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In vivo reduction of cell-free methemoglobin to oxyhemoglobin results in vasoconstriction in canines

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Abstract

BACKGROUND—Cell-free hemoglobin (Hb) in the vasculature leads to vasoconstriction and injury. Proposed mechanisms have been based on nitric oxide (NO) scavenging by oxyhemoglobin (oxyHb) or processes mediated by oxidative reactions of methemoglobin (metHb). To clarify this, we tested the vascular effect and fate of oxyHb or metHb infusions.

STUDY DESIGN AND METHODS—Twenty beagles were challenged with 1 h similar infusions of (200uM) metHb (n=5), oxyHb (n=5), albumin (n=5), or saline (n=5). Measurements were taken over 3 h.

RESULTS—Infusions of the two pure Hb species resulted in increases in mean arterial blood pressure (MAP), systemic vascular resistance index, and NO consumption capacity of plasma (all $p<0.05$) with the effects of oxyHb being greater than that from metHb (MAP; increase 0 to 3h; 27±6 % vs.7±2 %, respectively) (all $p<0.05$). The significant vasoconstrictive response of metHb (vs. albumin and saline controls) was related to *in vivo* auto-reduction of metHb to oxyHb, and the vasoactive Hb species that significantly correlated with MAP was always oxyHb, either from direct infusion or after *in vivo* reduction from metHb. Clearance of total Hb from plasma was faster after metHb than oxyHb infusion ($p<0.0001$).

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Dong Wang and Barbora Piknova contributed equally to writing the manuscript and should be considered co-first authors; Steven B. Solomon performed the experiments and helped write the manuscript; Irene Cortes-Puch made figures and helped with analysis; Steven J. Kern and Junfeng Sun did the statistical analysis; Christine Helms and Tamir Kanias did the laboratory analysis; Mark T. Gladwin, Daniel B. Kim-Shapiro, and Alan N. Schechter conceived of the experiments and helped write the manuscript; Charles Natanson designed the experiments and helped analyze the data and write the manuscript.

CONCLUSION—These findings indicate that greater NO consumption capacity makes oxyHb more vasoactive than metHb. Additionally, metHb is reduced to oxyHb post-infusion and cleared faster or is less stable than oxyHb. Although we found no direct evidence that metHb itself is involved in acute vascular effects, in aggregate, these studies suggest that metHb is not inert and its mechanism of vasoconstriction is due to its delayed conversion to oxyHb by plasma-reducing agents.

Keywords

methemoglobin; cell-free hemoglobin; nitric oxide; vasoconstriction; hemoglobin

INTRODUCTION

Cell-free hemoglobin (Hb) based blood substitutes (Hb-based oxygen carriers, HBOC) have been associated with hypertension,[1–5] myocardial infarctions,[6–10] and death.[10–12] In hemolytic diseases, cell-free Hb has been postulated to be responsible for impaired endothelial function and pathogenic abnormalities of the vasculature; however, some controversies still persist.[13–18] The exact mechanism(s) by which cell-free Hb causes endothelial dysfunction and vascular injury are unknown. Several hypotheses have been proposed: 1) Nitric oxide (NO) in the vasculature is scavenged by the ferrous (Fe^{2+}) oxygen-carrying form of cell-free Hb (oxyHb) forming ferric (Fe^{3+}) cell-free Hb (metHb). This fast and irreversible reaction results in NO oxidation to nitrate and a state of NO resistance producing systemic hypertension with potential vascular injury.[19, 20] 2) Cell-free oxyHb delivers oxygen prematurely to arterioles, resulting in systemic hypertension also with potential vascular injury.[2] 3) Cell-free oxyHb extravasates through the endothelial layer into various tissues where oxyHb either directly scavenges NO and/or oxyHb/metHb are catabolized to heme and globin, which may cause oxidative and inflammatory pathologies.[21, 22] 4) Circulating cell-free Hb is oxidized to ferryl Hb and reactive oxygen species which cause tissue injury.[1, 23, 24]

The effects of cell-free oxyHb have been extensively studied in animal models of sickle cell disease and experimental hemolysis.[4, 17, 20, 25–27] The effects of formation of metHb in the intact red blood cell have also been well characterized,[28] but the effects of cell-free metHb on vascular tone and redox reactions in animals have been less extensively studied. In two such preclinical studies, there were no significant increases in blood pressure associated with infusing cell-free metHb, but comparisons were not made to time-matched controls,[4, 25] and one of these studies ended after only five minutes.[25] At present, the *in vivo* effects of cell-free metHb have not been characterized.

The classic paradigm is that oxyHb released into plasma from RBC will undergo auto-oxidation and other oxidative reactions to form metHb and, eventually, other oxidative products (ferryl Hb, free heme, non-transferrin bound iron, etc.).[23, 29, 30] However, *in vitro* studies in human plasma suggest that high concentrations of naturally-occurring plasma antioxidants (i.e., urate, ascorbate, and glutathione) will maintain Hb in the reduced ferrous redox state.[31–33] The ascorbate levels in human plasma have been reported to be as high as ~50 μM.[34] Indeed, in patients with hemolytic diseases, such as sickle cell and paroxysmal nocturnal hemoglobinuria, and in the plasma of stored red blood cell units, most Hb remains oxyHb, suggesting active reduction and stability of the heme center in the Hb tetramer. Even though metHb formation has been implicated in the pathogenesis of heme-oxidative injury, its hemodynamic effects have not been thoroughly studied. Importantly, metHb is formed by reaction of cell-free oxyHb with various agents being developed or considered for therapeutic uses to improve NO bioavailability, including various metabolites of NO such as nitrite, Angelis salt and compounds or nitrosyl Hb.[35–37] Giving these NO donors

therapeutically in animal models of hemolysis associated with high levels of cell-free oxyHb results in high plasma levels of cell-free metHb being formed (50 to 150 μM).[35–38] The effects of high levels of circulating cell-free metHb are unknown and could be toxic and produce abnormal hemodynamic responses independent of any effect of cell-free oxyHb. To separate the effects of cell-free metHb and cell-free oxyHb, we gave pure infusions of each to different sets of animals and measured hemodynamics. We hypothesized the oxyHb would be more vasoconstrictive than metHb, as the former species would react with NO to convert it to nitrate (dioxygenation reaction) while metHb is known to react very slowly and to a minimal extent *in vivo* with NO. We found that high levels of circulating cell-free metHb has no measurable direct acute hemodynamic effects but surprisingly is indirectly vasoactive because it is reduced back to vasoconstrictive oxyHb *in vivo*.

MATERIALS AND METHODS

Experimental Design

All experiments were approved by the Animal Care and Use Committee of the Clinical Center at the National Institutes of Health. Twenty purpose-bred beagles (1–2 yr old, 10–14 kg) were studied over 3 h.

Four animals were randomized, each of 5 study days to one of four experimental groups: 1) cell-free metHb (n=5); 2) cell-free oxyHb (n=5); 3) albumin (n=5); and 4) 0.9% saline (n=5). We calculated cell-free Hb doses to produce 200 μM plasma levels at the end of a 60-min infusion. This plasma level in animals that previously received oxyHb is known to have marked hemodynamic effects.[20] Each study day, a new stock solution of metHb and oxyHb was used. To calculate the infusion rate assuming a specific blood volume of 0.08 l/kg body weight, the volume (l) of fluid to be infused within 1 h was calculated as: [0.08 (l/kg) × body weight (kg) × target plasma concentration (0.2mM) × (1-Hct)/concentration in Hb stock solution (2.3 to 3.53 mM)], where Hct is a fraction. At end of the infusion, mean (+/− SE) peak plasma metHb and oxyHb levels were 189 +/− 27 (n=5) and 188 +/− 28 μM (n=5), respectively. The volumes infused ranged from 36 to 73 ml. For controls, each study day, doses of albumin (25% Human Albumin, Talecris Biotherapeutics, NC) equivalent to the concentration (μM) and volume of cell-free Hb were infused over 1 h to one animal, as was an equivalent volume of 0.9% saline to another. Two control groups were used to account for colloid osmotic effects of oxyHb or metHb (albumin vs. 0.9% saline). Measurements during the 3-h experiment were obtained before infusions (metHb, oxyHb, albumin, 0.9% saline) started at time 0 h, during the infusions from 0 to 1 h, and for 2 h after the infusion was completed.

