**Title: Sodium Nitrite therapy attenuates hypertensive effects of HBOC-201 via nitrite reduction**

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**Supplementary Material**

**Materials and Methods**

All reagents were purchased from Sigma (St. Louis, MO, USA) except Mahma/NONOate (MNO) and L-NAME which were purchased from Axxora Platform (San Diego, CA, USA) Sodium nitrite was dissolved in phosphate buffered saline (PBS), pH 7.4. Indomethacin was dissolved in 100% ethanol, which was then diluted in vessel relaxation experiments to a final solvent concentration of 0.1% (v/v). Antifoam SE-15 was dissolved 1% in DMSO and added to a final solvent concentration of 0.00067% in the vessel bath. No effects of these solvents were observed on vasoactive responses at these concentrations (not shown). Human cell-free hemoglobin (hHb) was purified from healthy volunteers according to IRB-approved protocols as previously described[38]. Male Sprague-Dawley rats (200-250g) were purchased from Harlan (Indianapolis, IN, USA). HBOC-201 was obtained from Biopure Corporation, Cambridge, MA.

**Vessel Relaxation Studies**

For all vessel experiments thoracic aorta from male Sprague-Dawley rats were used as previously described[38]. To assess the effects of $O_2$ on nitrite- or MNO-mediated vasodilation, vessels were equilibrated with 95, 21, 2 or 0% $O_2$ gas mixtures (containing 5% $CO_2$ and balanced with $N_2$). Gas was delivered by mass flow controllers (Sierra Instruments, California, USA) set to 0.15L/min a requirement found to be necessary to achieve reproducible hemoglobin and HBOC fractional saturations between experiments. In all experiments vessels were pretreated with indomethacin (5μM) and $N^G$-monomethyl L-arginine (L-NMMA,100μM) to block cyclooxygenase and endothelial nitric oxide synthase, respectively. Vessels were pre-constricted to approximately 50-75% of maximal KCl constriction with L-phenylephrine (PE, 100nM). Upon lowering $O_2$ tensions, vessel tone can fluctuate before reaching a stable tone as previously indicated[43]. For all experiments reported here nitrite or MNO dose-responses were initiated after reaching a stable contractile tone. Nitrite- or MNO- dependent vasodilation was assessed by cumulative administration of increasing doses. Experiments were

performed in the absence or presence of either human hemoglobin (20µM heme) or HBOC-201 (20µM heme), which were added before initiating nitrite or MNO dose-response. Vasodilatory effects of cumulative MNO or nitrite additions were determined by measuring the delta tension and expressing this as a percent relaxation with respect to the maximal PE constriction. Cumulative dose-dependent relaxation curves were fitted to a sigmoidal function -using GraphPad- from which $EC_{50}$s were obtained.

Data collected in the context of assessing HBOC-201 concentrations during vasodilation experiments showed that as a function of time and in the presence of nitrite, but not MNO, HBOC-201 concentrations decreased. Concomitant to this decrease HBOC-201 protein precipitates were noticed in the vessel bioassay chambers. Since a varying heme concentration will affect NO-scavenging and NO-formation processes and hence the vessel response to vasoactive stimuli, in a sub-set of experiments we assessed the vasodilatory effect of single doses of nitrite in the absence and presence of HBOC-201. In these experiments, vessels were pre-contracted with PE and pre-equlibirated with the desired $O_2$ containing gas. HBOC-201 was then added and 2 minutes thereafter (allowing time for HBOC-201 mixing/equilibration) a single dose of nitrite was added and changes in vessel tension recorded. Using this protocol no significant loss of HBOC-201 was observed. This process was repeated using different vessel segments and different nitrite doses to assimilate a dose-dependence of nitrite-dependent vasodilation in the presence and absence of HBOC-201. Note that no significant loss of HBOC-201 was observed in MNO-dependent vasodilation experiments, nor was any loss of human Hb observed in any experiment.

