

Phosphodiesterase inhibition restores hypoxia-induced cerebrovascular dysfunction subsequent to improved systemic redox homeostasis: A randomized, double-blind, placebo-controlled crossover study

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Benjamin S Stacey¹ , Christopher J Marley¹,
Hayato Tsukamoto^{1,2} , Tony G Dawkins³, Thomas S Owens¹ ,
Thomas A Calverley¹, Lewis Fall¹, Angelo Iannetelli¹,
Ifan Lewis⁴, James M Coulson⁵, Mike Stemberge⁶ and
Damian M Bailey¹

Abstract

To what extent sildenafil, a selective inhibitor of the type-5 phosphodiesterase modulates systemic redox status and cerebrovascular function during acute exposure to hypoxia remains unknown. To address this, 12 healthy males (aged 24 ± 3 y) participated in a randomized, placebo-controlled crossover study involving exposure to both normoxia and acute (60 min) hypoxia ($\text{FiO}_2 = 0.14$), followed by oral administration of 50 mg sildenafil and placebo (double-blinded). Venous blood was sampled for the ascorbate radical ($\text{A}^{\bullet-}$: electron paramagnetic resonance spectroscopy) and nitric oxide metabolites (NO: ozone-based chemiluminescence). Transcranial Doppler ultrasound was employed to determine middle cerebral artery velocity (MCAv), cerebral delivery of oxygen (CDO_2), dynamic cerebral autoregulation (dCA) and cerebrovascular reactivity to hypo/hypercapnia ($\text{CVR}_{\text{CO}_2\text{HYPO/HYPER}}$). Cortical oxyhemoglobin (cO_2Hb) and oxygenation index (OI) were assessed using pulsed continuous wave near infra-red spectroscopy. Hypoxia decreased total plasma NO ($P = 0.008$), CDO_2 ($P = < 0.001$) and cO_2Hb ($P = 0.005$). In hypoxia, sildenafil selectively reduced $\text{A}^{\bullet-}$ ($P = 0.018$) and MCA_v ($P = 0.018$), and increased dCA metrics of low-frequency phase ($P = 0.029$) and $\text{CVR}_{\text{CO}_2\text{HYPER}}$ ($P = 0.007$) compared to hypoxia-placebo. Collectively, these findings provide evidence for a PDE-5 inhibitory pathway that enhances select aspects of cerebrovascular function in hypoxia subsequent to a systemic improvement in redox homeostasis and independent of altered vascular NO bioavailability.

Keywords

Cerebral autoregulation, cerebral blood flow, cerebrovascular reactivity, nitric oxide, oxidative stress, phosphodiesterase inhibition

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⁵Clinical Pharmacology, Therapeutics & Toxicology, Cardiff University, Cardiff, UK

⁶Cardiff School of Sport and Health Sciences, Cardiff Metropolitan University, Cardiff, UK

¹Neurovascular Research Laboratory, Faculty of Life Sciences and Education, University of South Wales, Pontypridd, UK

²Faculty of Sport Sciences, Waseda University, Shinjuku, Tokyo, Japan

³School of Health and Exercise Sciences, University of British Columbia, Kelowna, Canada

⁴University Hospital Wales, Cardiff, UK

Corresponding author:

Damian M Bailey, Neurovascular Research Laboratory, Alfred Russel Wallace Building, Faculty of Life Sciences and Education, University of South Wales, CF37 4AT, UK.

Email: damian.bailey@southwales.ac.uk

Introduction

Sildenafil was primarily developed for the treatment of hypertension and angina pectoris¹ owing to its highly selective ability to inhibit type-5 phosphodiesterase (PDE-5) and modulate the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP) pathway.² Sildenafil has since been of interest for providing neuroprotection,^{3–5} in light of its ability to cross the blood-brain barrier (BBB)⁶ and increase the expression of PDE-5 mRNA and protein in neurons, glial cells and cerebrovascular endothelial cells^{7–9} while also activating angiogenesis and neurogenesis.^{10–13} While limited data exists, sildenafil has been shown to increase basal cerebral blood flow (CBF) in healthy humans^{14,15} and more recently, lower the risk of Alzheimer's Disease particularly in those most frequently issued prescriptions for erectile dysfunction.¹⁶

The vascular protective benefits of sildenafil are likely attributed to its direct and indirect impact on redox status. The selective inhibition of PDE-5 has been shown to enhance endogenous antioxidant defenses given observed increases in the expression of erythrocyte superoxide dismutase and catalase activity^{17,18} and inhibition of nicotinamide adenine dinucleotide phosphate (NADPH) activity,^{2,19} collectively effecting a reduction in free radical and associated reactive oxygen species (ROS) formation preceding liberation of vascular NO.¹⁷ Sildenafil acts to increase the biological effect of NO by attenuating cGMP hydrolysis,²⁰ subsequently increasing intracellular cGMP concentration that mediates the NO-induced activation of cGMP-dependent protein kinases and ion channels.²¹

Exposure to the hypoxia of high-altitude promotes local elevations in oxidative-nitrosative stress (OXNOS), reflected by a free radical-mediated reduction in vascular NO bioavailability.²² This has been further associated with cerebrovascular dysfunction, namely attenuated vasoreactivity to hypercapnia²³ and select metrics of dynamic cerebral autoregulation (dCA).^{23,24} While sildenafil has been used prophylactically to attenuate the hypoxia-induced elevation in pulmonary artery systolic pressure, the primary risk factor for high-altitude pulmonary oedema,^{25,26} there is only one study that has explored its impact on the cerebrovasculature during inspiratory hypoxia. Accordingly, Chan, et al.¹⁸ reported a single dose (50 mg) of sildenafil increased cerebral oxygenation on days 1 and 3 following rapid ascent to 3,480 m. However, to what extent sildenafil could recover the aforementioned hypoxia-induced cerebrovascular dysfunction^{23,24} remains to be established.

In light of these findings, we conducted a randomized, double-blind, placebo-controlled crossover trial utilizing a functionally integrative translational

approach to examine the impact of PDE-5 inhibition on hypoxia-induced systemic OXNOS and corresponding implications for cerebrovascular function. We hypothesized that sildenafil would attenuate hypoxia-induced systemic OXNOS and promote improvements in select indices of cerebral hemodynamic function.

Methods

Ethics

The experimental protocol was approved by the Research Ethics Committees of the University of South Wales (#17070LSE) and Cardiff Metropolitan University (#16/8/01 R). All experimental procedures were carried out in accordance with the most recent (7th) amendment of the Declaration of Helsinki of the World Medical Association,²⁷ with the exception that it was not registered in a publicly accessible database, with verbal and written informed consent obtained from all participants.