On the day of the study, anesthesia was induced via mask inhalation using isoflurane (1–5%) and the animals were then intubated (6 mm, Rusch, Duluth, GA) and mechanically ventilated (Servo-I, Maquet, Wayne, NJ) (fractional inspired oxygen = 50%, positive end expiratory pressure = 5 cmH_20, ventilation rate = 15 breaths/minute, tidal volume = 20 ml/kg) for the duration of the study. A high FiO_2 was used to prevent hypoxemia from any cause during sedation and mechanical ventilation. Femoral arterial (20-gauge), external jugular venous (8-French) and radial venous (18-gauge) catheters (Maxxim Medical, Athens, TX) were placed percutaneously using aseptic techniques. Foley urinary catheters (Cook, Foley 8 Fr, 55 cm) were also placed in all animals using aseptic techniques to prevent bladder distention with hydration. After catheter placement, the anesthetic gas was discontinued and continuous infusions of midazolam (2.5–5 μg/kg/min infusion) and fentanyl (0.16 μg/kg/min infusion) were initiated and maintained for the duration of the study. Animals were continuously monitored for signs of distress and the infusions adjusted appropriately according to a protocol.[39]

Data Collection

MAP was obtained from the femoral artery catheter. A pulmonary artery thermodilution catheter (7-French, Abbott Critical Care, Chicago, IL) was introduced through the external jugular vein to measure central venous pressure (CVP) and to determine cardiac output (CO). The CO was measured by the thermodilution technique with 10 ml of sterile, room temperature 0.9% saline, injected through the proximal port of the triple lumen catheter, and the drop in temperature at the thermistor distal to the injection port was then recorded. CO was calculated using a CO module (Philips Medical Systems CO module, model M1012A). Three replicates were done at each time point and the average was used if there was less than 10% variance between measures. Systemic vascular resistance index was calculated (MAP − CVP) / (cardiac index) where cardiac index (CI) = CO / weight (kg). Hemodynamic measurements (MAP, CVP, and CO) and spectrophotometric-based quantification of cell-free Hb concentration, and chemiluminescence-based assays of NO consumption were obtained at 0-, 0.25-, 0.5-, 0.75-, 1.0-, 1.5-, 2.0-, 2.5-, and 3.0-h time points from arterial lines. Laboratory measurements (complete blood count, serum chemistries, and arterial blood gas analysis) were obtained from arterial lines at 0, 1, 2, 3 h. After the study was completed, while still sedated, all animals were euthanized (Beuthanol, 75 mg/kg IV).

Preparation of oxyHb and metHb solution from whole blood

Fresh blood was drawn into heparinized tubes from canines not enrolled in these studies and then centrifuged for 20 min at 3000*g*. Plasma was removed and red blood cells were washed 3 times using PBS and used immediately for preparation of oxyHb according to protocol developed by Rossi-Fanelli et al.[40] The oxyHb was then dialyzed against PBS for at least 24 h in dialysis bags with cutoff of 10kDa, and the 100% purity of resulting oxyHb was confirmed spectroscopically and on a Sephadex G25 column. After dialysis, oxyHb aliquots were frozen in liquid nitrogen and stored at −80 °C or used to prepare metHb. Excess of potassium hexaferricyanide was used to oxidize ferrous heme of oxyHb to ferric heme of metHb. To remove potassium ferricyanide, the metHb solution was also dialyzed against PBS for at least 24 h in dialysis bags with cutoff of 10kDa, and the 100% purity of resulting metHb was confirmed spectroscopically and on a Sephadex G25 column.

Plasma Nitric Oxide Consumption and Plasma Nitrite Level Determination Assay

The ability of cell-free Hb in the supernatant to scavenge NO was measured with a previously published and validated NO consumption assay with a NO chemiluminescence analyzer (Sievers, Boulder, CO).[41, 42] Additional experimental details are summarized in the online Supplemental Methods. Nitrite was measured using standard tri-iodide chemiluminescence assay.[43]

Cell-Free Plasma Hb Levels and Spectral Deconvolution of Species

Cell-free plasma Hb was measured only from plasma from arterial blood by conversion to cyanomet Hb with Drabkin's reagent and then by spectrophotometric measurement of absorbance at 540 nm (Beckman Coulter DU 800 UV/visible spectrophotometer, Brea, CA).[41, 42] Hb concentration and spectra were also measured on an Agilent 8453 UV-visible spectrophotometer (Agilent technologies, Santa Clara, CA) with 1-cm path-length cuvettes. Concentrations of oxyHb and metHb in plasma samples were analyzed by deconvolution of the spectrum into components from standard UV-visible spectra of human Hb composed of oxyHb, metHb, and deoxyHb in PBS buffer by a least-squares method, as described previously.[44]

STATISTICS

Unless noted otherwise, change from baseline values were used to account for baseline differences among animals. Linear mixed models (SAS PROC MIXED) were used to assess the effects of treatments over time. Random effects were included to account for the repeated measurements of each animal, and standard diagnostics were used to check model assumptions (e.g. normality, homoscedasticity). Log-transformation was used when necessary. Change-point linear regressions with changes in slopes fixed at the time of cessation of infusion (1 h) were used to estimate the rates of change in MAP, NO consumption, and Hb during and following infusion. Pearson's correlations (SAS PROC CORR) were used to show the correlation of change in MAP with Hb measurements at each time point. Linear mixed models were used to assess the relationship between Hb and percent change in MAP, accounting for repeated measures. SAS version 9.2 (Cary, NC) was used. All *p*-values are two-sided and considered significant at the $p \leq 0.05$ level.

RESULTS

Vasoconstrictive properties of cell-free Hb species vs. controls

Figure 1 shows the time course of vascular pressure changes of the four study groups. The albumin and saline groups were combined since they are similar. After the cell-free oxyHb (Fe^{2+}-O_2) infusion was completed (0 to 1 h), there were until the end of the experiment (1 to 3 h) significant elevations in mean MAP ($p < 0.0001$) and SVRI ($p < 0.0001$) compared to controls (albumin and saline). Unexpectedly, after cell-free ferric metHb (Fe^{3+}) infusions, there were also over this time period elevations in mean MAP ($p = 0.05$) and SVRI ($p = 0.04$) compared to control animals. However, despite infusing similar concentrations of Hb solutions over 1 h, the metHb infusions produced significantly less of an increase in MAP and SVRI compared to the oxyHb infusions ($p = 0.006$ and $p = 0.04$, respectively). For the rest of the Results section, we will focus only on changes in MAP, not SVRI, both to avoid redundancies in presentation, and because MAP is a direct measurement of vascular pressures whereas SVRI is calculated. The serial mean values for CI and HR which make up components of the calculation of the SVRI are shown by treatment group in the online Supplemental Figure 1 for completeness.