**Assessment of Hb or HBOC-201 concentration and redox/ligation state**

Aliquots of Hb or HBOC-201 were collected from vessel bioassay chambers after equilibration with different $O_2$ containing gases immediately before and after addition of either nitrite or MNO. Aliquots were collected as previously described[38] using gas-tight syringes and transferred to sealed gas-equilibrated cuvettes. UV-Vis spectra of samples were recorded between 450-700nm and concentrations of Hb or HBOC-201 together with redox and ligation state assessed by fitting measured spectra to previously acquired base spectra (oxy-, deoxy-, met-, ferryl-, nitrosyl- and metnitrite forms of both HBOC-201 and Hb) using a least squares method as previously described[26,36-38]. Standard spectra of Hb and HBOC-201 were previously generated and used for deconvolution of respective hemoglobins.

**Determination of the nitrite reductase activity of HBOC-201**.

Stock solutions of 30µM HBOC-201 in phosphate buffered saline (PBS) plus 100µM DTPA at pH 7.4 were progressively degassed under a stream of helium or nitrogen in order to obtain a range of different oxygen fractional saturations. The resulting solutions were transferred anaerobically to sealed spectrophotometer cuvettes using a gas tight syringe and equilibrated at 37$^{o}$C. UV-Vis spectra were collected between 450 and 700nm immediately before and then every 30 seconds during 15 minutes after the addition of excess sodium nitrite in order to attain pseudo-first order conditions (5mM, final concentration; nitrite stock solutions were prepared in degassed PBS). Concentrations of deoxyHBOC-201 as a function of time were determined by spectral deconvolution as described [26,36,37] using reference spectra. Rate constants for the reaction between nitrite and deoxyHBOC were calculated by dividing the slope of the initial linear portion of the curve by the initial concentration of deoxyHBOC and nitrite. Concentrations of nitrosylHBOC-201 were used as an index of nitric oxide production and were also determined by spectral deconvolution after 720 seconds of nitrite addition.

**Preparation of reference spectra for deconvolution**.
All reference spectra between 450-700 nm were obtained from HBOC-201 stocks prepared in PBS at pH 7.4 unless otherwise specified and expressed as the corresponding extinction coefficients. OxyHBOC-201 was prepared by equilibrating a stock solution under a 100% $O_2$ stream in a sealed quartz cuvette. DeoxyHBOC-201 was prepared by the addition of excess sodium dithionite to reduce and consume all oxygen in a sealed cuvette and nitrosylHBOC-201 was prepared by adding a 2:1 excess of Mahma-NONOate to the previously prepared deoxyHBOC-201 solution in 0.1M phosphate buffer. Met-HBOC-201 was prepared by the addition of excess potassium ferricyande to a HBOC-201 stock and met-nitriteHBOC-201 was then obtained by the addition of excess sodium nitrite to oxidized HBOC-201. Finally ferryl-HBOC-201 was prepared by treatment of a metHBOC-201 with excess hydrogen peroxide for 5 minutes followed by separation in a PD10 column (Sephadex G25). Standard spectra for deconvolution of cell-free human hemoglobin were acquired as previously described[38].

**Nitric oxide detection by chemiluminescence**. Direct formation of NO was monitored by ozone-based chemiluminescence using a nitric oxide analyzer (NOA 280i Sievers) as described previously[26]. Sodium nitrite solutions at 1 mM in PBS containing 100μM DTPA and 50μL GE Antifoam (Sievers) pH 7.4 were equilibrated at 37$^o$C under anoxic conditions in a sealed chamber directly connected to the analyzer before the addition of HBOC-201 solutions through a rubber septum. Nitric oxide formation was confirmed by the addition of the NO-scavenger CPTIO (Sigma)

**Cyclic GMP measurement.** Rat aortic rings in KH buffer were equilibrated with gas mixtures containing 0% and 95% $O_2$ (supplemented with 5% $CO_2$) at 37$^o$C and pH 7.45 in the presence of 5μM indomethacin, 100μM L-NMMA, 100μM isobutylmethylxanthine (IBMX), 100nM PE and 0.00067% (v/v) SE-15 antifoam in vessel bioassay chambers. HBOC-201 was added to a final concentration of 20 μM and equilibrated for 1 min before the addition of 25 μM sodium nitrite. Rings were collected after 10 minutes, blotted dry, weighed and frozen in liquid nitrogen. cGMP was measured in ring homogenates using an enzyme-linked immunoassay kit following the manufacturer's indications (Cayman Chemical, Ann Arbor, MI. USA.).