Participants

Twelve healthy, physically active young (26 ± 12 y) males with a body mass index (BMI) of $26 \pm 3 \text{ kg} \cdot \text{m}^{-2}$ were recruited into the study (see power calculations in statistical analysis). All participants were free of medication, non-smokers and abstained from taking nutritional supplements, including oral antioxidants and anti-inflammatories. Participants were specifically asked to refrain from physical activity, caffeine and alcohol for a period of 48 h prior to formal experimentation, to minimize biological variation.²⁴ They were also encouraged to follow a low-nitrate/nitrite ($\text{NO}_3^-/\text{NO}_2^-$) diet for 96 h prior to the study, with specific instructions to avoid fruits, salads and cured meats.²⁸

Design

The study adopted a randomized, double-blind, placebo-controlled crossover design (Figure 1). All participants completed two trials (oral administration of sildenafil and placebo) and were exposed to both normoxia ($\text{FiO}_2 = 0.21$) and normobaric hypoxia ($\text{FiO}_2 = 0.14$) during both visits. Measurements were conducted in a $\sim 120 \text{ m}^3$ environmental chamber maintained at 21°C and 50% relative humidity (Weiss Technik UK Limited, Ebbw Vale, UK). Participants attended the laboratory following a 12 h overnight fast and ingested a tablet containing either 50 mg sildenafil (sildenafil citrate, [Viagra], Pfizer, UK) or 50 mg placebo (Placebo-lactate, Placebo-world, UK) which had an identical appearance, taste and smell in a double-blind, randomized manner by the toss of a fair coin. Following a 50 min normoxic resting period

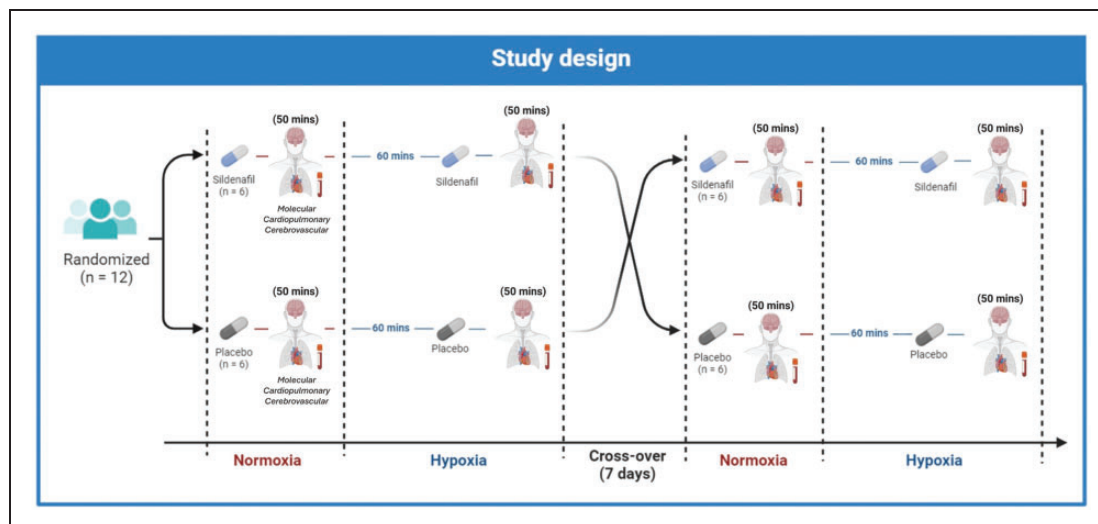


Figure 1. Study design. The study adopted a randomized, double-blind, placebo-controlled crossover design. All participants completed two trials (sildenafil and placebo) and were exposed to both normoxia ($\text{FiO}_2 = 0.21$) and normobaric hypoxia ($\text{FiO}_2 = 0.14$) during both visits. Participants were administered either 50 mg (oral) sildenafil or 50 mg placebo which was followed by a 50 min resting period in normoxia before data collection (blood sample, cardiopulmonary and cerebrovascular assessments). Participants were then exposed to hypoxia for 60 min. A second tablet of either 50 mg sildenafil or 50 mg placebo was then administered followed by another 50 min rest period prior to a second bout of data collection. Each trial was separated by a 7-day recovery period.

(allowing for sildenafil to reach peak plasma concentration²⁹) venous blood samples were obtained, followed immediately by cardiopulmonary and cerebrovascular measurements. Participants then re-entered the environmental chamber (hypoxia) and rested (seated) for 60 min. A second tablet of either 50 mg sildenafil or 50 mg placebo was subsequently administered (to maintain peak plasma sildenafil concentration) followed by another 50 min rest prior to a second bout of blood/hemodynamic data collection. Each trial was separated by a 7-day washout period.

Measurements

Molecular function

Blood was collected from an indwelling cannula into Vacutainers[®] (Becton, Dickinson and Company, Oxford, UK) before centrifugation at 600 *g* (4°C) for 10 min. Plasma (K-EDTA) and red blood cell (RBC) supernatant were decanted into cryogenic vials (Nalgene[®] Labware, Thermo Fisher Scientific Inc, Waltham, MA, USA), immediately snap-frozen in liquid nitrogen and subsequently stored at -80°C. Prior to batch analysis, each sample were thawed at 37°C in the dark for 3 min. Whole blood was also assayed for hemoglobin (Hb) and hematocrit (Hct). Hb was measured photometrically (HemoCue 201+, Radiometer, UK) and Hct was prepared via ultracentrifugation (Hawksley and Sons Ltd, Sussex, UK) and

measured using a Hawksley Micro Hematocrit Reader (Hawksley and Sons Ltd, Sussex, UK).

Free radicals. The ascorbate free radical ($\text{A}^{\bullet-}$) was employed as a direct measure of global free radical formation.^{30,31} Plasma (1 mL) was injected directly into a high-sensitivity multiple-bore sample cell (AquaX, Bruker Daltonics Inc., Billerica, MA, USA) housed within a TM_{110} cavity of an EPR spectrometer operating at X-band (9.87 GHz). Samples were recorded by cumulative signal averaging of 10 scans using the following instrument parameters: resolution, 1024 points; microwave power, 20 mW; modulation amplitude, 0.65 G; receiver gain, 2×10^5 ; time constant, 40.96 ms; sweep rate, 0.14 G/s; sweep width, 6 G; centre field, 3486 G. Spectra were filtered identically (moving average, 15 conversion points) using WINEPR software (Version 2.11, Bruker, Karlsruhe, Germany) and the double integral of each doublet was determined using commercial software (OriginLab Corps, MA, USA). The intra- and inter-assay CVs were both <5%.³¹

NO metabolites. Ozone-based chemiluminescence (Sievers NOA 280i, Analytix Ltd, Durham, UK) was employed to detect NO liberated from plasma and RBC samples via chemical reagent cleavage as previously described in detail.^{32,33} This facilitated detection of total plasma (combined concentration of nitrite $[\text{NO}_2^-] + \text{S-nitrosothiols } [\text{RSNO}]$) and total RBC-bound (combined concentration of

NO_2^- + S-nitrosohemoglobin [SNO-Hb] and iron nitrosylhemoglobin [HbNO]) NO metabolites. Plasma (200 μL) was injected into tri-iodide (I_3) reagent for the measurement of total plasma NO. The original I_3 reagent was subsequently modified with the addition of potassium hexacyanoferrate [$\text{K}_3\text{Fe}^{\text{III}}(\text{CN})_6$] to limit Hb/cell free heme [$\text{Fe}(\text{II})$] auto-capture of NO for the analysis of total RBC-bound NO.³⁴ Briefly, RBC samples were lysed 1:4 with EDTA (0.5 mM final concentration, pH 7.0), incubated for 5 min on ice and subsequently injected (400 μL) into the modified I_3 reagent. The signal output (in mV) from the analyzer was plotted against time using Origin 8 (OriginLab Corps, Massachusetts, USA) and smoothed using a 150-point averaging algorithm. The Peak Analysis package was used to calculate area under the curve (mV/s), that was subsequently converted to a concentration, using standard curves of known concentrations of sodium nitrite, measured at identical injection volumes. The intra-assay and inter-assay coefficients of variation for plasma and RBC-bound NO metabolites were <10%.³²