Nitric oxide (NO) consumption potential of plasma after infusions of cell-free Hb species vs. controls

In order to better understand the weaker vasoconstrictive effect of metHb vs. oxyHb, we examined the NO consumption potential of plasma from these animals (Figure 2). This assay uses the fact that oxyHb is a very potent NO scavenger and that presence of any traces of oxyHb in plasma will result in loss of plasma NO. In practice, a chemiluminescence NO detector is used to measure changes in the steady-state NO in a bath with a NO donor present. If, with the addition of plasma, NO is scavenged, the steady state level of NO decreases which is observed as a drop in voltage in the detector of the nitric oxide analyzer (Figure 2A). This voltage drop indicates the presence of cell-free oxyHb (or potentially other NO-scavenging species such as ceruloplasmin) in plasma.[41, 45] We measured elevated NO consumption ability of plasma in samples collected after oxyHb and metHb infusions compared to controls with infused albumin and saline (both $p < 0.0001$) (Figure 2B). As expected, increase in NO consumption ability of plasma was highest with oxyHb. Unexpectedly, plasma from metHb infusions was also able to consume NO, albeit at a10-fold lower level than the infused oxyHb-containing plasma ($p = 0.009$) (Figure 2B), consistent with the decreased vasoconstrictive properties associated with metHb infusions in Figure 1. Since metHb is able to react only with very low affinity and very slowly with

NO,[46] we further pursued characterization of other Hb species present in plasma after metHb infusion.

Correlations between vascular pressure changes and levels of Hb species

Using a spectral deconvolution of absorption spectra, we quantified Hb species present in plasma. These results were used to determine the correlation between increased MAP and Hb species in plasma. We found that the "active" Hb species (more strongly correlated), was always plasma oxyHb levels, either infused directly or reduced *in vivo* (converted) in metHb-infused animals.

Infused cell-free oxyHb levels as well as the fraction in vivo oxidized to metHb and percent changes in MAP

Figure 3A shows oxyHb levels in plasma as a function of time—levels increased progressively during the 1-h infusion ($p < 0.0001$ for slope) and then monotonically decreased over the 2 h after the infusion stops ($P < 0.0001$ for slope). The concentration of cell-free oxyHb oxidized in plasma to metHb is plotted in Figure 3B and the levels of metHb progressively increased during the 1-h oxyHb infusion ($p = 0.002$ for slope) and remained elevated and unchanged during the last 2 h of the experiment. Figure 3C shows the MAP similarly increasing throughout the 3-h experiment (27% increase from 0 to 3 h, $p<0.0001$).

Correlations of MAP with infused oxyHb levels and the fraction oxidized to metHb

Next we examined if there was a correlation between oxyHb concentration in plasma and increases in MAP during infusion when levels of oxyHb were increasing (Figure 3). Using mixed models, we determined during infusion there was overall a significant positive relationship between increasing oxyHb levels and increases in MAP ($p = 0.03$ for slope) (Figure 4A, left side). Moreover, during infusion, there was at each time point studied a similar positive correlation between increases in MAP and oxyHb plasma levels (0.25, 0.50. 0.75, 1.0 h), ($r = +0.79$ to $+0.91$) (Figure 4A, right side). This strong positive correlation during the infusion occurred over a wide range of oxyHb values; near the start of the infusion (0.25 h), plasma concentrations in the five animals studied ranged from ~40 to 90 μM, and by the end of the infusion (1 h), they varied from ~90 to 250 μM. However, once the oxyHb infusion ended and oxyHb levels fell, the correlations between oxyHb plasma levels and increases in MAP at each time point measured became weaker ($r = +0.80$ to -0.06) (see online Supplemental Figure 2A right side) and overall non-significant ($p = 0.62$ for slope) (see online Supplemental Figure 2A left side). Finally, using similar analysis with mixed models, there was no significant relationship between the levels of oxyHb oxidized to metHb and percent increase in MAP throughout the experiment ($p = 0.12$ for slope, see online Supplemental Figure 3).

In summary, we found the relationship between oxyHb plasma levels and MAP was very strong during infusion of oxyHb over a wide range of plasma levels (Figure 4A, top panels). After the infusion ended and levels were decreasing, likely in part because the elimination of oxyHb from plasma became more prominent, the correlation progressively weakened and became overall non-significant (see online Supplemental Figure 2A). In contrast, metHb levels converted from infused oxyHb (product of oxyHb oxidation in plasma) were not correlated with changes in MAP throughout the experiment (see online Supplemental Figure 3).

Infused metHb levels and the fraction reduced to oxyHb and percent changes in MAP

Figure 5A shows cell-free metHb concentration in plasma as a function of time during the 3-h experiment. There is a progressive increase in plasma metHb levels during infusion (0 to 1

h) ($p < 0.0001$ for slope) and the metHb concentration in plasma monotonically decreased after the infusion stopped (1 to 3 h; $p < 0.0001$ for slope). Spectral deconvolution to determine the fraction *in vivo* reduced to oxyHb showed during the 1-h metHb infusion, oxyHb levels in the plasma progressively increased ($p < 0.0001$ for slope) and after the metHb infusion stopped, oxyHb levels continued to rise ($p = 0.03$ for slope) (Figure 5B). Figure 5C shows the time dependence of MAP changes throughout the 3-h experiment. There was an increase in MAP during and after the infusion of metHb (7% total increase; 0 to 3 h; $p < 0.0001$ for slope).

Correlations of MAP with infused metHb levels and the fraction of metHb reduced (converted) to oxyHb in vivo

Next, we looked for the presence of a relationship between infused Hb species and MAP during metHb infusions in a manner similar to that of infused oxyHb. Using mixed models, we found that there was no overall significant relationship between infused metHb plasma levels and MAP during the infusion ($p = 0.19$ for slope) (Figure 4B, left side). Moreover, there was actually a negative or very weak positive correlation between MAP and metHb plasma levels at each time point studied during infusion (0.25, 0.50, 0.75, 1.0) ($r = -0.29$ to $r = 0.14$) (Figure 4B, right side). Using mixed models, the relationship with MAP from 1 to 3 h was also non-significant when the metHb infusion was stopped and metHb levels were decreasing ($p = 0.18$ for slope, see online Supplemental Figure 2B).

The *in vivo* reduced from metHb to oxyHb plasma levels were ~10-times lower than the infused plasma metHb levels. The converted oxyHb plasma levels followed a different time course pattern than infused metHb levels, peaking 1 h after the metHb infusion stopped (the 2 h time point) and remaining elevated and not decreasing until the end of the experiment (2 to 3 h) (Figure 5A and B). The pattern of changes over time in oxyHb (reduced from metHb) at these low plasma levels (6.3 to 20.5 μM) paralleled the changes in MAP over time (Figure 5B and C). To further test this, we examined the relationship between changes in MAP and changes in converted oxyHb levels in each animal and found there was a moderate to strong positive relationship in each animal ($r = 0.97$ to $r = 0.44$) (Figure 6) that was using mixed models overall statistically significant (slope: 0.60 ± 0.13, $p = 0.01$).

In summary, the following findings indicate that the increase in MAP after metHb infusions is mediated through converted oxyHb: 1) infused metHb levels do not correlate with MAP but infused oxyHb levels do correlate with MAP; 2) as reduction of infused metHb into oxyHb progresses over time, it correlates with observed increases of MAP over the 3-h experiment; and 3) the increases in converted oxyHb plasma levels across animals are significantly related to the increases in MAP, but the increases in converted to metHb levels across animals are not significantly related to MAP.

Plasma nitrite levels after cell-free Hb solution infusions vs. controls

To determine if intravascular scavenging of NO by oxyHb (infused or converted) was responsible for the increases in MAP, we measured plasma nitrite levels in animals at several time points. Nitrite can be converted to NO and is also a biomarker for NO production by endothelial NOS.[47] The mean nitrite levels were similar in animals receiving oxyHb and metHb infusions, compared to controls ($p = 0.28$ and 0.07, respectively) (Figure 7). Furthermore, in all four treatment groups, nitrite concentration did not significantly change throughout the experiment; the concentrations in plasma ranged on average from ~120 to 250 nM throughout (all, $p > 0.05$).