**Trauma-Hemorrhage and resuscitation model**
C57Bl/6 male mice were anesthetized initially by inhalation of 5% isofluorane in air, and then reduced to minimal concentration for maintenance (~1%). All invasive procedures were performed under standard aseptic conditions and according to UAB IACUC approved protocols. The abdomen and groins were shaved and washed with povidone-iodine (10%). A 2-cm midline laparotomy was performed to induce soft-tissue trauma. The incision was closed in two layers (fascia/muscle and skin) and bathed in 1% lidocaine for analgesia. Both femoral arteries were cannulated with femoral catheters (Braintree Scientific, Braintree, MA). Systemic arterial pressure was continuously monitored through one arterial line while hemorrhage and resuscitation was performed via the second arterial line. The animals were bled over 30 minutes to a mean arterial pressure (MAP) of 25 ± 5 mm Hg. This blood pressure was maintained for a further 60 minutes by additional bleeding as required. At the end of the 90 minute hemorrhagic shock period, animals were resuscitated over a period of 30 minutes with either lactate ringers (LR) or HBOC-201 formulation equal to total bleedout volume (~60% of total blood volume). Nitrite was administered in a bolus form (100uL i.v; nitrite stock solutions were 0.1mM, 0.3mM, 1mM, 100mM) immediately prior to initiation of HBOC-201

infusion. Nitrite doses indicated in Figure 6 correspond to amount of nitrite administered. One higher dose of nitrite (100µl of 1M stock solution) was also evaluated in preliminary experiments (n=2) but this induced death within the resuscitation phase and therefore excluded from analysis (not shown). All experiments were carried out for at least 120 minutes post-resuscitation and at the end of the study period, mice were anesthetized by inhalation of 5% isofluorane in air, followed by exsanguination by cardiac puncture and cervical dislocation, in accordance with UAB IACUC guidelines. For all experiments Mean Arterial Pressure (MAP) was continuously recorded using a BPA400 Analyzer (Micro-Med Inc, Louisville, KY). Arterial blood samples were obtained from the femoral arterial catheter at designated intervals for nitrite measurement and assessment of $PaO_2$, $PaCO_2$ , fractional saturation and methemoglobin.

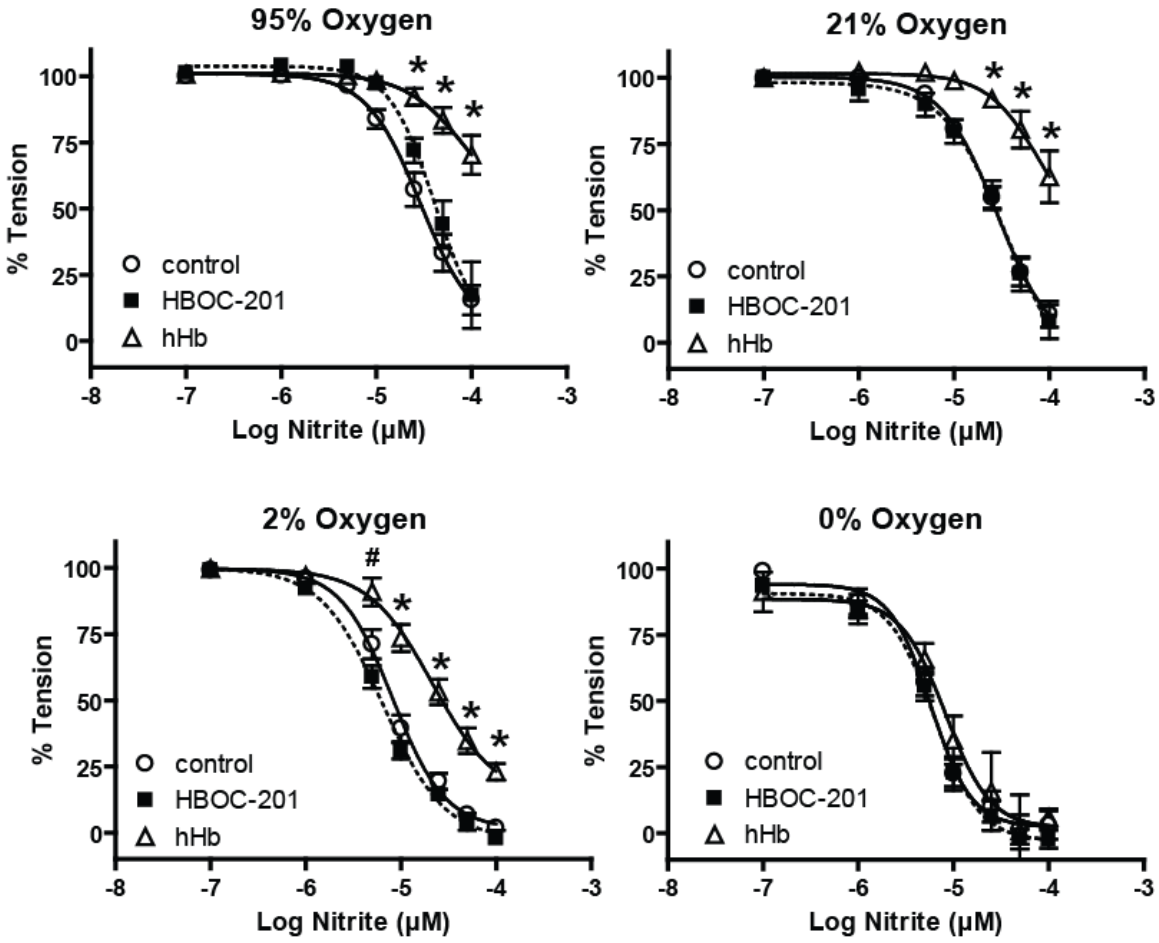
**Measurement of Nitric oxide metabolites**