Cardiopulmonary function

Beat-by-beat arterial blood pressure was assessed via finger photoplethysmography and arterial volume clamping (Finometer PRO, Finapres Medical Systems, Amsterdam, Netherlands) and used to calculate mean arterial blood pressure (MAP) after calibrating values to the average of three automated brachial blood pressure measurements (Life Source, A&D Medical, model: UA767FAM), taken over a 5 min resting baseline period. Heart rate (HR) was assessed using a 3-lead electrocardiogram (ECG, ADI BioAmp ML132, ADInstruments, Colorado Springs, CO, USA). Respiratory flow was measured with a pneumotachometer (model HR 800 L, Hans Rudolph, Shawnee, KS) and expired gases (end-tidal partial pressures of CO_2 and O_2 [P_{ETCO_2} and P_{ETO_2}]) were sampled continuously via capnography (model ML206, ADInstruments, Colorado Springs, CO, USA). Stroke volume (SV) was estimated from the arterial blood pressure waveform using the modelflow (MF) method.³⁵ Cardiac output (\dot{Q}) was calculated mathematically as the product of HR and SV. Total peripheral resistance (TPR) was calculated as $\frac{\text{MAP (mmHg)}}{\dot{Q} (\text{L} \cdot \text{min}^{-1})}$. Peripheral oxygen saturation (SpO_2) was measured using fingertip pulse oximetry (Nonin 9550 Onyx II, Nonin Medical, Inc., Plymouth, MI, USA). All cardiopulmonary variables were sampled continuously at 1 kHz using an analogue-to-digital converter (Powerlab, 16/30; ADInstruments, Colorado Springs, CO, USA) and data were interfaced with LabChart (Version 7.3), and analyzed offline.

Cerebrovascular function

Cerebral haemodynamics. The proximal segment of the right middle cerebral artery (MCA) was insonated using a 2 MHz-pulsed transcranial Doppler (TCD) ultrasound system (Multi-Dop X4; DWL Electroniche Systeme, Singen, Germany). Following standardized search techniques,³⁶ the Doppler probe was secured over the middle trans-temporal window using a custom fit headband device (Spencer Technologies) to securely insonate the MCA and measure blood velocity (MCAv). Indices of cerebrovascular conductance (CVCi) and cerebrovascular resistance (CVRi) were calculated as mean MCAv/MAP and MAP/mean MCAv, respectively. Pulsatility index (PI) was calculated as: systolic MCAv – diastolic MCAv/mean MCAv and subsequently normalized to the prevailing MAP ($\text{PI}_{\text{Norm}} = \text{PI}/\text{MAP}$). Cerebral O_2 delivery (CDO_2) was calculated as mean MCAv \times arterial oxygen content (CaO_2) where CaO_2 was estimated as: $\text{CaO}_2 (\text{mL/dL}) = \text{Hb (g/dL)} \times 1.34 \left(\frac{\text{SaO}_2 (\%) }{100} \right)$.²⁴

Cerebral oxygenation. Pulsed continuous-wave near-infrared spectroscopy (NIRS, Oxymon Mk III; Artinis Medical Systems BV, Zetten, The Netherlands) was used to monitor changes in cerebral oxygenation at optical densities of 780 and 850 nm.³⁷ One set of NIRS optodes (5.0 cm source detector spacing, consistent with recent recommendations) was placed on the skin over the left frontal prefrontal cortical region of the forehead between Fp1 and F3, consistent with the anatomical landmarks of the International 10–20 system for EEG placement.³⁸ Optodes were secured in place using double-sided adhesive tape and positioned underneath the custom fit headband device. Concentration changes (Δ) in oxyhemoglobin (O_2Hb) and deoxyhemoglobin (HHb) were calculated using the modified Beer–Lambert Law³⁹ incorporating a differential pathlength factor (DPF) of 5.93% for cerebral tissue.⁴⁰ We also calculated the difference between $\Delta[\text{O}_2\text{Hb}]$ and $\Delta[\text{HHb}]$ as a surrogate ‘oxygenation index’ (OI), consistent with NIRS studies that have also employed similar continuous-wave devices (Grassi *et al.* 2003). Finally, $\Delta[\text{THb}]$ was calculated as the sum of $\Delta[\text{O}_2\text{Hb}]$ and $\Delta[\text{HHb}]$ signals and used as a surrogate for changes in regional blood volume (Van Beekvelt *et al.* 2001). All NIRS signals were normalized to reflect changes relative to a 5 min resting baseline control in normoxia following administration of the placebo (arbitrarily defined as $0 \mu\text{mol} \cdot \text{L}^{-1}$). Signals were recorded at 50 Hz, displayed in real time and stored on a computer for offline analysis. A 60 s average at the end of a five min resting baseline was calculated in each condition.

Dynamic cerebral autoregulation (dCA). Following 10 min of seated rest, 5-min segments of MAP and MCAv data were obtained for spectral analysis of spontaneous oscillations to assess dCA via transfer function analysis (TFA).⁴¹ Beat-to-beat MAP and MCAv mean signals were calculated across each cardiac cycle, linearly interpolated, and resampled at 4 Hz in accordance with formal recommendations of the Cerebrovascular Research Network.^{42,43} Spontaneous MAP and MCAv mean power spectrum density and the mean value of TFA coherence, gain, and phase were band averaged across the very low frequency (VLF: 0.02–0.07 Hz, 50 to 14.3-s cycles) and low frequency (LF: 0.07–0.2 Hz, 14.3 to 5-s cycles) ranges where CA is considered to be most operant.⁴¹ The squared coherence function reflects the fraction of output power (i.e., MCAv mean) that can be linearly related to the input power (i.e., MAP) at each frequency. Similar to a correlation coefficient, this value varies between 0 and 1 with 1 indicating that all the variability in MCAv is linearly explained by the variability in MAP. To ensure that robust phase and gain estimates were entered for subsequent analysis, we averaged only those gain and phase (positive to eliminate wrap-around) values where the corresponding coherence was ≥ 0.34 , applied to single harmonics only. An increase in gain and reduction in phase were taken to reflect reduced dCA, indicative of a more pressure-passive relationship between MAP and MCAv.²⁴

Cerebrovascular reactivity to carbon dioxide (CVR_{CO2}). Following the 5 min resting baseline for TFA, the inspired air was rapidly changed to 5% CO₂ with 21% O₂ (balanced nitrogen) from a 200 L Douglas Bag attached to Falconia tubing (Cranleigh, UK) connected to the inspiratory port of a 2-way nonrebreathing valve (Hans Rudolph, 2400 series) for 3 min. Following 5 min of recovery breathing ambient air, participants were instructed to hyperventilate at 15 breaths/min for 3 minutes. From this, CVR_{CO2} to hypercapnia (CVR_{CO2Hyper})/hypocapnia (CVR_{CO2Hypo}) were calculated as the percent change in MCAv from baseline per mmHg change in P_{ET}CO₂ recorded during the final 30 s (average taken) of the respective challenge when steady-state had been achieved. We also derived the CVR_{CO2Range} as a useful indication of the cerebral circulation's combined ability to respond to differential changes in CO₂, calculated as the change in MCAv divided by the change in P_{ET}CO₂ across the hypocapnia-hypercapnia range.

