heart Rate). HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). No statistically significant changes were noted in any comparisons, however, as with pulse pressure (Figure 6), a slight upward trend can be seen in the graph.

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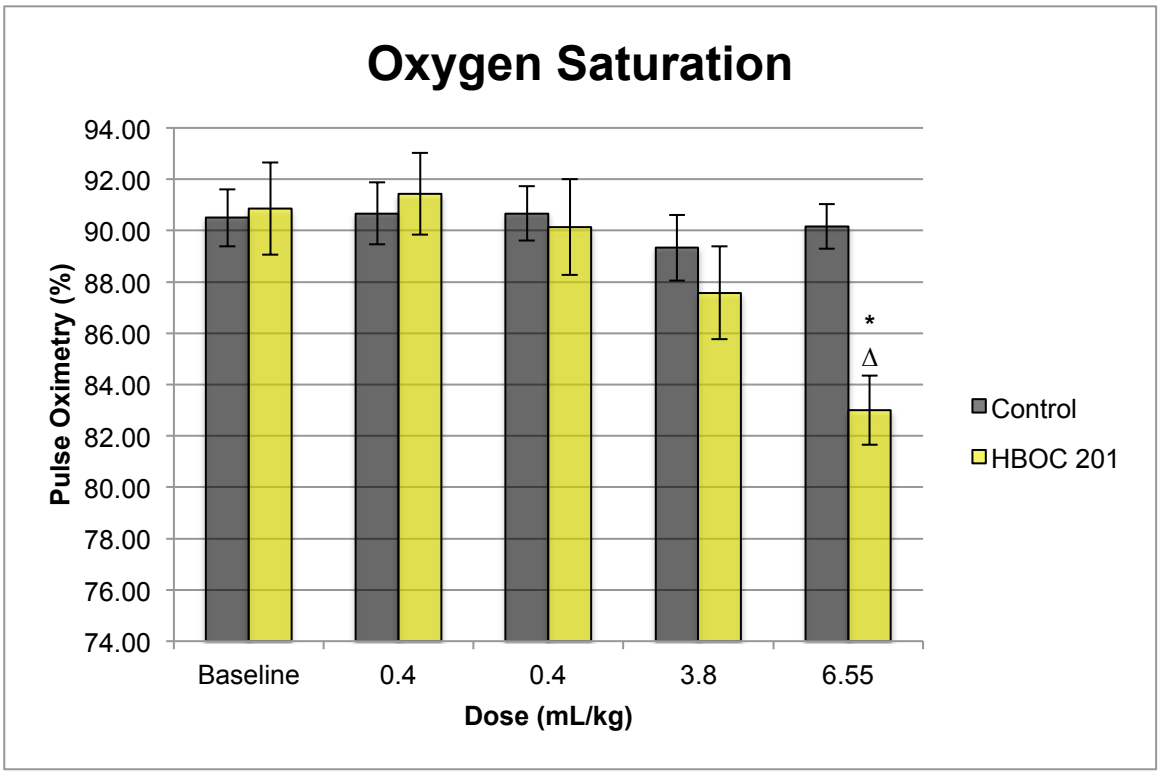

Figure 8. Dose-Response Graph (Oxygen Saturation). HBOC-201 (N = 7) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 6). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). * denotes statistically significant difference ($p < 0.05$) from baseline. Δ denotes statistically significant difference ($p < 0.05$) from control at the current dose.

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Microvascular Parameters

Arteriolar Diameter

Due to the wide range of vessel sizes (30-80 μm), data for arteriolar diameter changes are reported using percent (%) change from baseline (Figure 9). A positive change indicates an increase in arteriolar diameter, whereas a negative change indicates a decrease. Arterioles were divided into three groups, those most distal (HBOC-201: 4.40 ± 1.29, N = 12; 5.9% HSA: 1.89 ± 2.00, N = 16), proximal (HBOC-201: 4.77 ± 2.81, N = 12; 5.9% HSA: 5.86 ± 2.44, N = 16), and a distance between the two extremes (HBOC-201: 7.35 ± 2.51, N = 12; 5.9% HSA: -2.04 ± 1.75, N = 13), with each vessel being compared to others in its group. Diameter changes were also compared across location groups and doses, as well as overall treatment by itself (HBOC-201 v. 5.9% HSA). No significant differences in arteriolar diameter were noted across any groups.

