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# Cerebral Vasomotor Reactivity to Carbon Dioxide Using the Rebreathe Technique: Assessment of Within-day and Between-day Repeatability

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Running Head: Repeatability of cerebral vasomotor reactivity

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## Abstract

The cerebral vasodilator response to increased arterial carbon dioxide ($CO_2$) concentration, termed cerebral vasomotor reactivity (CVMR), is used to assess cerebral vascular function. We sought to assess the within-day and between-day repeatability of CVMR to rebreathing-induced hypercapnia. Twelve healthy adults performed a *within-day short interval* protocol (17±2 minutes between trials), ten performed *a within-day long interval* protocol (145±16 minutes between trials), and seventeen performed a *between-day* protocol (5±2 days between visits). Repeatability of the slope of the percent change in middle cerebral artery mean blood velocity (%$MCAv_{mean}$) and cerebral vascular conductance index (%CVCi), to the change in partial pressure of end-tidal $CO_2$ ($P_{ET}CO_2$) between the two trials/days was assessed. *Within-day short interval* %$MCAv_{mean}$ slope demonstrated fair to excellent repeatability (intraclass correlation, ICC=0.92 [95% confidence interval 0.72–0.98]; $p<0.001$) while %CVCi slope showed more variability (ICC=0.84 [0.47–0.95]; $p=0.002$]). *Within-day long interval*, %$MCAv_{mean}$ (ICC=0.95 [0.80–0.99]) and %CVCi (ICC=0.94 [0.71–0.99]) slopes showed good to excellent and fair to excellent repeatability respectively ($p<0.001$ for both). For *between-day* trials, better repeatability was observed for %CVCi (ICC=0.85 [0.57–0.95]; $p<0.001$) compared to %$MCAv_{mean}$ (ICC=0.76 [0.33–0.91]; $p=0.004$) slope. These findings indicate repeatable *within- and between-day* CVMR responses to rebreathe induced hypercapnia. However, a longer interval may be better for *within-day* repeat trials, particularly for CVCi measures.

## New and Noteworthy

The cerebral vasodilator response to increases in arterial carbon dioxide concentration, termed cerebral vasomotor reactivity, provides an index of cerebral vascular function/health. Reduced responses are present in populations with elevated cerebral vascular & neurocognitive disease risk / overt disease. Cerebral vasomotor reactivity is often assessed during rebreathing induced hypercapnia. This study determined that the day-to-day and between-day variability in this response is repeatable, thereby providing important methodological information to the scientific community.

**Keywords:** cerebrovascular function, cerebral vascular reactivity, middle cerebral artery blood velocity, rebreathing-induced hypercapnia, transcranial doppler ultrasound

## Introduction

Cerebral blood flow is tightly regulated to meet the metabolic demands of the brain. One intrinsic mechanism that ensures this precise regulation is the alteration of cerebral vascular tone in response to changes in arterial blood gases (1, 2). Assessment of cerebral vasomotor reactivity (CVMR) to increases in arterial carbon dioxide ($P_aCO_2$) concentration is a common methodological approach that provides an index of cerebral vascular function/health. Importantly, attenuated responses to increases in $P_aCO_2$ is predictive of future risk of stroke and higher mortality (3, 4). In addition, CVMR is blunted in several clinical conditions such as diabetes (5, 6), hypertension (6), Alzheimer's disease and related dementias (7–10), and in populations with elevated risk for various cerebral vascular and neurocognitive conditions such as obesity (11), hypercholesterolemia (12), and non-Hispanic Black individuals (13, 14).