Plasma nitrite, S-nitrosothiols and N-nitroso compounds were measured as previously described[54]. Blood (150µl) was collected at i) pre-initiation of hemorrhage, ii) at 90min after initiation of experiment (i.e. at end of shock period), iii) at 105-110min after initiation of experiment (corresponding to 20 min post addition of nitrite and onset of resuscitation) and iv) 240 min after initiation of experiment (corresponding to end of experiment). Upon collection, RBC and plasma were immediately separated by centrifugation (2000g, 1min). Plasma was then mixed 1:1 with a solution containing NEM (1mM final concentration), DTPA (100µM, final concentration) and incubated at room temperature 2mins before snap freezing in liquid nitrogen. Samples were then thawed on ice and in the dark within 4hr of collection and nitrite, S-nitrosothiol and C-/N-nitroso species measured.

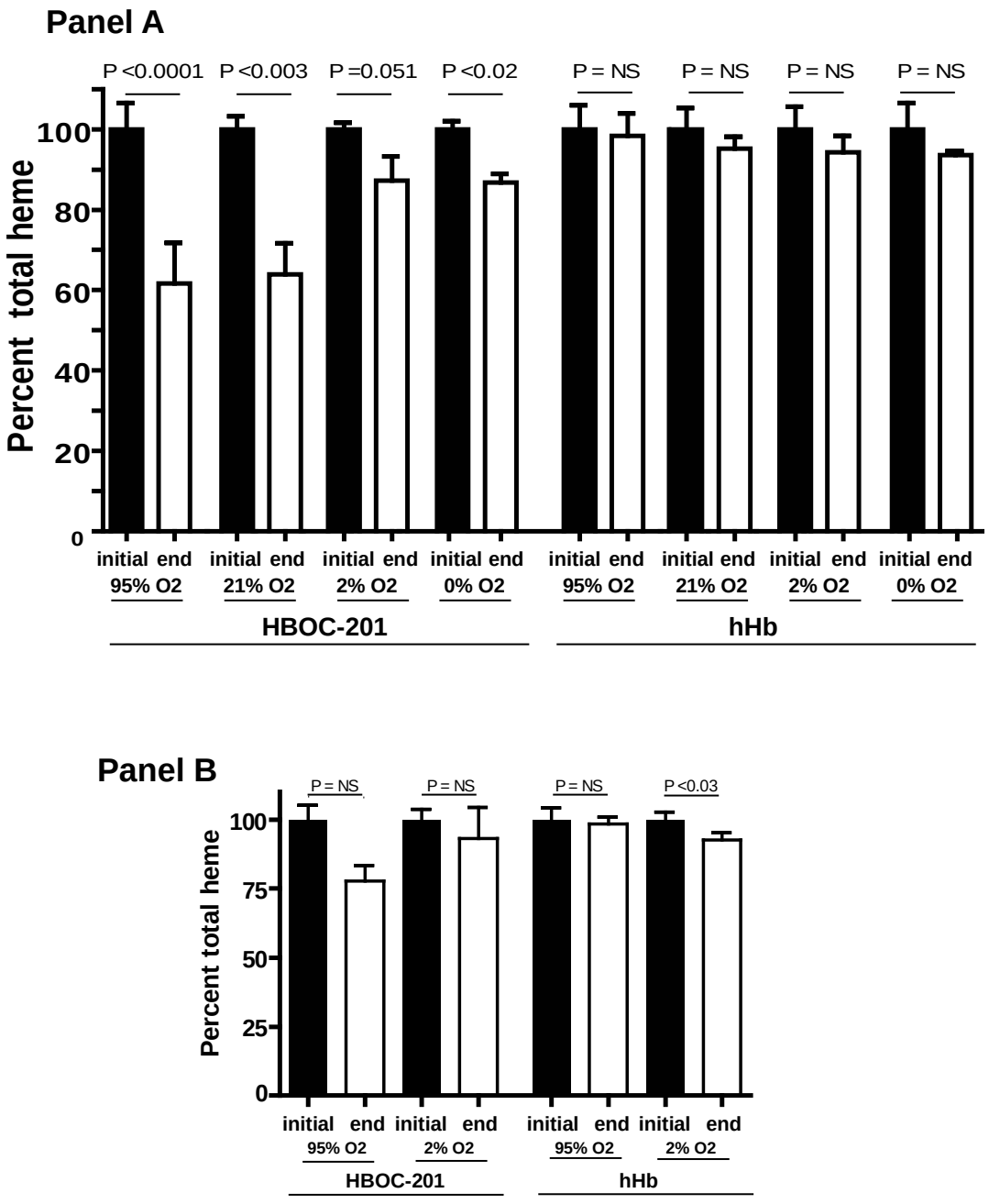
**Supplementary Results**

**Effects of HBOC-201 and hHb in nitrite-dependent vasodilation**

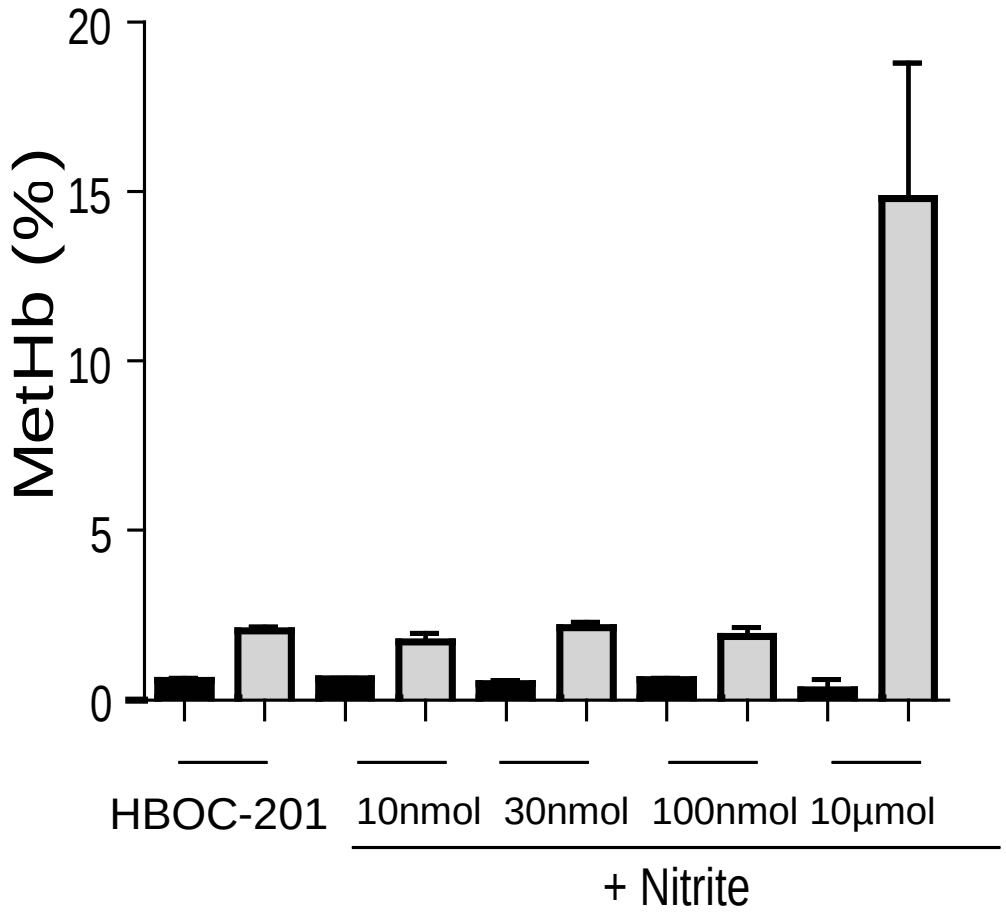
hHb inhibited nitrite dependent vasodilation at all oxygen tensions that resulted in hHb oxygenation (95%, 21% and 2% oxygen), a result that can be explained by NO-scavenging as previously reported. However, when hHb was significantly deoxygenated (FS ~25%)) at 0% oxygen, hHb did not affect nitrite-dependent vasodilation. We have suggested previously that this effect may be explained by deoxyhemoglobin mediated nitrite-reductase