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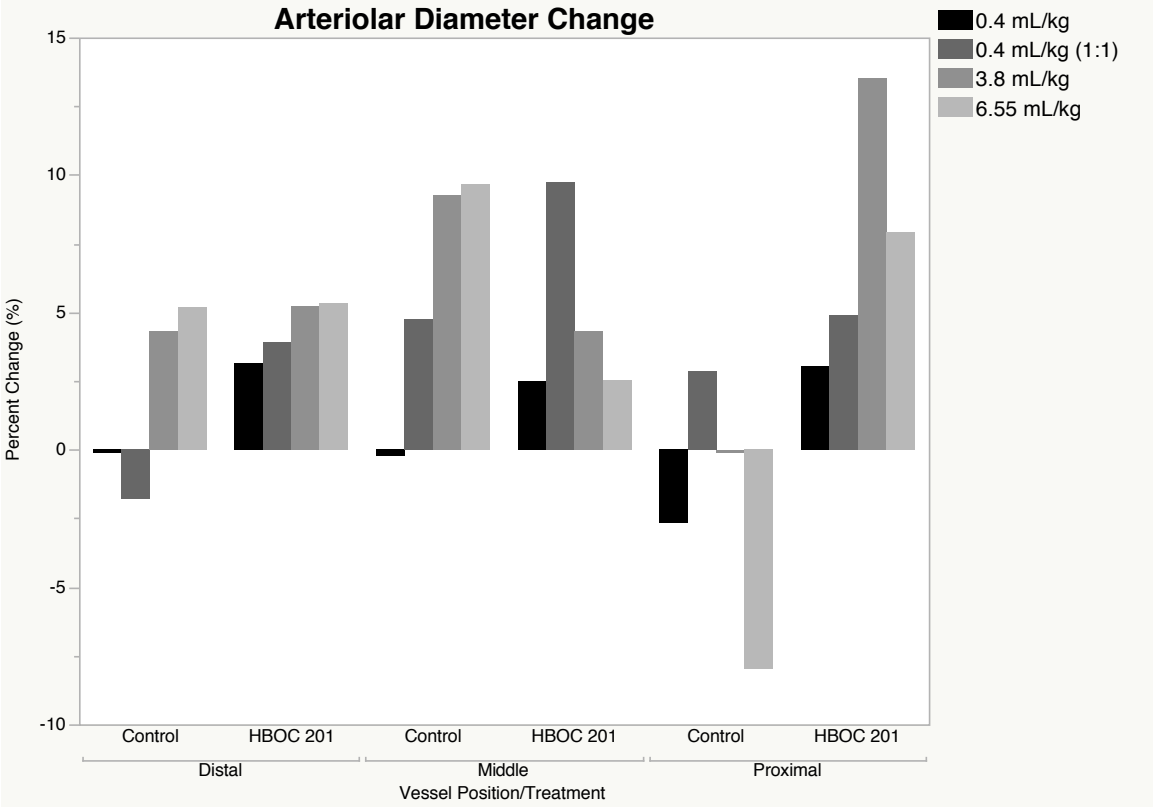

Figure 9. Dose Response Graph (Vascular Resistance). Results expressed as % change due to wide variation in vessel sizes. HBOC-201 (N = 9) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 11). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). Three vessel sites were chosen in each muscle preparation, with N = 4 (5.9% HSA) and N = 3 (HBOC-201). No statistically significant differences were noted from baseline, or between treatment groups.