Statistical analysis

Power calculations. Prospective power calculations were determined using G*Power (V.3.1.9.4). Assuming

comparable differences (15%) and corresponding effect size (0.60) previously observed in basal plasma A⁺ ($\eta^2 = 0.54$)⁴⁴ between normoxia and hypoxia, our primary end-outcome variable for OXNOS, the present study required a (minimum) sample size of 20 participants (10 per group) in order to achieve adequate power ($1-\beta = 0.80$ at $P < 0.05$). We chose to further inflate this by 20% during recruitment ($n = 24$ or 12 per group) given the potential for loss to follow-up or incomplete data collection.

Inferential analyses. Data were analyzed using the Statistics Package for Social Scientists (IBM SPSS Statistics Version 29.0). Shapiro-Wilk W tests (all $P > 0.05$) confirmed that all data sets were normally distributed. A 2-way (Inspirate: normoxia vs. hypoxia \times Drug: placebo vs. sildenafil) repeated measures analysis of variance (ANOVA) was used to detect differences between conditions and presented alongside effect sizes (partial eta squared: η_p^2) and estimates of statistical power (β). Where interaction effects were detected, post-hoc comparisons were made using paired samples t -tests with Bonferroni correction. Significance was established at $P < 0.05$ for all two-tailed tests and data expressed as mean \pm standard deviation (SD).

Results

Compliance and randomization

A total of 12 participants completed both placebo and sildenafil trials with no loss to follow-up. Randomization led to 6 participants completing the sildenafil trial first with 6 participants completing the placebo trial first (Figure 1).

Molecular function

During the placebo trial, hypoxia did not impact A⁺. However, A⁺ was decreased following sildenafil in hypoxia, when compared to hypoxia-placebo ($P = 0.018$, Figure 2). Hypoxia decreased plasma NO in both the placebo and sildenafil trials ($P = 0.008$, Figure 2).

Cardiopulmonary function

Hypoxia increased Hb ($P < 0.001$), Hct ($P = 0.004$), \dot{Q} ($P = 0.003$) and \dot{V}_E ($P < 0.001$), and reduced P_{ET}O₂ ($P < 0.001$), P_{ET}CO₂ ($P < 0.001$), SpO₂ ($P < 0.001$) and caO₂ ($P < 0.001$) across the placebo and sildenafil trials. Sildenafil increased HR in the normoxia and hypoxia trial ($P = 0.001$) but had no effect ($P > 0.05$) on other cardiopulmonary variables (MAP, SV, \dot{Q} , TPR, P_{ET}O₂/CO₂ or \dot{V}_E) (Table 1).

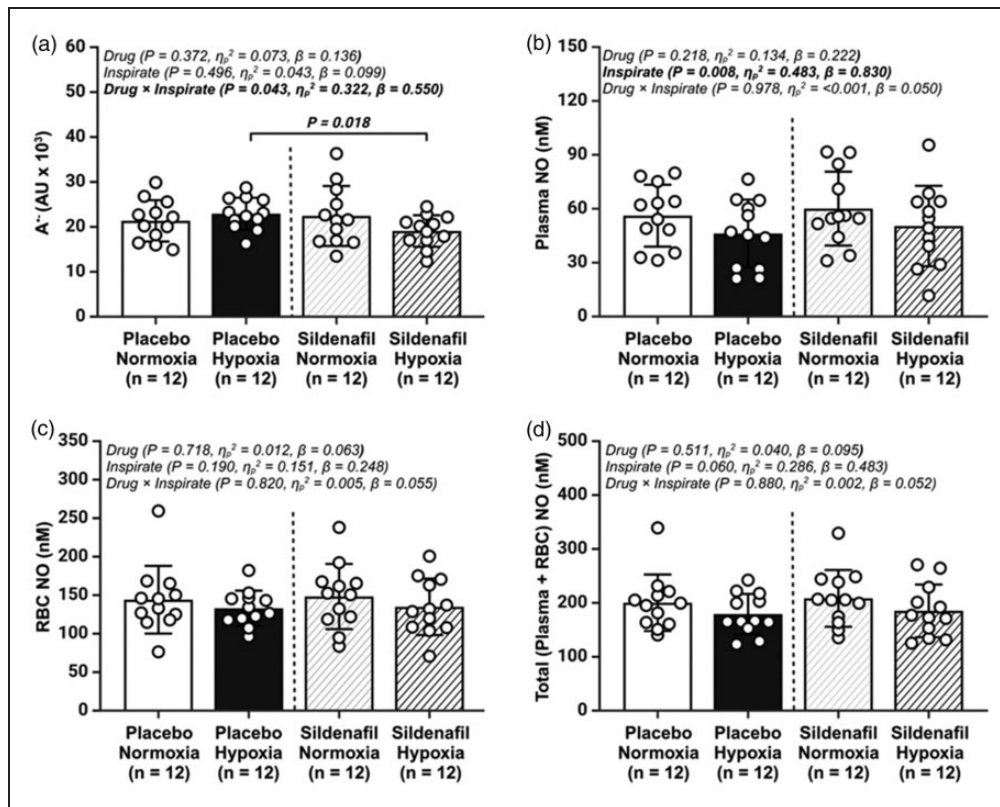


Figure 2. Basal metrics of systemic oxidative-nitrosative stress (OXNOS). (a) plasma ascorbate free radical ($A^{\bullet-}$); (b) total plasma nitric oxide (NO) concentration (nitrite + S-Nitrosothiols); (c) total red blood cell (RBC) NO (nitrite + S-Nitrosohemoglobin + hemoglobin-bound NO); and (d) total (plasma + total RBC) NO concentrations; AU, arbitrary units; Values are mean \pm SD. $n = 12$ for all parameters.

Cerebrovascular function

Haemodynamics: Hypoxia reduced CDO_2 across both placebo and sildenafil trials ($P < 0.001$, Table 2). Compared to hypoxia-placebo and normoxia-sildenafil, hypoxia-sildenafil decreased MCA_V ($P = 0.018$ and $P = 0.011$, respectively; Table 2).

Cortical oxygenation: As anticipated, hypoxia reduced cO_2Hb ($P = 0.005$) and OI ($P < 0.001$), whereas $cHHb$ increased ($P = 0.025$) across both placebo and sildenafil trials (Table 2).