Different techniques are utilized to manipulate $P_aCO_2$ for the assessment of CVMR, including acetazolamide administration (15, 16), breath holding (17, 18), administration of a fixed inspired $CO_2$ concentration (16, 19), dynamic end-tidal forcing (20, 21), and prospective end-tidal targeting (22, 23). We and others have utilized the rebreathing technique to assess CVMR in healthy individuals and in various populations with elevated risk for/prevalence of various neurocognitive conditions and cerebral vascular diseases (14, 19, 24–30). In this approach, participants rebreathe their expired air from a rebreathing bag for a fixed time duration or until a target increase in end-tidal partial pressure of $CO_2$ ($P_{ET}CO_2$), an index of $P_aCO_2$, is reached (14, 25, 26, 31–33). The rebreathing technique has been utilized in crosssectional (9, 14, 24, 32) as well as short-term (13, 28, 34) and long-term (27, 30) interventional studies. However, to the best of our knowledge, the repeatability of CVMR determined using this protocol both within a study visit and between study visits has not been thoroughly investigated. One previous study reported a poor relationship (intraclass correlation: ICC 0.31) between middle cerebral artery blood velocity and rebreathing induced hypercapnia (35). However, this study did not report cerebral vascular conductance, which is the more appropriate variable when assessing cerebral vascular responses as it normalizes for the impact of hypercapnia on arterial blood pressure (36). Also, the repeatability of several techniques each separated by 5 minutes were assessed thereby introducing the possibility of a carryover effect between trials (35). Therefore, in light of the importance of rigor and reproducibility, combined with the scarcity of published data establishing the repeatability of CVMR to rebreathing-induced hypercapnia, it is critical to assess the variability among repeat measures within an individual for both internal and external validation of studies utilizing this technique.

Accordingly, we investigated the within-day and between-day repeatability of CVMR during rebreathing-induced hypercapnia. Within-day repeatability was assessed between two rebreathing-induced hypercapnic trials separated by ~ 15 minutes (*within-day short interval*) and ~ 145 minutes (*within-day long interval*). The short interval represents a time interval that could be used in scenarios where the acute effect of a perturbation (e.g., exercise, baroreceptor unloading, cognitive challenge etc.) (37, 39) is of interest or a repeat trial is necessary due to methodological issues encountered in the first trial. The long interval was selected as this is an approximate time frame that is often used in interventional studies (e.g., the effect of eating a meal (13, 34), pharmacological intervention, whole-body heat therapy (28) etc.).

## Materials and Methods

### *Study population*

Twenty-two healthy young adults volunteered to participate. Twelve individuals (age 25 ± 5 yr; BMI 25.5 ± 3.8 kg/m$^2$; six male) performed the *within-day repeatability - short interval* protocol, ten individuals (age 24 ± 4 yr; BMI 25.8 ± 4.8 kg/m$^2$; ten male) participated in the *within-day repeatability - long interval* protocol, and seventeen individuals (age 24 ± 4 yr; BMI 25.8 ± 4.4 kg/m$^2$; 11 male) participated in the *between-day repeatability* protocol. Some of the data collected for the *within-day long interval* protocol and *between-day* protocol have been previously published as part of a larger data set (34); however, the aims of this previous publication differed from the current investigation. All participants were non-smokers, were not on any prescription medication, and reported having no cardiovascular, cerebrovascular, metabolic, or neurological diseases. Female participants reported on their last menstrual period date. All female participants except one were in the same phase of the menstrual cycle for both visits during the between visit assessments. Experiments were performed in a temperature-controlled (20°C–22°C) dimly lit room. Participants reported to the laboratory following a minimum 4 hr fast and were instructed to abstain from caffeine and over-the-counter medication for at least 12 hrs, and alcohol and strenuous exercise for at least 24 hrs before the study. After receiving a detailed verbal and written explanation of the experimental protocol, participants provided informed written consent. All experimental procedures conformed to the Declaration of Helsinki, except for registration in a database, and were approved by the Institutional Review Board at the University of Texas at Arlington (IRB 2016-0740 and 2020-0050).