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Discussion

The results of this study suggest a plateau in the effect of HBOC-201 in regard to dose-response. As the dose of HBOC-201 increased, the MAP also increased, but only to a certain point. This serves to demonstrate a sigmoid curve rather than a direct linear relationship between dose and cardiovascular response. While very little statistical significance was found, there is an obvious trend in the data for MAP that suggests a closer association between dose and pressure. Results for the second lowest dose of HBOC-201, while not statistically significant, appears to produce a higher MAP than the HSA control. This is consistent with recent, similar studies that suggest no significant difference in MAP between the control and HBOC treatments (Song et al., 2013; Song, Nugent, Moon-Massat, & Pittman, 2014).

No statistically significant differences were found with either the pulse pressure or heart rate parameters; however, slight trends were noticed in the graphed data. A slight and gradual increase in both parameters can be seen, though both the HSA control and HBOC-201 groups tended to mimic each other in magnitude of response. One point of interest is the fact that the average heart rate appeared consistently higher in the HSA control group than in the HBOC-201 group, though not significantly so.

Pulse oximetry was the only other parameter to show a significant change. The HSA control appeared to stay fairly consistent throughout the experiment with regard to SpO_2, while the HBOC-201 treatment group showed a dramatic and obvious decrease after the two highest doses. These data are consistent

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with previous studies showing a decrease in oxygen saturation after administration of a cell-free HBOC solution(Hughes et al., 1996). The artificially decreased affinity for oxygen of HBOC-201 appears to be the cause of lower pulse oximetry readings, however, previous studies have shown a much lower magnitude of change compared to the results described here. The use of a murine model may have contributed to the increased magnitude of change seen in this study, but further research will have to be performed in order to determine this effect.

Interestingly, as stated before, there was an observed lack of arteriolar response to HBOC-201 administration. Coupled with the lack of change in heart rate, it is curious to note the much more obvious increase in MAP. Control of MAP can be reduced to three main components: cardiac output, systemic vascular resistance (SVR), and blood volume. This model attempts to control for, and measure, each component of this system in some way. Cardiac output can be determined by multiplying heart rate and stroke volume, of which we directly measured heart rate in two ways (non-invasive pulse oximetry and arterial catheter placement). Systemic vascular resistance was directly measured by intravital microscopy of skeletal muscle arterioles, which leads to indirect calculation of regional blood flow, even though flow was not directly measured. Finally, blood volume changes were accounted for by administration of a control solution of iso-oncotic 5.9% human serum albumin to prevent fluid shift into or out of the interstitial space after administration. The 5.9% HSA was also used as the diluent for varying the HBOC-201 concentrations.

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Based on the results of this study, and the lack of change in several parameters that directly affected blood pressure, we are led to infer that an increase in stroke volume may have occurred that resulted in an overall increase in MAP. Cardiac output was not directly measured in this set of experiments, though the placement of a flow probe or thermodilution catheter may provide better insight into the cause of the blood pressure increase(Phillips et al., 2012; Swan et al., 1970).

As originally stated, several doses of HBOC-201 were used, representing 2, 22, 230, and 780 mg/kg. While the doses were converted to a consistent volume relative to the HSA control solution, one variable remained unaccounted for. One central venous catheter was used for administration of each dose, with each syringe being replaced by the next with a higher dose. Unfortunately, the dead space in the catheter and hub was not insignificant, accounting for approximately 0.6 mL of volume. The average volume of the first infusion was 0.14 mL, and the average total volume infused was 3.95 mL. Inadvertent and unavoidable inter-infusion doses of 0.6 mL occurred with each syringe change, adding a total of 1.2 mL to the total dose. These inter-infusion doses were consistent across all subjects, occurring with approximately the same volume and time between each syringe change. Unfortunately, it is currently unknown if or how these extra boluses affected the above-described parameters.