Plasma total Hb levels

To confirm all animals actually received equivalent micromolar quantities of oxyHb or metHb during the 1-h infusion, we measured plasma total Hb concentrations serially. During infusion until the end of infusion, from 0 to 1 h, these two groups of animals had similar significant rate increases in plasma total Hb levels (Figure 8). However, total Hb concentration overall in plasma decreased post-infusion (from 1 to 3 h) at a faster rate in animals receiving metHb compared to those receiving oxyHb infusions ($p < 0.0001$ for the difference in slopes), indicating that cell-free metHb is cleared faster or is less stable in plasma than cell-free oxyHb.

Other chemistries

For completeness, in an online Supplement Figure 4 (Panels A–K) it is shown that there were no significant abnormalities throughout the experiment in complete blood counts (Panels A–D), serum electrolytes (Panels E–H), and blood gases (Panels I–K) and no significant differences comparing treatment groups in each of these panels (Panels A–K) to explain these results (all, p=ns).

DISCUSSION

Cell-free metHb infusions in canines produced unexpected and, to the best of our knowledge, not previously described elevations in MAP and SVRI compared to control animals (Figure 1). At similar concentrations, cell-free oxyHb produced more marked increases in vascular pressures and tone than metHb. Cell-free metHb solution infusions were not only associated with progressive increases in plasma metHb levels, as expected (Figure 5A), but formation of the reduced species—oxyHb—in plasma was observed (Figure 5B). The infused metHb level was not positively correlated with MAP, but when plotted against the amount of oxyHb present (reduced from infused metHb), MAP and oxyHb plasma levels were correlated (Figure 6). These data indicate the increase in MAP after metHb infusion is mediated through reduction of infused metHb to oxyHb, resulting in NO scavenging and increases in vascular pressure. In human plasma, reducing agents such as urate, ascorbic acid, and glutathione were shown to be responsible for the conversion of cell-free metHb to oxyHb.[48–50] In canines and humans, levels >20 μM of ascorbic acid have been reported in plasma and the processes we describe here could apply to both species.[34, 51]

We found that animals receiving cell-free metHb infusions were forming oxyHb in the plasma (Figure 5B) on a micromolar basis at a rate faster than animals that received cell-free oxyHb infusions were forming metHb (Figure 3B). This was surprising since the conversion of cell-free oxy- to metHb occurs through an auto-oxidation reaction and a dioxygenation reaction with NO (a reaction which is rate-limited by diffusion).[52–54] This reaction with reducing agents has not been established as dominating over auto-oxidation or NO reactivity. Moreover, from the time the infusions stopped until the end of the experiment, converted plasma oxyHb levels remained higher (18 to 23 μM) in animals that received cell-free metHb (Figure 5B) than the metHb levels (8 to 12 μM) in the animals that received cell-free oxyHb (Figure 3B).

If a NO deficit in the luminal space of the vasculature is causing increases in MAP, then these two variables should be strongly correlated. As expected, there was a strong correlation between the level of oxyHb in plasma and MAP levels during the 1-h oxyHb infusions, measured every 15 min over a wide range of gradually increasing plasma levels from 90 to 250 μM (Figure 4, top panels). Unexpectedly, after the cell-free oxyHb infusion ended and the plasma oxyHb levels were decreasing over the ensuing 2 h (but still in the same range, 90 to 250 μM), the correlation with MAP became non-significant. Despite this

loss of correlation after the infusion ended, the MAP continued to steadily rise over the next 2 h at the same rate as during the infusion (Figure 3C). The loss of correlation over time but continued rise in MAP could be explained in part by the fact that once the infusion stops, elimination of oxyHb from the systemic circulation becomes more prominent and the relationship between NO and oxyHb after this point becomes more complex. However, levels of oxyHb remain high enough vascularly or perivascularly to continue to increase MAP.

Based on *in vitro* experiments, the rate of oxyHb reaction with NO is limited only by diffusion, so any free NO in the plasma will be quickly scavenged in the presence of cell-free oxyHb.[54] Recently, Hall and Garthwaite reported that there is immense variability of reported NO concentrations, depending on the method used and tissue/model studied, ranging from a few pM to ~1 μM.[55] The authors based their estimate of the functional concentrations of NO on studies using soluble guanyl cyclase (sGC) as an innate NO sensor and concluded that 100 pM to 5 – 10 nM of NO could be considered as a functional NO range. However, there is one other source of NO present in the vasculature plasma-nitrite. The circulating nitrite levels varied from 100 to 250 nM levels in our animal experiments (Figure 8). Notably, even taking into account the highest estimate of 10 nM of cell-free NO in plasma from published sources[55] and plasma nitrite levels measured in our experiments, there is still a large excess of oxyHb over nitrite and NO in both of our experimental setups: in the case of oxyHb infusions, 200 μM oxyHb compared to 100 to 250 nM available NO to be scavenged; and in the case of metHb infusions, 20 μM of oxyHb compared to 100 to 250 nM nitrite available to be scavenged. Without existence of some kind of "protective compartmentalization," NO in plasma should be completely scavenged in a very short time after being released either from endothelial cells or red blood cells (RBCs)—probably on the order of milliseconds. Previous work has suggested that the extent of the effect of intravascular oxyHb on the concentration of NO at the smooth muscle decreases as the concentration of Hb increases to high enough levels.[56] The fact that vascular pressures markedly differ even with very high plasma oxyHb levels in both oxyHb- and metHb-infused animals in excess of plasma NO available to be scavenged can only be explained if the effect of cell-free Hb is not limited to the luminal space and potentially occurs more perivascularly.

One possibility is that extravasation of cell-free Hb beyond the endothelium scavenges endothelial NO within the smooth muscle layer where NO levels may be higher, or there are other effects such as oxidative changes in the tissues. Hb has a molecular weight similar to albumin which has a wide extravascular circulation.[57, 58] This extravasation of oxyHb into the extravascular circulation would explain why during the oxyHb infusion in these experiments intravascular plasma oxyHb levels and blood pressure were strongly correlated (i.e., intravascular and extravascular plasma levels should be correlated because they are similarly increasing during the infusion time). However, MAP and oxyHb levels gradually become less correlated after the infusion ends. After stopping the infusion, elimination of Hb from the intravascular space becomes a more significant factor. Because of this, post-infusion relationships between MAP and oxyHb in the extravascular and intravascular space may not be in step with one another and become more complex. This potentially explains the gradual loss of a significant correlation between MAP and plasma oxyHb levels, despite MAP still increasing.

The mechanism(s) of the multiple different toxic effects of cell-free Hb reported remain not fully understood. In terms of vasculopathy, multiple lines of clinical evidence support a role for NO scavenging by cell-free Hb in various disease processes.[59] We have recently shown in a canine model of pneumonia that, after transfusion, older blood releases *in vivo* cell-free oxyHb over days in large quantities (50 to 150 μM on average), producing pulmonary

hypertension, pulmonary vascular necrosis, and worsened gas exchange, associated with increased mortality.[60] Further, giving NO donors can prevent the vaso-occlusive disease that occurs over 7–14 days following cerebral hemorrhage.[61] This is the time when cerebral clots break up and cell-free Hb is potentially released near vascular tissue, causing vasospasm through NO scavenging. Notably, it is also possible this beneficial effect is not related to NO donors oxidizing these low levels of cell-free oxyHb but to the direct vasodilation effect of giving a NO donor. Also of note, in clinical trials, therapy with HBOCs associated with high circulating levels of modified cell-free Hb were associated with a 3-fold increased incidence of acute myocardial infarctions.[10] If there is a loss of NO-related coronary vasodilatory tone and increased systemic pressures, patients are at risk for myocardial ischemic events.[62–65] Both of these effects can potentially be precipitated by HBOCs scavenging NO. Other potentially important mechanisms, as discussed in the Introduction, mediated by oxidative reactions of metHb, may also be critical in producing these vascular toxicities.[1, 2, 21–24, 50]