Autoregulation: A total of 4 participants were excluded from the VLF estimates for gain and phase, owing to a VLF coherence < 0.34 . LF phase was increased under hypoxia-sildenafil when compared to the placebo-hypoxia ($P = 0.029$; Figure 3(c)) and normoxia-sildenafil ($P = 0.011$; Figure 3(c)) trials. In normoxia, sildenafil reduced LF phase ($P = 0.019$; Figure 3(c)) when compared to the placebo trial. LF coherence was also reduced in the hypoxia-sildenafil ($P = 0.023$; Figure 3(a)). Despite differences in mean MCA_V across conditions, no differences were observed for normalized gain in either the VLF or LF range. **Vasoreactivity:** CVR_{CO_2HYPER} was lower in the hypoxia-placebo trial when compared to the normoxia-

placebo trial (2.2 ± 1.1 vs. $3.1 \pm 1.1\% \cdot mmHg^{-1}$; $P = 0.042$; Figure 4(a)). However, hypoxia-sildenafil increased CVR_{CO_2HYPER} when compared to the hypoxia-placebo trial (3.4 ± 1.1 vs. $2.2 \pm 1.1\% \cdot mmHg^{-1}$; $P = 0.007$; Figure 4(a)). CVR_{CO_2RANGE} was higher in the sildenafil trials in both normoxia and hypoxia ($P = 0.023$), when compared to the placebo trials (Figure 4(c)). No differences were observed for CVR_{CO_2HYPO} or CVR_{CO_2RANGE} (all $p > 0.05$; Figure 4(b) and (c)).

Discussion

Taking a functionally integrative translational approach, the present study has identified two important findings. First, sildenafil selectively reduced systemic free-radical formation in hypoxia yet, contrary to original expectations, this was not accompanied by a reciprocal elevation in vascular NO bioavailability consistent with (attenuated) systemic OXNOS. Second, despite a selective reduction in MCA_V that likely reflected enhanced (hypoxic) vasodilation given the attenuated hypoxia-induced cerebral deoxygenation and clearly independent of hyperventilation-induced

Table 1. Cardiopulmonary function.

Drug	Placebo	
	Normoxia	Hypoxia
Inspirate		
Cardiovascular		
Hb (g · dL ⁻¹)	14.5 ± 1.5	14.8 ± 1.4
Drug (P = 0.659, $\eta_p^2 = 0.018$, $\beta = 0.070$); Inspirate (P = <0.001, $\eta_p^2 = 0.749$, $\beta = 0.999$); Drug × Inspirate (P = 0.761, $\eta_p^2 = 0.009$, $\beta = 0.059$)		
Hct (%)	45 ± 3	47 ± 2
Drug (P = 0.779, $\eta_p^2 = 0.007$, $\beta = 0.058$); Inspirate (P = 0.005, $\eta_p^2 = 0.533$, $\beta = 0.897$); Drug × Inspirate (P = 0.117, $\eta_p^2 = 0.208$, $\beta = 0.343$)		
SBP (mmHg)	133 ± 18	132 ± 11
Drug (P = 0.823, $\eta_p^2 = 0.005$, $\beta = 0.055$); Inspirate (P = 0.766, $\eta_p^2 = 0.008$, $\beta = 0.059$); Drug × Inspirate (P = 0.837, $\eta_p^2 = 0.044$, $\beta = 0.054$)		
DBP (mmHg)	65 ± 10	64 ± 8
Drug (P = 0.505, $\eta_p^2 = 0.041$, $\beta = 0.097$); Inspirate (P = 0.828, $\eta_p^2 = 0.044$, $\beta = 0.055$); Drug × Inspirate (P = 0.457, $\eta_p^2 = 0.051$, $\beta = 0.109$)		
MAP (mmHg)	88 ± 11	86 ± 6
Drug (P = 0.721, $\eta_p^2 = 0.012$, $\beta = 0.063$); Inspirate (P = 0.952, $\eta_p^2 = <0.001$, $\beta = 0.050$); Drug × Inspirate (P = 0.720, $\eta_p^2 = 0.120$, $\beta = 0.063$)		
HR (bpm)	61 ± 10	65 ± 11
Drug (P = 0.169, $\eta_p^2 = 0.164$, $\beta = 0.269$); Inspirate (P = 0.001, $\eta_p^2 = 0.653$, $\beta = 0.985$); Drug × Inspirate (P = 0.278, $\eta_p^2 = 0.106$, $\beta = 0.181$)		
SV (mL)	102 ± 7	101 ± 3
Drug (P = 0.708, $\eta_p^2 = 0.013$, $\beta = 0.064$); Inspirate (P = 0.299, $\eta_p^2 = 0.097$, $\beta = 0.169$); Drug × Inspirate (P = 0.866, $\eta_p^2 = 0.003$, $\beta = 0.053$)		
Q (L · min ⁻¹)	6.19 ± 0.96	6.61 ± 1.10
Drug (P = 0.163, $\eta_p^2 = 0.169$, $\beta = 0.277$); Inspirate (P = 0.003, $\eta_p^2 = 0.564$, $\beta = 0.929$); Drug × Inspirate (P = 0.253, $\eta_p^2 = 0.117$, $\beta = 0.197$)		
TPR (mmHg · min · L ⁻¹)	14.70 ± 3.91	13.55 ± 3.29
Drug (P = 0.198, $\eta_p^2 = 0.146$, $\beta = 0.240$); Inspirate (P = 0.077, $\eta_p^2 = 0.256$, $\beta = 0.428$); Drug × Inspirate (P = 0.996, $\eta_p^2 = <0.001$, $\beta = 0.050$)		
Pulmonary		
SpO ₂ (%)	99 ± 1	88 ± 4
Drug (P = 0.210, $\eta_p^2 = 0.139$, $\beta = 0.229$); Inspirate (P = <0.001, $\eta_p^2 = 0.919$, $\beta = 1.000$); Drug × Inspirate (P = 0.199, $\eta_p^2 = 0.145$, $\beta = 0.239$)		
caO ₂ (mL · dL ⁻¹)	19.8 ± 2.0	18.2 ± 2.4
Drug (P = 0.990, $\eta_p^2 = <0.001$, $\beta = 0.050$); Inspirate (P = <0.001, $\eta_p^2 = 0.901$, $\beta = 1.000$); Drug × Inspirate (P = 0.289, $\eta_p^2 = 0.102$, $\beta = 0.175$)		
V _E (L · min ⁻¹)	19 ± 6	26 ± 4
Drug (P = 0.989, $\eta_p^2 = <0.001$, $\beta = 0.050$); Inspirate (P = <0.001, $\eta_p^2 = 0.915$, $\beta = 1.000$); Drug × Inspirate (P = 0.104, $\eta_p^2 = 0.380$, $\beta = 0.365$)		
P _{ET} O ₂ (mmHg)	89 ± 4	51 ± 3
Drug (P = 0.729, $\eta_p^2 = 0.011$, $\beta = 0.062$); Inspirate (P = <0.001, $\eta_p^2 = 0.995$, $\beta = 1.000$); Drug × Inspirate (P = 0.121, $\eta_p^2 = 0.205$, $\beta = 0.336$)		
P _{ET} CO ₂ (mmHg)	41 ± 3	38 ± 3
Drug (P = 0.588, $\eta_p^2 = 0.126$, $\beta = 0.062$); Inspirate (P = <0.001, $\eta_p^2 = 0.995$, $\beta = 1.000$); Drug × Inspirate (P = 0.825, $\eta_p^2 = 0.205$, $\beta = 0.336$)		

Values are mean ± SD; SBP: systolic blood pressure; DBP: diastolic blood pressure; MAP: mean arterial pressure; HR: heart rate; SV: stroke volume; Q: cardiac output; TPR: total peripheral resistance; Hb: hemoglobin; Hct: hematocrit; SpO₂: arterial oxyhemoglobin saturation; caO₂: arterial oxygen content; V_E: minute ventilation; P_{ET}O₂/P_{ET}CO₂: end-tidal partial pressure of oxygen/carbon dioxide. n = 12 for all parameters.