### *Experimental Protocol*

Participants were instrumented with a standard lead II electrocardiogram (model Q710, Quinton, Bothell, WA or CardioCard; Nasiff Associates; Central Square, NY, USA) to continuously measure heart rate and a pneumobelt (Pneumo- trace II 1132, UFI, Morro Bay, CA) to monitor respiration. Intermittent brachial artery blood pressure (BP) was obtained using an automated sphygmomanometer (Welch Allyn; Skaneateles Falls, NY or Tango M2; SunTech Medical; Morrisville, NC). Beat-to-beat arterial BP was measured using finger photoplethysmography following return-to-flow calibration (Finometer PRO, Finapres Medical Systems, Amsterdam, The Netherlands). Mean middle cerebral artery blood velocity (MCAv$_{mean}$) was measured using a 2-MHz transcranial doppler (TCD) ultrasound probe (Multigon Industries Inc., Yonkers, NY) which was held in place over the temporal window using a headband. Participants wore a nose clip and breathed through a mouthpiece attached to a 3-way valve (Hans Rudolph Inc., Kansas City, KS) connected to a 5-L rebreathing bag pre-filled with the participants expired air. A sampling line attached to the mouthpiece and a finger pulse oximeter were used to measure $P_{ET}CO_2$ and arterial oxygen saturation ($SpO_2$) respectively via a capnograph (Capnocheck Plus, Smiths Medical, Dublin, OH). All MCAv$_{mean}$ signals were obtained by personnel who are trained with this technique. Furthermore, within each participant all signals were obtained by the same individual with consultation with other research personnel to ensure the highest quality signal was acquired.

The rebreathing-induced hypercapnia protocol was performed as described previously (24, 32, 34). Briefly, participants breathed room air through the mouthpiece for a minimum 3-minute baseline period. Then the valve was turned such that participants were exposed to rebreathing for 3 minutes. If they did not reach a $\Delta P_{ET}CO_2$ of at least +15 mmHg during that time and participant tolerated well, the rebreathing was continued up to a $\Delta P_{ET}CO_2$ of +15 mmHg. The rebreathe was terminated earlier, if the participant signaled to stop (range of time of the rebreathe for all trials: 102 – 305 minutes). Importantly, data analyses within an individual all comparisons were conducted up to a similar degree of hypercapnia between trials. Once terminated, the valve was then switched back to allow the participant to breathe room air for up to 3 minutes of recovery. Throughout the rebreathe protocol, 100% oxygen was continuously administered into the 5-L bag to maintain arterial normoxia ($S_pO_2$ ~97%) (29). Heart rate, respiration, beat-to-beat BP, $MCAv_{mean}$, $P_{ET}CO_2$, and $SpO_2$ were continuously recorded throughout the protocol at 1000Hz using Powerlab (ADInstruments, Colorado Springs, CO, USA) for offline analysis. The rebreathing protocol was repeated for each experiment as follows:

*Within-day repeatability – short interval:* The rebreathing protocol was performed twice with ~ 15-minute interval from end of the first trial to the start of the second trial. This time interval was chosen as it represents a common interval between experimental protocols when making repeat measurements (e.g., flow mediated dilation) or following acute perturbations (e.g., handgrip exercise, baroreceptor unloading) (37, 39). The participant rested quietly and remained instrumented during the interval.

*Within-day repeatability – long interval:* The rebreathing protocol was performed twice with ~ 145-minute interval from end of the first trial to the start of the second trial. This time interval was chosen as it is a period that is often utilized to assess the impact of an intervention (13, 28, 34). During this period, the instrumentation was removed for subject comfort. Aside from a restroom break (if needed), the participant rested quietly during the interval.

*Between-day repeatability:* participants performed the same experimental protocol on two days separated by up to seven (range 3 – 7) days. Trials were performed at the same time of day (11 ± 12 minute difference between days) with identical pre-experiment instructions given.