In brief, the results of this study demonstrate that HBOC-201 does not have any more effect on several cardiovascular parameters than an iso-oncotic volume control. This is in direct contrast with previous studies using many

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different models, (Caron, Malfatti, Aguejouf, Faivre-Fiorina, & Menu, 2001; Freilich et al., 2009; Vogel, Dennis, Cassidy, Apstein, & Valeri, 1986; Winslow et al., 1998; Winslow, 2004) though compared to systemic studies, few microvascular studies have been performed to shed light on this issue. These results do correspond, however, to similar, recently published studies on rat mesentery and skeletal muscle microvascular effects(Song et al., 2013; Song et al., 2014). The lack of difference between the HBOC-201 and iso-oncotic volume control seems to point to volume expansion as the main culprit behind the hypertensive effect, and not a property of HBOC-201, itself. As the NO scavenging hypothesis relies on this specific property of HBOC-201, it is clear that this effect is minimal at best, if present at all. Several past studies utilized other volume controls, such as isotonic or hypertonic crystalloids and colloids, similar to those used in clinical practice. This, however, allows for much broader variation in fluid dynamics in the body, as these fluids may rapidly leave the vascular space, which an endogenous iso-oncotic fluid would likely avoid (Mazzoni, Borgstrom, Arfors, & Intaglietta, 1988; Quinlan, Martin, & Evans, 2005).

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Appendix

Arteriolar Diameter (% Change)	Treatment	N	0.4 mL/kg			0.4 mL/kg (1:1)			3.8 mL/kg			6.55 mL/kg		
			Mean	Std Dev	p-value	Mean	Std Dev	p-value	Mean	Std Dev	p-value	Mean	Std Dev	p-value
Distal	Control	4	-0.12 ± 1.73	3.47	0.93	-1.81 ± 1.90	3.79	0.90	4.32 ± 6.93	13.86	1.00	5.18 ± 3.69	7.39	1.00
	HBOC 201	3	3.13 ± 2.56	4.43		3.91 ± 2.94	5.09		5.24 ± 3.24	5.61		5.32 ± 3.08	5.33	
Middle	Control	4	-0.24 ± 3.30	6.61	0.97	4.75 ± 4.54	9.07	0.94	9.27 ± 5.44	10.88	0.98	9.67 ± 6.05	12.10	0.96
	HBOC 201	3	2.50 ± 2.54	4.40		9.74 ± 7.18	12.44		4.32 ± 3.53	6.11		2.51 ± 9.25	16.02	
Proximal	Control	3	-2.68 ± 1.55	3.09	0.58	2.87 ± 2.52	4.37	1.00	-0.11 ± 0.34	0.59	0.49	-8.00 ± 6.06	10.50	0.55
	HBOC 201	3	3.05 ± 2.45	4.25		4.90 ± 2.99	5.17		13.54 ± 4.61	7.98		7.92 ± 8.43	14.60	
Average	Control	11	-1.02 ± 1.27	4.40	0.49	1.85 ± 1.95	6.47	0.50	4.91 ± 3.14	10.42	0.56	3.22 ± 3.55	11.76	0.47
	HBOC 201	9	2.89 ± 1.26	3.79		6.18 ± 2.56	7.69		7.70 ± 2.41	7.24		5.25 ± 3.80	11.40	

Table 3. Summary statistics for vascular resistance. HBOC-201 (N = 9) doses converted to mL/kg to correspond to 5.9% HSA iso-oncotic volume control (N = 11). Concentrations of HBOC-201 were as follows: Dose 1, 2 mg/kg (~1 μM); Dose 2, 22 mg/kg (~10 μM); Dose 3, 230 mg/kg (~100 μM); Dose 4, 780 mg/kg (~300 μM). Three vessel sites were chosen in each muscle preparation, with N = 4 (5.9% HSA) and N = 3 (HBOC-201). No statistically significant differences were noted from baseline, or between treatment groups.

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Vita

Alan Robert Ottarson was born in Gloucester, Virginia. He graduated from West Point High School in 2009 and received a BS in Biology from Virginia Commonwealth University in 2012. He entered the Master of Science program in the Department of Biology at VCU in 2012, where he began work on his thesis research under Dr. Roland Pittman in the School of Medicine's Department of Physiology and Biophysics. During his time in graduate school, Alan taught introductory biology laboratory classes for Biology majors and non-majors, and graduated in the summer of 2014.

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