Table 2. Cerebrovascular function.

Drug	Placebo		Sildenafil	
	Normoxia	Hypoxia	Normoxia	Hypoxia
Cardiovascular				
sMCA _v (cm·s ⁻¹)	114 ± 18	115 ± 18	121 ± 21	109 ± 16†
Drug (P = 0.748, $\eta_p^2 = 0.010$, $\beta = 0.061$); Inspirate (P = 0.020, $\eta_p^2 = 0.400$, $\beta = 0.695$); Drug × Inspirate (P = 0.024, $\eta_p^2 = 0.385$, $\beta = 0.666$)				
dMCA _v (cm·s ⁻¹)	36 ± 7	35 ± 6	34 ± 11	30 ± 7
Drug (P = 0.164, $\eta_p^2 = 0.168$, $\beta = 0.275$); Inspirate (P = 0.069, $\eta_p^2 = 0.270$, $\beta = 0.452$); Drug × Inspirate (P = 0.162, $\eta_p^2 = 0.169$, $\beta = 0.278$)				
MCA _v (cm·s ⁻¹)	62 ± 10	62 ± 10	63 ± 12	57 ± 8*†
Drug (P = 0.318, $\eta_p^2 = 0.091$, $\beta = 0.160$); Inspirate (P = 0.018, $\eta_p^2 = 0.413$, $\beta = 0.717$); Drug × Inspirate (P = 0.036, $\eta_p^2 = 0.342$, $\beta = 0.587$)				
PI (AU)	1.26 ± 0.14	1.29 ± 0.12	1.38 ± 0.30	1.39 ± 0.22
Drug (P = 0.163, $\eta_p^2 = 0.169$, $\beta = 0.277$); Inspirate (P = 0.471, $\eta_p^2 = 0.048$, $\beta = 0.105$); Drug × Inspirate (P = 0.600, $\eta_p^2 = 0.026$, $\beta = 0.078$)				
CVRI (mmHg·cm·s ⁻¹)	1.43 ± 0.20	1.43 ± 0.24	1.38 ± 0.30	1.55 ± 0.45
Drug (P = 0.670, $\eta_p^2 = 0.017$, $\beta = 0.069$); Inspirate (P = 0.345, $\eta_p^2 = 0.081$, $\beta = 0.147$); Drug × Inspirate (P = 0.267, $\eta_p^2 = 0.111$, $\beta = 0.188$)				
CVCI (cm·s ⁻¹ ·mmHg ⁻¹)	0.71 ± 0.10	0.72 ± 0.12	0.76 ± 0.21	0.70 ± 0.22
Drug (P = 0.666, $\eta_p^2 = 0.018$, $\beta = 0.069$); Inspirate (P = 0.544, $\eta_p^2 = 0.034$, $\beta = 0.088$); Drug × Inspirate (P = 0.402, $\eta_p^2 = 0.064$, $\beta = 0.125$)				
CDO ₂ (mL·cm·s ⁻¹)	1233 ± 240	1119 ± 220	1239 ± 240	1042 ± 168
Drug (P = 0.379, $\eta_p^2 = 0.071$, $\beta = 0.134$); Inspirate (P = <0.001, $\eta_p^2 = 0.734$, $\beta = 0.999$); Drug × Inspirate (P = 0.165, $\eta_p^2 = 0.167$, $\beta = 0.274$)				
Δ cO ₂ Hb (μmol·L ⁻¹)	0.0 ± 0.0	-5.5 ± 6.0	4.4 ± 8.3	-3.2 ± 6.6
Drug (P = 0.102, $\eta_p^2 = 0.245$, $\beta = 0.370$); Inspirate (P = 0.005, $\eta_p^2 = 0.559$, $\beta = 0.893$); Drug × Inspirate (P = 0.423, $\eta_p^2 = 0.065$, $\beta = 0.118$)				
Δ cHHb (μmol·L ⁻¹)	0.0 ± 0.0	3.3 ± 5.5	0.5 ± 8.2	5.1 ± 7.7
Drug (P = 0.594, $\eta_p^2 = 0.029$, $\beta = 0.079$); Inspirate (P = 0.025, $\eta_p^2 = 0.409$, $\beta = 0.660$); Drug × Inspirate (P = 0.523, $\eta_p^2 = 0.042$, $\beta = 0.092$)				
Δ ctHb (μmol·L ⁻¹)	0.0 ± 0.0	-2.2 ± 10.6	4.9 ± 15.2	1.9 ± 13.7
Drug (P = 0.264, $\eta_p^2 = 0.123$, $\beta = 0.188$); Inspirate (P = 0.438, $\eta_p^2 = 0.061$, $\beta = 0.113$); Drug × Inspirate (P = 0.854, $\eta_p^2 = 0.004$, $\beta = 0.053$)				
OI (%)	0.0 ± 0.0	-9.2 ± 5.9	2.8 ± 6.2	-9.5 ± 4.1
Drug (P = 0.369, $\eta_p^2 = 0.074$, $\beta = 0.138$); Inspirate (P = <0.001, $\eta_p^2 = 0.879$, $\beta = 1.000$); Drug × Inspirate (P = 0.182, $\eta_p^2 = 0.156$, $\beta = 0.256$)				

Values are mean ± SD; MCA_v: middle cerebral artery blood velocity; sMCA_v: systolic MCA_v; dMCA_v: diastolic MCA_v; PI: pulsatility index; CVRI: cerebrovascular resistance index; CVCI: cerebrovascular conductance index; CDO₂: cerebral delivery of oxygen; Δ cO₂Hb: change in cortical oxy-hemoglobin concentration; Δ cHHb: change in cortical deoxy-hemoglobin concentration; Δ ctHb: change in cortical total hemoglobin concentration; OI: oxygenation index. *P < 0.05 vs. normoxia; †P < 0.05 vs. normoxia - sildenafil. n = 12 for all parameters.