### ***Data analysis***

Heart rate, beat-to-beat mean arterial pressure (MAP), $P_{ET}CO_2$, respiratory rate, $MCAv_{mean}$, and cerebral vascular conductance index (CVCi = $MCAv_{mean}$/MAP) were averaged during the final minute or for a minimum of 10 respiratory cycles of the baseline period, whichever was longer. Finometer-derived beat-to-beat MAP was calibrated to the average of two automated baseline BP measurements to ensure that Finometer-derived BP measurements matched the absolute value of BPs obtained from the automated sphygmomanometer. CVMR was quantified as the linear regression slope of $\Delta MCAv_{mean}\%$ and ΔCVCi% vs. $\Delta P_{ET}CO_2$ from baseline using breath-by-breath data. Importantly, within each individual, these data were calculated up to the highest common $\Delta P_{ET}CO_2$ that was reached during both trials (Trial 1 & Trial 2) for each condition.

### *Statistical Analysis*

Data were analyzed using statistical software (SPSS v25 and MedCalc® v22.026). Group data are presented as mean ± SD. Paired sample t-tests were used to compare outcome measures between trials/visits. Repeatability was assessed using intraclass correlation (ICC; two-way mixed effects, average measures, and absolute agreement) and 95% confidence intervals (95% CI). The relative degree of repeatability was quantified as follows: ICC<0.50 = poor, 0.50 to 0.75 = fair, 0.76 to 0.90 = good, and 0.91 to 1.00 = excellent (38). Data are also presented using Bland-Altman plots with calculated bias and 95% limits of agreement, and correlation plots with correlation coefficient (r) and coefficient of determination ($R^2$). The typical error of measurement (TEM and TEM%; i.e., within person standard error of the measurement) (40) and coefficient of variation (CV%) with 95% CI are also presented as additional indices of repeatability. Significance level was set at $\alpha < 0.05$.

## Results

Mean data and reproducibility indices (paired t-test, ICC, TEM, CV) for all resting cardiovascular, respiratory, and cerebrovascular measures are presented in Table 1. Resting heart rate, MAP, respiratory rate, $MCAv_{mean}$, CVCi, and $P_{ET}CO_2$ were not different for within-day or between-day trials ($p>0.05$ for all). Representative data from one participant for the change in arterial BP, $MCAv_{mean}$, and $P_{ET}CO_2$ during the rebreathing-induced hypercapnia, and the %CVCi slope derived during each protocol are presented in Figure 1.

### *Within-day repeatability - short interval*

The interval between the two trials was 17±2 minutes. The cardiorespiratory and cerebrovascular responses to the highest common level of hypercapnia reached during the 2 trials (within each individual) are shown in Table 2. There were no differences for the $MCAv_{mean}$ slope (3.15±1.50 vs. 3.11±1.38 %/mmHg; $p=0.865$, effect size = 0.05) or CVCi slope (1.64±1.26 vs. 2.03±1.12 %/mmHg; $p=0.135$, effect size = 0.5) between trial 1 and trial 2. The repeatability of $MCAv_{mean}$ slope between trial 1 and trial 2 ranged from fair to excellent (Figure 2A and C; TEM = 0.6; TEM% = 18.4; CV [95%CI] = 17.5% [10.3% to 22.4%]) while the CVCi slope was more variable and ranged from poor to excellent (Figure 2B and D; TEM = 0.6; TEM% = 32.5%; CV [95%CI] = 46.1% [16.6 to 63.1]). Slopes for absolute changes are presented in Table 3.

### *Within-day repeatability - long interval*

The interval between the two trials was 145±16 minutes. The cardiorespiratory and cerebrovascular responses to the highest common level of hypercapnia reached during the 2 trials (within each individual) are shown in Table 2. $MCAv_{mean}$

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and CVCi slopes were not different between trial 1 and trial 2 ($MCAv_{mean}$: 3.21±1.09 vs. 3.23±1.11 %/mmHg; p=0.930; effect size = 0.04; CVCi: 2.15±1.06 vs. 2.45±1.07 %/mmHg; p=0.072; effect size = 0.7). For $MCAv_{mean}$ and CVCi slopes, the repeatability ranged from good to excellent (Figure 3A and C; $MCAv_{mean}$ slope: TEM = 0.3; TEM% = 10.8; CV [95%CI] = 10.4% [6.2% to 13.3%]) and fair to excellent (Figure 3B and D; CVCi slope: TEM = 0.3; TEM% = 14.1; CV [95%CI] = 40.2% [0.0% to 69.0%]) respectively. Slopes for absolute changes are presented in Table 3.