In previous experiments, we showed inhaled NO, 80 parts per million in canines, which by itself has miniscule or no measureable effects on systemic blood pressure, completely eliminates by oxidizing oxyHb the hypertensive effects associated with intravascular free water-induced hemolysis and release of cell-free oxyHb.[20] Yu et al. have done experiments in knockout mice without endothelial nitric oxide synthase (eNOS, enzyme that produces NO), which are hypertensive compared to wild type animals. Notably, HBOCs increase vascular pressure in wild type mice, but completely lose this ability in these eNOS knockout mice.[25] The present study shows that cell-free metHb infusions, after being reduced to oxyHb, increase vascular pressures. Since the cell-free metHb is reduced *in vivo* in the plasma to oxyHb, and then increases vascular pressure, it is difficult to ascribe these hypertensive effects to red cell membranes or other impurities in the process of formation of *ex vivo* cell-free Hb (Figure 5B). Overall, the above data show that NO scavenging ability of the oxyHb molecule at a minimum are at least responsible for some of the hypertensive vascular effects and potentially the vasculopathies associated with cell-free Hb in various disease states.[10, 60, 61]

It should also be emphasized cell-free metHb is cleared faster and/or is less stable than cell-free oxyHb in plasma (Figure 8). This is an unexpected finding, as the classic mechanism of Hb clearance is through binding to haptoglobin and subsequent internalization through CD 163 receptor and clearance from plasma by either macrophages or liver hepatocytes.[21] This clearance mechanism is not known to discriminate between oxyHb and metHb. We therefore speculate clearance is not increased but may be due to metHb dissociating faster favoring formation of dimers and heme dissociating faster from dimers.[23, 66] With a higher percentage of dimers present, this might increase clearance by haptoglobin. Alternatively, since heme has a different absorption spectra than metHb, this could give the appearance of faster clearance given our use of spectroscopic methods to measure metHb in the plasma. The underlying reasons and exact mechanism for the faster clearance of metHb levels using spectroscopic assays from plasma requires further investigation.

We are aware of limitations to the interpretation of our findings. We are measuring short-term effects on hemodynamics by cell-free oxyHb. Although we speculate that over the long term this vasoconstrictive effect of cell-free Hb may produce permanent vascular injury, we may be observing only a short-term effect of oxyHb on a normal functioning endothelium with oxyHb scavenging endothelial NO in these experiments. Our control albumin is about the same molecular weight as Hb in its normal state, a tetramer, but some Hb could break down into dimers or monomers, making our albumin control slightly less comparable in terms of oncotic pressure to cell-free Hb.

This is the first systematic experimental study of metHb infusions that includes necessary time-matched controls. The major finding of this study is that vascular effects of cell-free metHb exist and they are related to reduction of cell-free metHb to cell-free oxyHb, likely by plasma-reducing agents. To the best of our knowledge, this finding has not been reported in any study in animals or humans. The results of this study also indicate metHb has no direct vascular effects during the time period studied and the results do not support the hypothesis that hypertension associated with cell-free Hb is directly and mainly mediated by oxidative reactions. However, in aggregate, metHb should not be considered inert and in the application of any therapies producing metHb in the plasma, this can result in potentially injurious vascular hypertensive effects by being reduced to oxyHb.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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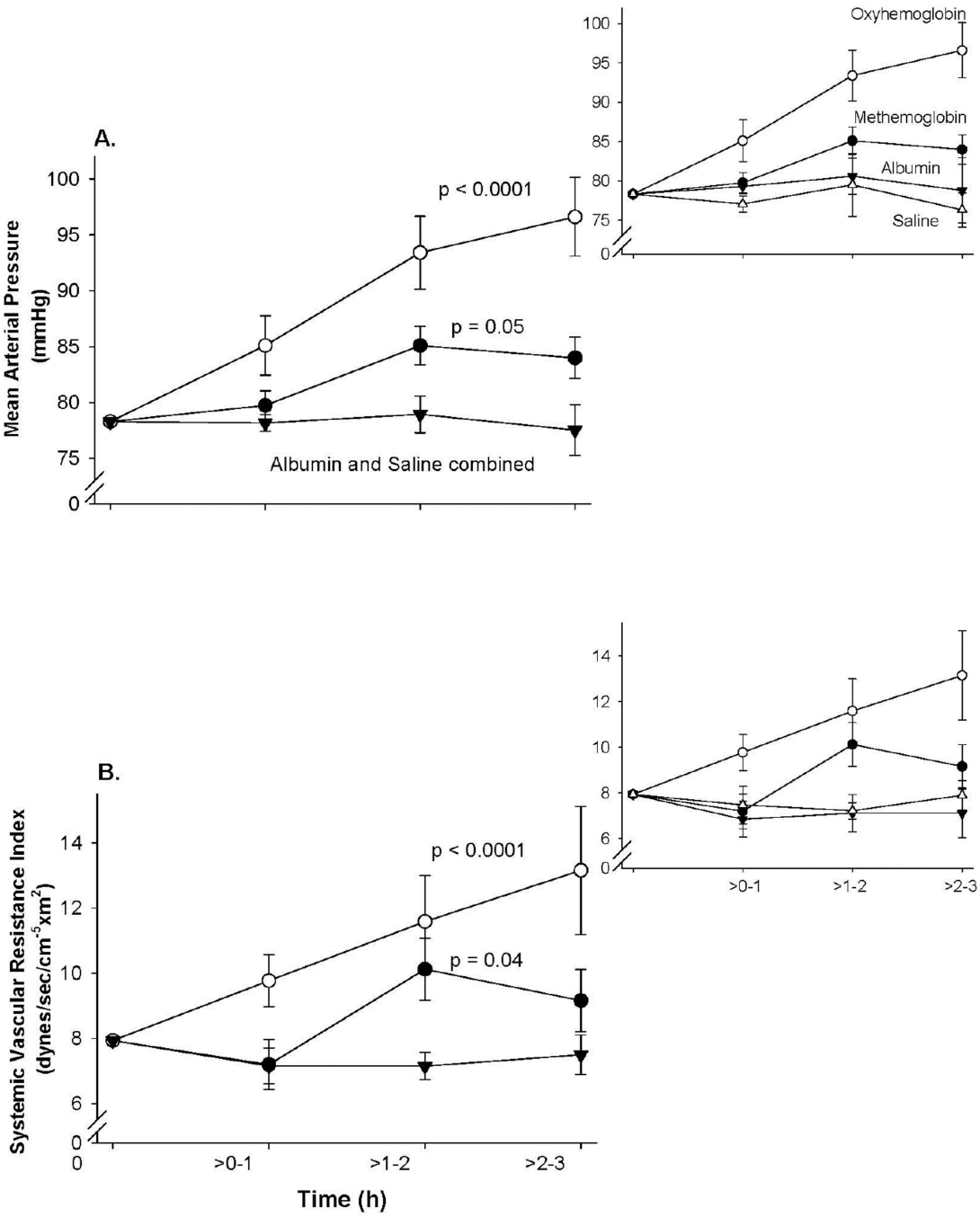


Figure 1. Changes in MAP and SVRI

Serial mean (± SE) changes in (A) MAP, (B) SVRI in animals receiving oxyHb (n=5), metHb (n=5), albumin (n=5), or saline (n=5) are plotted. Hemodynamic values are plotted from a common origin representing the mean values for all animals at time 0. The inset above and to the right shows the individual serial changes for albumin and saline controls compared to the other two treatment groups. P-value represents changes over time compared to the combined controls.