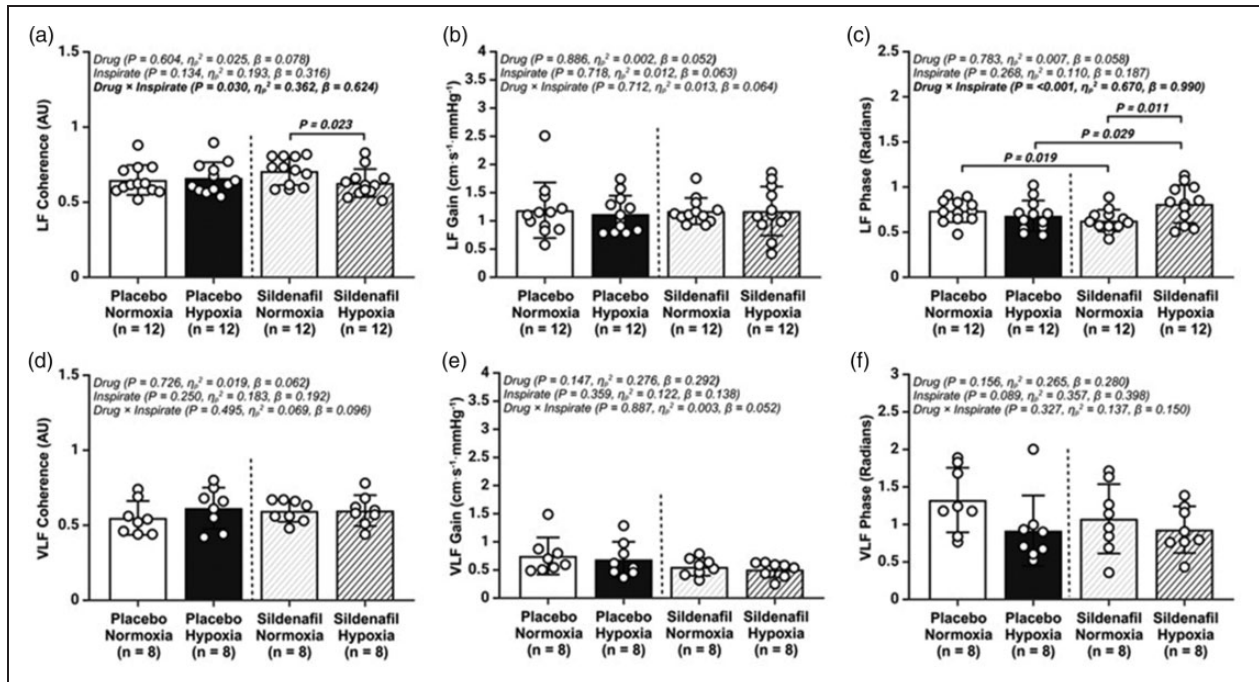


Figure 3. Transfer function analysis of the cerebral pressure-flow relationship during spontaneous oscillations in mean arterial blood pressure (MAP) and middle cerebral artery velocity (MCAv). (a) low frequency (LF) Coherence; (b) LF Gain; (c) LF Phase; (d) very low frequency (VLF) Coherence; (e) VLF Gain and (f) VLF Phase; AU, arbitrary units; Values are mean \pm SD. $n = 12$ for LF parameters, whereas $n = 8$ for VLF data.

hypocapnia-mediated cerebral vasoconstriction (i.e. PET_{CO₂} invariant), sildenafil increased LF phase and restored the hypoxia-induced attenuation in CVR_{CO₂Hyper}. Collectively, these findings provide evidence for a PDE-5 inhibitory pathway that enhances select aspects of cerebrovascular function in hypoxia, subsequent to a systemic improvement in redox homeostasis that is independent of systemic NO bioavailability.

Molecular function

Owing to the low reduction potential for the A^{•-}/ascorbate monanion (AH⁻) couple, any oxidizing radical (R[•]) present within the systemic/cerebral circulation will react with AH⁻ to form A^{•-} (R[•] + AH⁻ → A^{•-} + R-H, $E^0 = 282$ mV).³⁰ The EPR spectroscopic detection of A^{•-} thus provides the first direct and unequivocal evidence for reduced systemic free radical formation in hypoxia following PDE-5 inhibition. Despite the inverse relationship observed between free-radical formation and NO bioavailability under conditions of acute, albeit more severe, hypoxia,^{22,25,45} this did not translate to a corresponding elevation in systemic NO bioavailability following sildenafil administration in hypoxia. Despite lower total plasma NO bioavailability observed under conditions of hypoxia (primarily attributed to lower NO₂⁻), it should be noted that the vasodilatory properties of NO₂⁻ in hypoxia are greatly

enhanced.^{46,47} Coupled with sildenafil, a highly selective inhibitor of cGMP-specific PDE-5 that enhances NO-mediated smooth muscle relaxation, it is plausible that this may result in a 'supercharged' milieu to optimize cerebral bioenergetic homeostasis.

Cerebrovascular function

The functional recovery of hypoxia-induced cerebrovascular impairments following sildenafil administration, taking the form of elevated CVR_{CO₂HYPER} and a less pressure-passive dCA response (increased LF phase) are equally novel findings. Not only does sildenafil have the capacity to cross the blood-brain-barrier⁶ and act directly upon the central nervous system where PDE-5 has been localized to neurons and glial cells,⁶ sildenafil can also target PDE-5 within cerebrovascular endothelial cells.⁹ Accordingly, the NO/cGMP signal pathway has been shown to inhibit neuroinflammation, increase CBF and promote neurogenesis and synaptic plasticity.^{48,49}

Additionally, these findings may be attributed to sympathetic hyperactivity following sildenafil administration in hypoxia,^{50,51} given that ganglion blockade with trimethaphan diminishes dCA⁵² and cerebral vasomotor reactivity⁵³. Although sympathetic nervous activity (SNA) was not assessed directly in the present study, we did observe an elevation in heart rate with

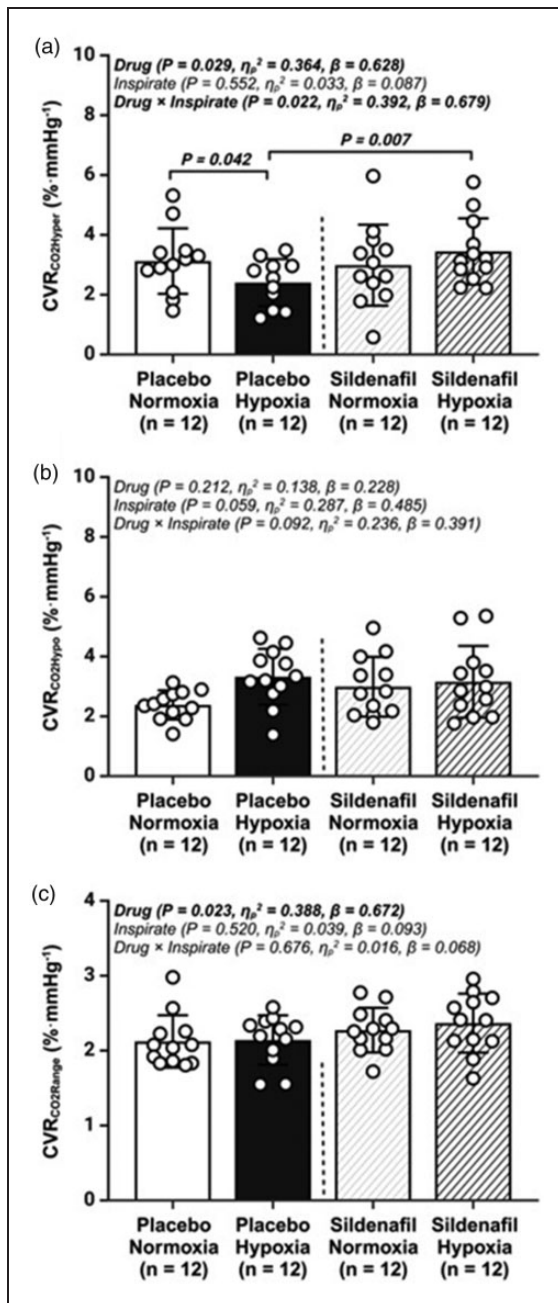


Figure 4. Cerebrovascular reactivity to carbon dioxide (CVR_{CO2}). (a) cerebrovascular reactivity to hypercapnia (CVR_{CO2Hyper}); (b) cerebrovascular reactivity to hypocapnia (CVR_{CO2Hypo}) and (c) cerebrovascular reactivity to carbon dioxide range (CVR_{CO2Range}); Values are mean \pm SD. $n = 12$ for all parameters.