### *Between-day repeatability*

The duration between the two visits was 5±2 days. The cardiorespiratory and cerebrovascular responses to the highest common level of hypercapnia reached during the two trials (within each individual) are shown in Table 2. $MCAv_{mean}$ slope between visit 1 and visit 2 was not different (2.95 ± 1.09 vs. 2.97 ± 1.07 %/mmHg; p=0.899; effect size = 0.03). Likewise, the CVCi slope was also not different between visits (visit 1: 1.89 ± 1.05 vs. visit 2: 1.85 ± 0.91 %/mmHg; p=0.829; effect size = 0.06). The repeatability of the $MCAv_{mean}$ slope was somewhat variable and ranged from poor to excellent (Figure 4A and C; TEM = 0.7; TEM% = 23.0; CV [95%CI] = 29.0% [0.0% to 43.9%]) while the CVCi slope ranged from fair to excellent (Figure 4B and D; TEM = 0.5; TEM% = 27.6; CV [95%CI] = 42.1% [0.0% to 61.5%]). Slopes for absolute changes are presented in Table 3.

## Discussion

Many study designs dictate that a given physiological measurement is conducted multiple times within a study visit or between study visits. Rebreathing induced hypercapnia, which is used to assess cerebral vascular function, is an example of a research technique that is routinely conducted multiple times within a given study visit as well as between study visits. In the present study we found good repeatability of %MCAv$_{mean}$ and %CVCi slopes. Notably, the relationships were stronger when the trials were separated by a longer (~ 145 minutes) interval compared to a shorter (~ 17 minutes) interval within the same day. Furthermore, good repeatability of CVMR outcomes between two visits within the span of one week was observed, with %CVCi slope showing better repeatability compared to the %MCAv$_{mean}$ slope. Collectively, these findings provide support for the repeatability of this methodological approach and will be informative for future study designs incorporating assessments of CVMR. In this regard, a longer interval appears better for *within-day* repeat rebreathing trials.

### *Within-day Repeatability*

Assessment of changes in cerebral blood flow/velocity and CVCi to alterations in $P_{ET}CO_2$ is an established method of evaluating cerebral vascular function in health and disease (3, 4, 13, 14, 5–12). The repeatability of CVMR during other methodological approaches used to manipulate $P_aCO_2$ (e.g. acetazolamide administration, breath hold, and various methods of hypercapnic gas inhalation) has been previously reported (41, 42), whereas limited data are available for rebreathing-induced hypercapnia. To our knowledge, only one study has attempted to examine  repeatability of CVMR to rebreathing-induced hypercapnia (35). In this study, the ICC for MCAv responses when trials were performed within one hour was 0.31, which was interpreted as fair repeatability based on previous recommendations (43). However, it is notable that the increase in $P_{ET}CO_2$ ($\Delta P_{ET}CO_2$) in this previous study was only 7.5 mmHg (35). Therefore, repeatability of the response to higher $\Delta P_{ET}CO_2$ levels that are commonly reported using this approach (24, 26, 32) was not established. Moreover, CVC, which is an established measure of cerebral vascular tone and thus a primary marker of cerebral vascular function, was not reported (35). This is critical due to the potential impact of hypercapnia on arterial BP (36, 44). Lastly, the repeatability of several techniques was assessed with an interval of 5-minutes between the different trials (35), and therefore, a carryover effect from the previous trial likely affected the results.