activity resulting in NO-generation which counters NO-scavenging processes. HBOC-201 showed a similar reactivity at oxygen tensions which results in deoxygenation. Surprisingly however, at 95% and 21% oxygen where oxygenated HBOC-201 is the predominant ligation state populated, nitrite-dependent vasodilation was not affected (**supplementary Figure 1**). In the course of these experiments it was noted that during vasodilation experiments, the concentration of HBOC-201 was decreasing specifically in the presence of nitrite and more so at higher oxygen tensions. **Supplementary Fig 2** quantitates this effect and demonstrates that the HBOC-201 concentration decreases by approximately 40% during the course of nitrite-dependent vasodilation experiments at both 95% and 21% oxygen (**Supplementary Fig 2A**). This decrease in heme will in turn decrease the rate of both NO-scavenging and nitrite oxidation kinetics and in turn may explain the apparent lack of inhibition of nitrite-dependent vasodilation by oxyHBOC-201. **Supplementary Fig 2A** also shows that no loss of human Hb is observed during these studies. **Supplementary Fig 2B** shows that no loss of HBOC-201 occurs during MNO-vasodilation experiments.




**Supplementary Figure 1: Effects of Hb and HBOC-201 on cumulative nitrite-dependent vasodilation at different oxygen tensions.** Nitrite-dependent vasodilation was assessed in the absence or presence of either HBOC-201 (20μM) or hHb (20μM) at the indicated oxygen tensions. Lines show best fits using sigmoidal fitting alogirithms and data represent mean ± SEM (n=4-9). In some cases errors are smaller than symbol size. *P =< 0.001, #P =< 0.01, hHb relative to control. P-values calculated by 2-way RM-ANOVA with Bonferroni post-test.




**Supplementary Figure 2: Effects of nitrite or MNO on HBOC-201 and hHb concentrations during vessel bioassay experiments.** Aliquots of either HBOC-201 or hHb were taken from vessel bioassay chambers perfused with different oxygen tensions (as indicated) and total heme determined by UV-Vis spectroscopy coupled with spectral deconvolution. Samples were taken both prior to initiation of either nitrite (Panel A) or MNO (Panel B) dose-dependent vasodilation studies and also at the end of the cumulative dose response. Initial and end labeling correspond to these two samplings respectively. Data are presented as percent heme relative to respective initial concentration. Initial heme concentrations were 21.6 ± 0.5µM and 21.2 ± 0.2µM for MNO and nitrite experiments respectively. The average time between initial and end sampling for MNO experiments was 28.1 ± 3.4 min (mean ± SEM, n=9) and for nitrite experiments was 38.1 ± 1.4 min (mean ± SEM, n=17).




**Supplementary Figure 3: Effects of nitrite on metHb formation during resuscitation with HBOC-201 in a model of trauma hemorrhage.** metHb levels pre-hemorrhage (black bars) and post-resuscitation (grey bars). Data show mean ± SEM (n=2-7).