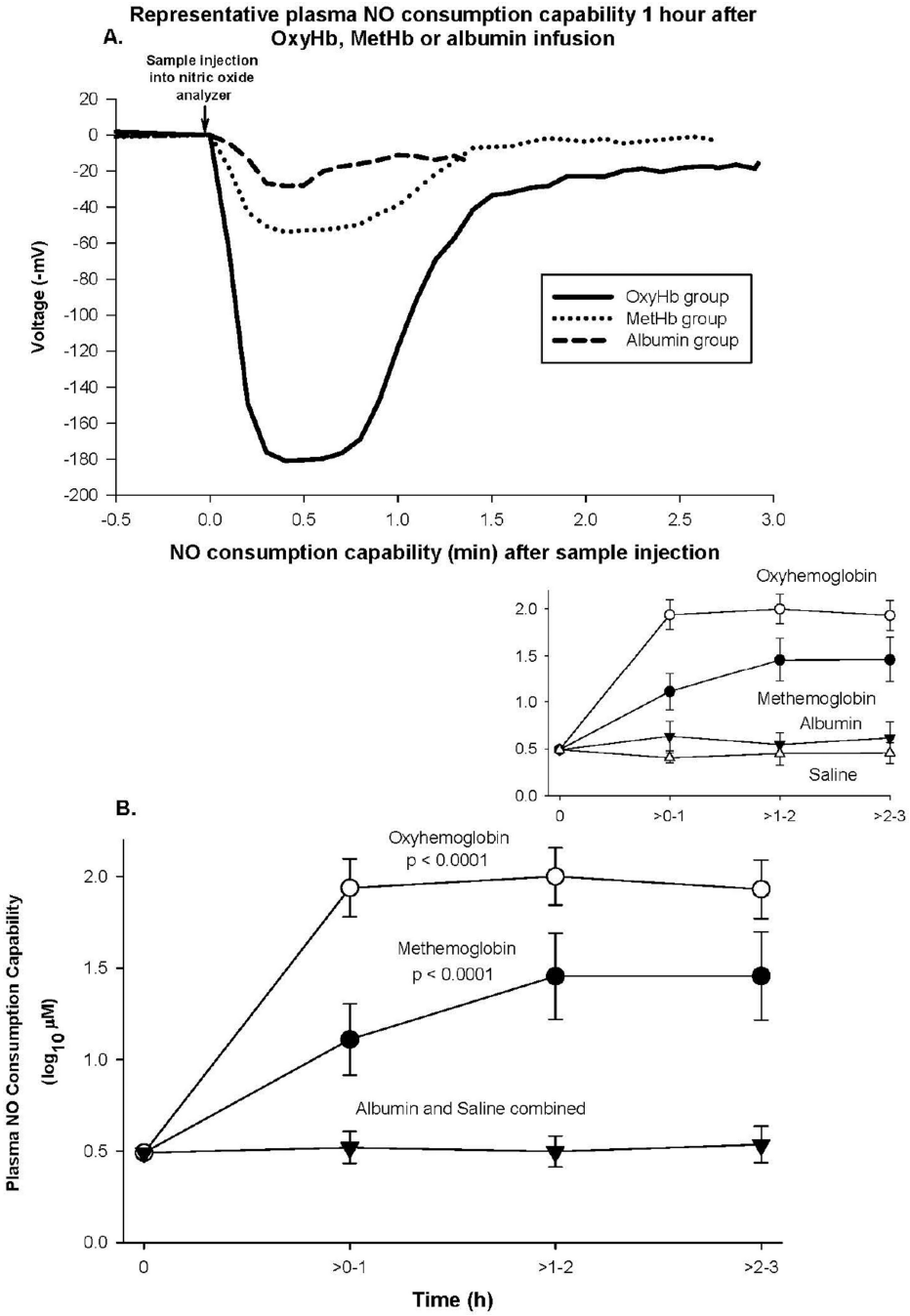


Figure 2. NO consumption

Panel A represents plasma NO consumption capability obtained from animal 1 h after infusion of various Hb species or albumin. Panel B represents the format similar to Figure 1, except now mean (± SE) log10 NO consumption capability of plasma is plotted.

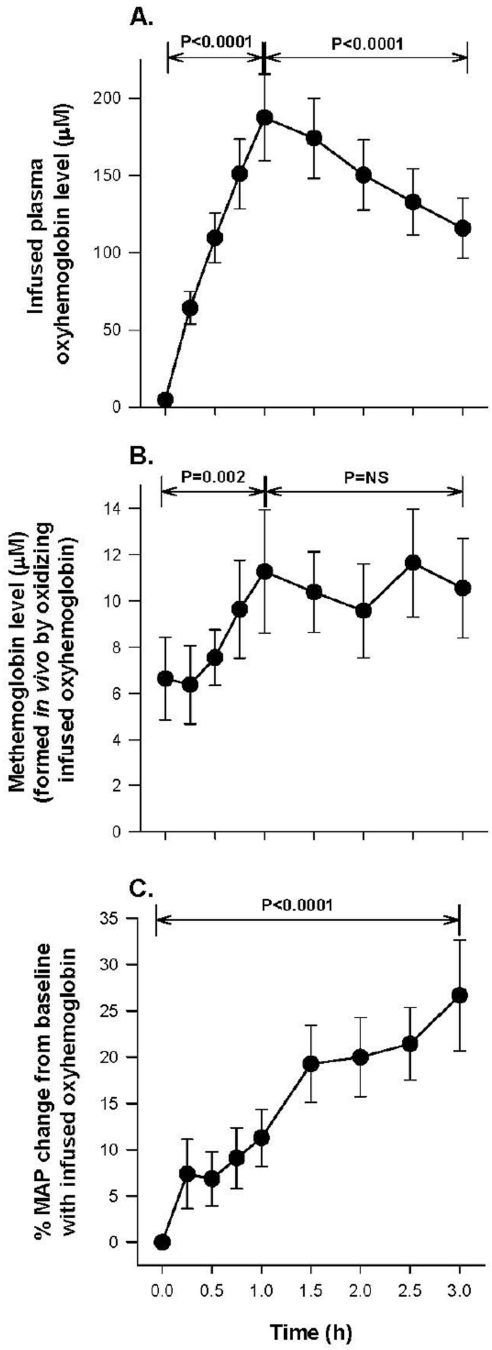


Figure 3. Hb species and percent changes in MAP during oxyHb infusions

Panel A shows serial mean (± SE) values of oxyHb levels. Panel B shows serial mean (± SE) metHb levels formed by oxidizing a fraction of the oxyHb infusion *in vivo*. Panel C shows the mean (± SE) percent increase in MAP during the oxyHb infusion. All P values compare changes over the time period indicated by brackets.