We found that within-day repeatability for %$MCAv_{mean}$ slope was greater than previously reported when trials were performed with an interval of either 25 or 150 minutes (35). Interestingly, while both time periods yielded repeatable results, we found that the longer interval of 145 minutes between trials resulted in a higher level of repeatability than the shorter-interval of 17 minutes. Interindividual variability in the time to return to baseline ~~“recover”~~ from the first trial during the shorter-interval may have contributed to this difference. Although ~17 minutes appeared to be sufficient for reproducibility in most participants, it is possible a residual effect from the first trial was present in some subjects despite $MCAv_{mean}$ and MAP being back to pre-trial values. We also found that the repeatability of the %CVCi slope was largely similar to %$MCAv_{mean}$ slope following the *within-day long interval* protocol, whereas it was lower than the %MCAv slope following the *within-day short interval* protocol. While the reason for this is uncertain, this indicates that an interval between trials longer than ~17 minutes may be advantageous, when possible. While we did not assess time intervals between trials shorter than ~17 minutes, the present data indicates that this time interval is a reasonable target when longer intervals are not logistically possible. In this regard, the longer interval of 145 minutes investigated in the current study appears to be ideal when repeat trials are performed in interventional studies.

### *Between-day Repeatability*

In the current study, the two between-day trials were performed within one week (range: three to seven days). The relatively wide confidence limits of the ICC indicate variability in the response across individuals. Although we attempted to match the experimental conditions as much as possible within our limits, it is impossible to ensure the exact same physiological conditions in human research. For example, a number of factors can contribute to varying day-to-day physiological responses in cerebral and peripheral vascular function including: diet (34), stress exposure (45, 46), activity/sedentary behaviors (47, 48), sleep quantity & quality (49, 50), etc. Therefore, some degree of variability in the hemodynamic response is expected when performed on separate days. However, based on our data, the %CVCi slope showed better repeatability compared to the %$MCAv_{mean}$ slope, indicating that %CVCi slope is a more reliable measure of CVMR to rebreathing-induced hypercapnia when performing experiments on two separate days. Collectively, while keeping the limitations in mind, these data indicate that the rebreathing technique can be utilized in studies that need to assess cerebral vascular function on multiple study visits.

### *Comparisons to other Methodological Approaches*

The ICC of %MCAv$_{mean}$ slope following rebreathing-induced hypercapnia in the current study in the long-interval protocol was substantially greater than the ICC reported in a recent study that induced hypercapnia by administering 6% $CO_2$ and repeated trials with a similar interval (2.5 hrs) (42). Our ICC values are also higher than those reported for repeatability of MCAv response to 5% $CO_2$ inhalation and breath hold tests where the two trials were performed with an interval of one hr and 24 hrs (35). Collectively, these findings suggest that the reliability of CVMR to rebreathing-induced hypercapnia is higher than the CVMR to a breath holding test or steady state $CO_2$ inhalation.

### *Methodological Considerations*

There are considerations that are worth noting. First, sex hormones can exert influences on vascular function (51, 52). Although one female was tested in different phases of her cycle for the between day trials, removal of this individual did not change the interpretation of the data. Moreover, despite evidence of a difference in baseline cerebral blood flow (51, 53), evidence on differential cerebral vasodilator response across the menstrual cycle is lacking (54). Thus, further evaluation of repeatability across phases of the menstrual cycle is warranted. Second, TCD is inherently limited by the fact that it is not able to image the vessel and thus calculations of vessel diameter are not possible. Furthermore, this limitation is exacerbated at higher magnitudes of hypercapnia similar to those achieved in this study, which induces vasodilation of the middle cerebral artery (55). However, this limitation is minimized in the current study design because all comparisons between trials within conditions were made within each individual (i.e. they were their own control) at a similar degree of hypercapnia achieved among the trials being compared. Therefore, if there was an effect of hypercapnia on diameter (55) it would be expected to be similar between conditions. Thirdly, for the within-day long interval protocol, there was a standard deviation of 16 minutes for the duration between trials. However, this was unavoidable and was primarily due to logistics involved with re-instrumenting some participants and optimizing the TCD and Finometer signal for the second trial.