sildenafil, indicative of increased sympathetic tone.⁵⁴ Equally, whilst we did not observe any changes in TFA Gain estimates in either the low or very-low frequency bands, the use of Phase estimates alone has been shown to be a more reliable measure of dCA than Gain in clinical studies.^{43,55–57}

It should be noted that while our findings under conditions of normoxia are supported by previous research,⁵⁸ there does exist a discrepancy with that of Jahshan, et al.⁵⁹ who demonstrated sildenafil to increase CVR_{CO2HYPER} without affecting basal cerebrovascular tone or static/dynamic indices of CA. These discrepancies may be related to differences in drug dosage/timing notwithstanding technical nuances associated with the analytical assessment of CVR_{CO2HYPER} and dCA. In our study, we administered 50 mg of sildenafil in normoxia and employed a 3-min inhalation of 5% CO₂ with 21% O₂ (balanced nitrogen) for the assessment of CVR_{CO2HYPER} and 5-min of spontaneous oscillations in MAP/MCAv for dCA. In contrast, Jahshan, et al.⁵⁹ combined 100 mg of sildenafil (in normoxia), with a carbogen mixture consisting of 5% CO₂ with 95% O₂ to assess CVR_{CO2HYPER} and graded intravenous infusions of phenylephrine to assess dCA. Extending prior research findings employing nitric oxide (NO) donation/blockade,^{41,60,61} Jahshan, et al.⁵⁹ concluded that sildenafil-induced amplification of the NO-cGMP signaling pathway contributes solely to CVR_{CO2}, without affecting the static and dynamic pressure-dependent mechanisms of CA. That we demonstrate tentative evidence of improved dCA [elevated (LF) Phase] is novel, albeit constrained by the interpretive deficiencies associated with spontaneous (as opposed to forced) oscillations and consistent with our prior contention that dCA is subject to redox-regulation.^{24,45} Further research is warranted to further explore functional integration of the NO-CVR_{CO2}-CA axis.

Clinical importance

The findings from this study are clinically relevant given that free radical-mediated impairments in cerebrovascular function caused by arterial hypoxaemia precede myriad infectious and degenerative neurological disorders.⁶² While this is the first investigation to explore the cerebrovascular implications of sildenafil under controlled conditions of acute inspiratory hypoxia utilizing a placebo-controlled cross-over design, these findings are commensurate with research in those with existing cerebrovascular impairments. In patients with pulmonary hypertension that are characterized by lower CBF and cerebrovascular reactivity, sildenafil enhanced visually-evoked flow responses indicating improved neurovascular coupling.¹⁵ More recently, a single dose (50 mg) of sildenafil has been shown to increase global CBF and CMRO₂ in patients with Alzheimer's Disease¹⁰ and increase CVR_{CO2} in traumatic brain injury (TBI) patients.⁶³ The protective effects of sildenafil in these patients could be attributed to lower oxidative stress and enhanced potentiation of

endothelial derived NO in sufficiently damaged microvasculature proving to be the unifying mechanism to explain these clinically relevant findings.

Limitations

The present study has several limitations that warrant consideration. First, MCAv represents an indirect surrogate measure of CBF and given that MCA diameter appears to increase in hypoxia⁶⁴, we cannot rule out the possibility that we underestimated the volumetric changes incurred by hypoxia and sildenafil. In support, the selective reduction observed in MCAv during the sildenafil hypoxic trial may reflect an increase in CBF subsequent to enhanced vasodilation, highlighting the importance of duplex imaging of global CBF (flow and velocity metrics of internal carotid and vertebral arteries) in future studies. Second, there has been an ongoing debate regarding the best method to quantify dCA and in particular, the use of spontaneous versus forced oscillations on blood pressure as the TFA input. The present study utilized spontaneous oscillations in blood pressure which is arguably more reflective of basal activity, safer to reproduce in a clinical population and importantly, does not challenge either the autonomic or cardiorespiratory systems.⁶⁵ However, this approach assesses the minimal level of blood pressure variations leading to lower TFA coherence and diminished linearity within the cerebral pressure-flow relationship.⁶⁶ Alternatively, it has been recommended that the TFA input should utilize forced oscillations in blood pressure (i.e. repeated squat-stands) to increase the signal-to-noise ratio,⁴³ arguing that this reflects everyday challenges associated with large swings of blood pressure (i.e. bending down, standing up, walking upstairs) and that owing to the high coherence (e.g. close to 1.0), the changes in phase and gain are interpretable.⁶⁶ However, we have consistently identified that >50% of young, healthy participants experience vasovagal syncope and associated cephalalgia during forced oscillations at an $F_{I}O_2$ of 0.12 (DM Bailey et al, unpublished observations), hence why we constrained our analyses to spontaneous oscillations only. Lastly, these findings are constrained to that of normobaric hypoxia with comparison to chronic high-altitude (hypobaric hypoxic) accordingly difficult owing to different physiological responses.^{67,68} However, we, among others, have demonstrated that acclimatization to high-altitude hypoxia increases basal systemic nitric oxide bioavailability^{33,69} which is responsible, at least in part for, increasing cerebral substrate delivery and preserving bioenergetic function consistent with the conservation of mass principle.⁷⁰ An improvement in cerebral redox homeostasis may represent the unifying mechanism that explains the

functional improvements in cerebral oxygenation documented by Chan, et al.¹⁸ following sildenafil administration after chronic exposure to high-altitude.

Conclusion

The present study has demonstrated that PDE-5 inhibition has the potential to restore redox homeostasis, namely attenuate systemic free radical formation without altering vascular NO bioavailability and recover select impairments in cerebral hemodynamic function following acute exposure to poikilocapnic hypoxia. These findings are clinically relevant given that free radical-mediated impairments in cerebrovascular function caused by arterial hypoxaemia precede myriad infectious and degenerative neurological disorders.

Data availability statement

Original data arising from this research are available directly from Professor Damian Miles Bailey upon reasonable request.

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Authors' contributions

All experiments were conducted in the Neurovascular Research Laboratory at the University of South Wales. B.S.S and D.M.B conceived and designed the research. B.S.S, C.J.M, H.T, T.G.D, T.S.O, T.A.C, L.F, A.I, I.L, J.M.C, M.S and D.M.B contributed to the acquisition, analysis and interpretation of data. B.S.S and D.M.B drafted the manuscript, and all authors revised it critically and contributed intellectual content. B.S.S, C.J.M, H.T, T.G.D, T.S.O, T.A.C, L.F, A.

I, I.L., J.M.C., M.S. and D.M.B. approved the final version of the manuscript, agree to be accountable for all aspects of the work, and will ensure that any questions concerning the accuracy or integrity of any part of this work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

ORCID iDs

Benjamin S Stacey  <https://orcid.org/0000-0003-1005-7038>
 Hayato Tsukamoto  <https://orcid.org/0000-0001-5512-0497>

Thomas S Owens  <https://orcid.org/0000-0003-0097-0616>

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