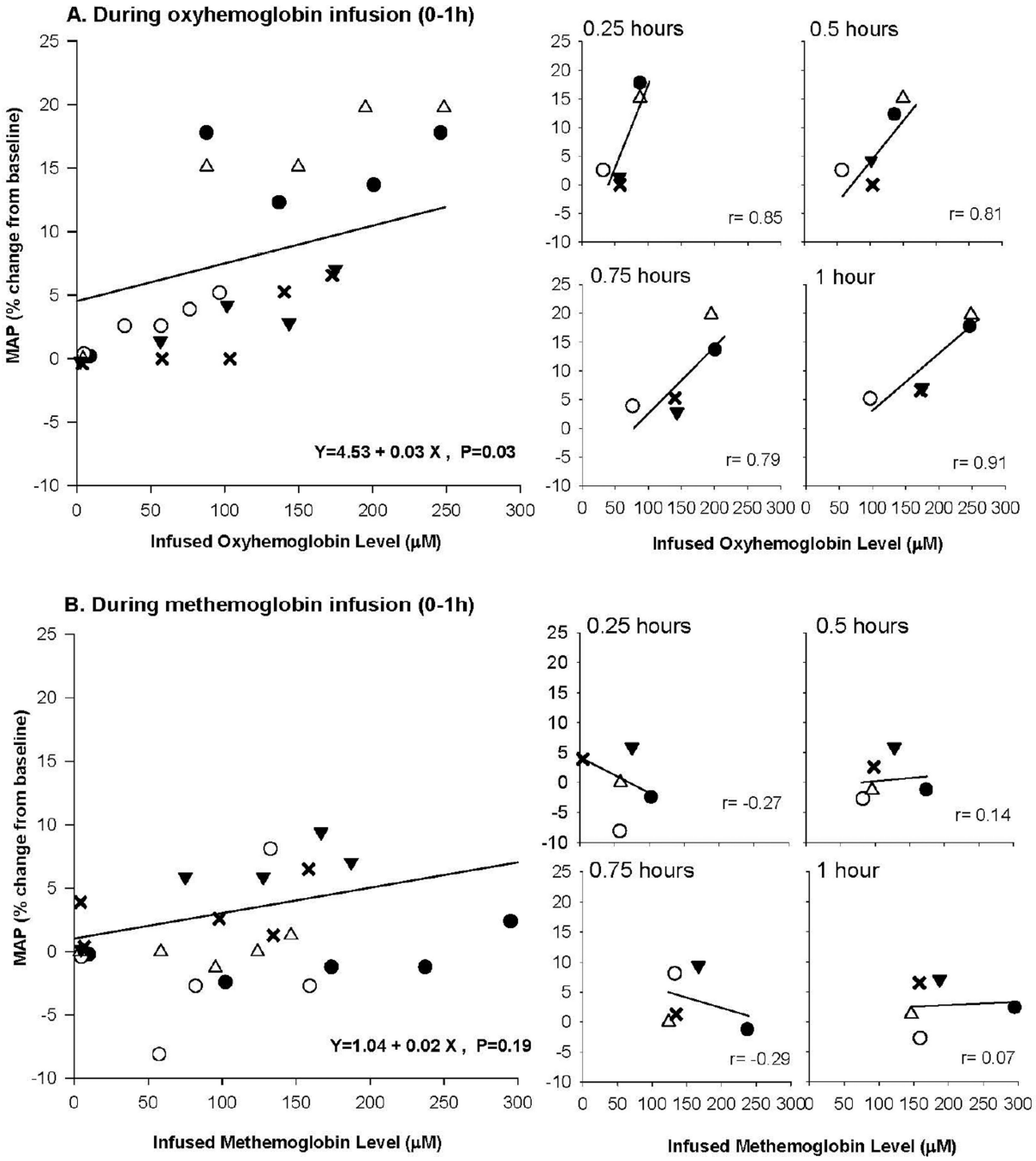


Figure 4. Comparison of percent change in MAP and infused oxyHb plasma levels

The top four panels labeled A on the left shows the association of MAP and oxyHb levels during infusion 0 to 1 h. Each of the 5 animals is depicted by a different symbol (open circle, open triangle, closed circle, inverted closed triangle, and X), and the regression line was estimated using a mixed model. On the top panel to the right are shown at serial time points in animals during the oxyHb infusion, the correlation between MAP and plasma oxyHb levels. (The symbols are the same for each animal, as in the figure to the left.) The bottom panels labeled 5B are similar in format to the 5A top panels, except now infused metHb levels are shown from 0 to 1 h.

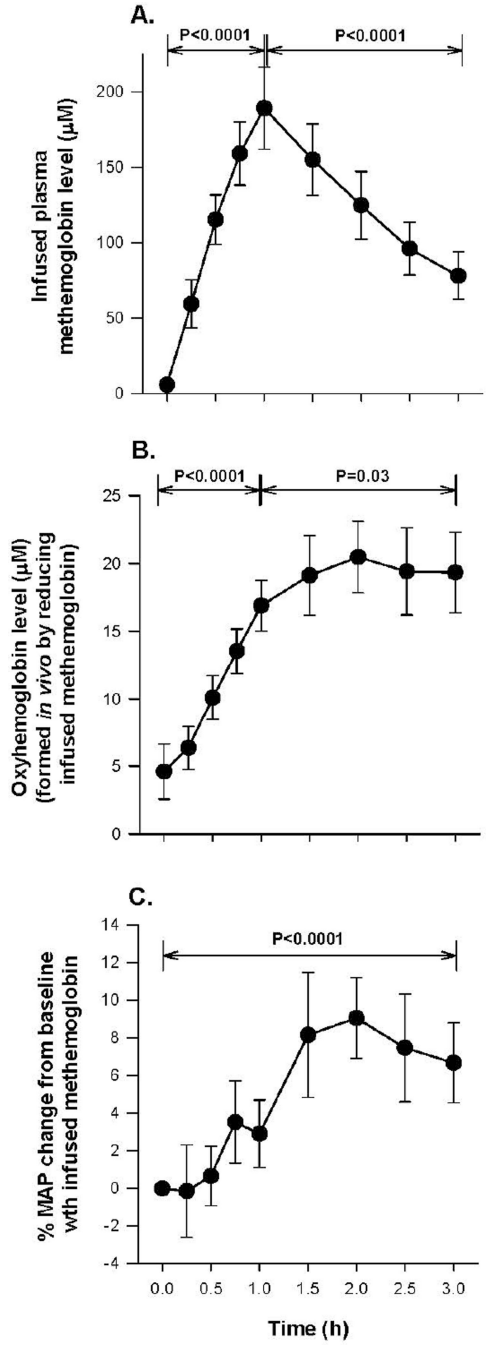


Figure 5. Hb species and percent changes in MAP during metHb infusions

The format is the same as Figure 3, except now Hb species and percent changes in MAP are shown during the metHb infusion. Of note, Panel B now shows the cell-free fraction of metHb that was reduced to cell-free oxyHb during infusion.

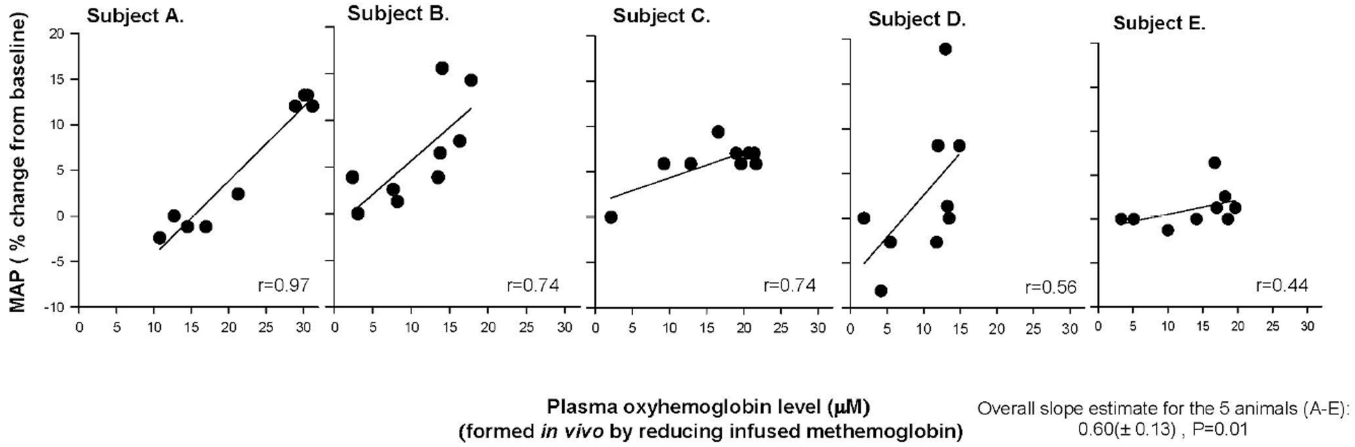
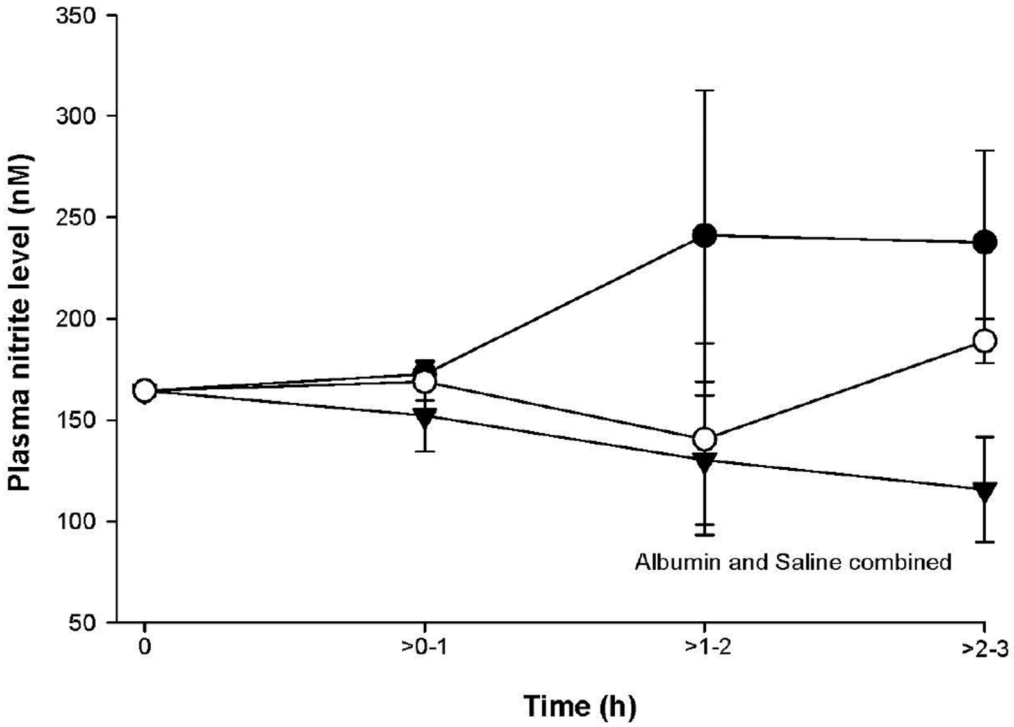


Figure 6. Comparison of the percent change in MAP and the fraction of metHb that was reduced (converted) to cell-free oxyHb during infusion

Panels A to E show in each of these animals over the 3-h experiment the relationship between MAP and converted oxyHb levels. The overall slope of the lines is shown in the lower right-hand corner—analyzing this overall relationship between MAP, and converted oxyHb plasma levels using mixed models.



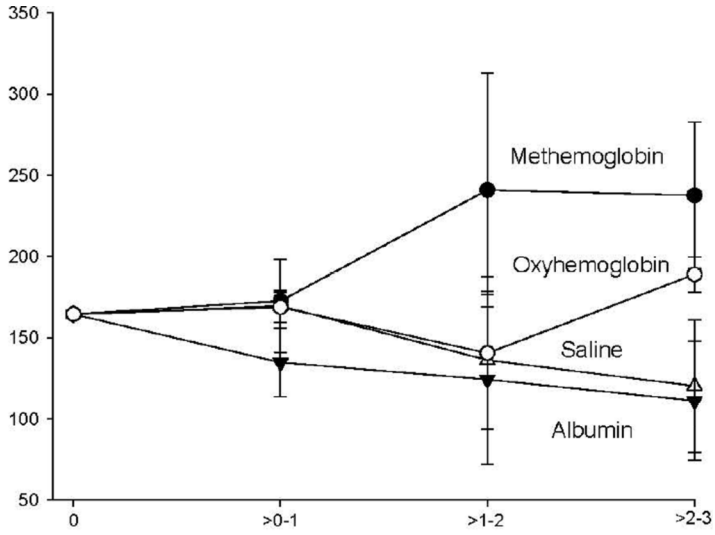


Figure 7. Plasma nitrite level

The format is similar to Figure 1 and 2, except now mean (± SE) nitrite level in plasma is plotted.

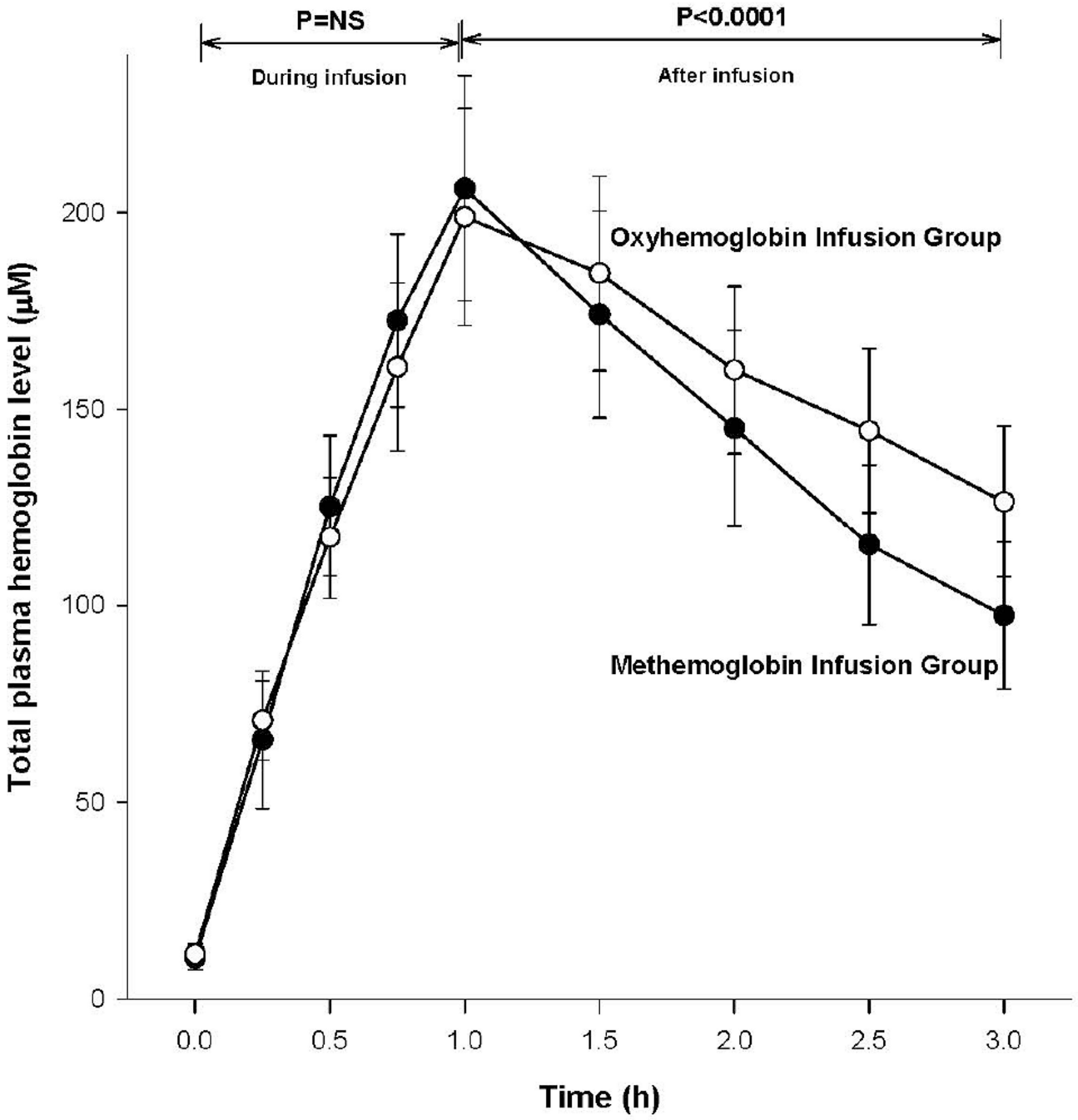


Figure 8. Serial mean (± SE) changes in total Hb levels in animals receiving oxyHb (open circles) and metHb (closed circles)

All p-values compare changes in these two treatment groups over the time period indicated by the brackets.