## PERSPECTIVES AND SIGNIFICANCE

The current investigation demonstrates within- and between-day repeatability of the cerebral vascular vasodilator responses to rebreathing induced hypercapnia. Indeed, while a 25-minute interval between trials conducted in the same day yields repeatable responses, the degree of variability is minimized when the interval period is extended. Given the need to ensure rigor and reproducibility in studies as emphasized by the National Institutes of Health (https://grants.nih.gov/reproducibility/index.htm), it is important to identify the inherent variability of different physiological measures and techniques and how this may impact the overall interpretation of data within a study and between studies. Collectively, the findings of the current study demonstrate that rebreathing-induced hypercapnia can be reliably used to assess cerebral vascular function. Moreover, this study in young healthy adults provides proof of concept for reliability of cerebrovascular response to rebreathing-induced hypercapnia and provides the foundation for future investigations on older adults and clinical populations.

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## DISCLOSURE/CONFLICT OF INTEREST

The Authors declare that there is no conflict of interest

## AUTHOR CONTRIBUTIONS

D.N., R.M.B., P.J.F., and Z.T.M. conceived and designed research; D.N., R.J.S., Z.T.M., J.C.P., Z.T., and A.O. performed experiments; D.N., R.J.S., and Y.K. analyzed data; D.N., R.J.S., Z.T.M., Y.K., P.J.F., and R.M.B., interpreted results of experiments; D.N. prepared figures; D.N. drafted manuscript; D.N., R.J.S., Z.T.M., J.C.P., Z.T., A.O., Y.K., P.J.F., and

R.M.B. edited and revised manuscript; D.N., R.J.S., Z.T.M., J.C.P., Z.T., A.O., Y.K., P.J.F., and R.M.B. approved final version of manuscript.

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## FIGURE LEGENDS

**Figure 1.** Representative data for arterial blood pressure (ABP), middle cerebral artery mean blood velocity ($MCAv_{mean}$), end-tidal carbon dioxide ($P_{ET}CO_2$) concentration, and slope of cerebral vascular conductance index (CVCi) for one participant in the *within-day repeatability short interval* protocol (panel A and B for trial 1 and trial 2 respectively), *within-day repeatability long interval* protocol (panel C and D for trial 1 and trial 2 respectively), and *between-day repeatability* protocol (panel E and F for visit 1 and visit 2 respectively).

**Figure 2.** Bland-Altman plots (A and B) and correlations (C and D) of slopes for middle cerebral artery mean blood velocity ($MCAv_{mean}$) and cerebral vascular conductance index (CVCi) for *within-day repeatability - short interval* protocol (n = 12; 6 males). The mean bias of the Bland-Altman plot is represented by the solid line (± SD). The thick dotted line represents the 95% limits of agreement of the bias and the thin dotted line at y = 0 represents perfect repeatability. r, Pearson's correlation coefficient between trial one and trial two. $R^2$, coefficient of determination.

**Figure 3.** Bland-Altman plots (A and B) and correlations (C and D) of slopes for middle cerebral artery mean blood velocity ($MCAv_{mean}$) and cerebral vascular conductance index (CVCi) for *within-day repeatability - long interval* protocol (n = 10; all males). The mean bias of the Bland-Altman plot is represented by the solid line (± SD). The thick dotted line represents the 95% limits of agreement of the bias and the thin dotted line at y = 0 represents perfect repeatability. r, Pearson's correlation coefficient between trial one and trial two. $R^2$, coefficient of determination.

**Figure 4.** Bland-Altman plots (A and B) and correlations (C and D) of slopes for middle cerebral artery mean blood velocity ($MCAv_{mean}$) and cerebral vascular conductance index (CVCi) for *between-day repeatability* protocol (n = 17; 11 males). The mean bias of the Bland-Altman plot is represented by the solid line (± SD). The thick dotted line represents

the 95% limits of agreement of the bias and the thin dotted line at y = 0 represents perfect repeatability. r, Pearson's correlation coefficient between trial one and trial two. $R^2$, coefficient of determination.

Figure 1

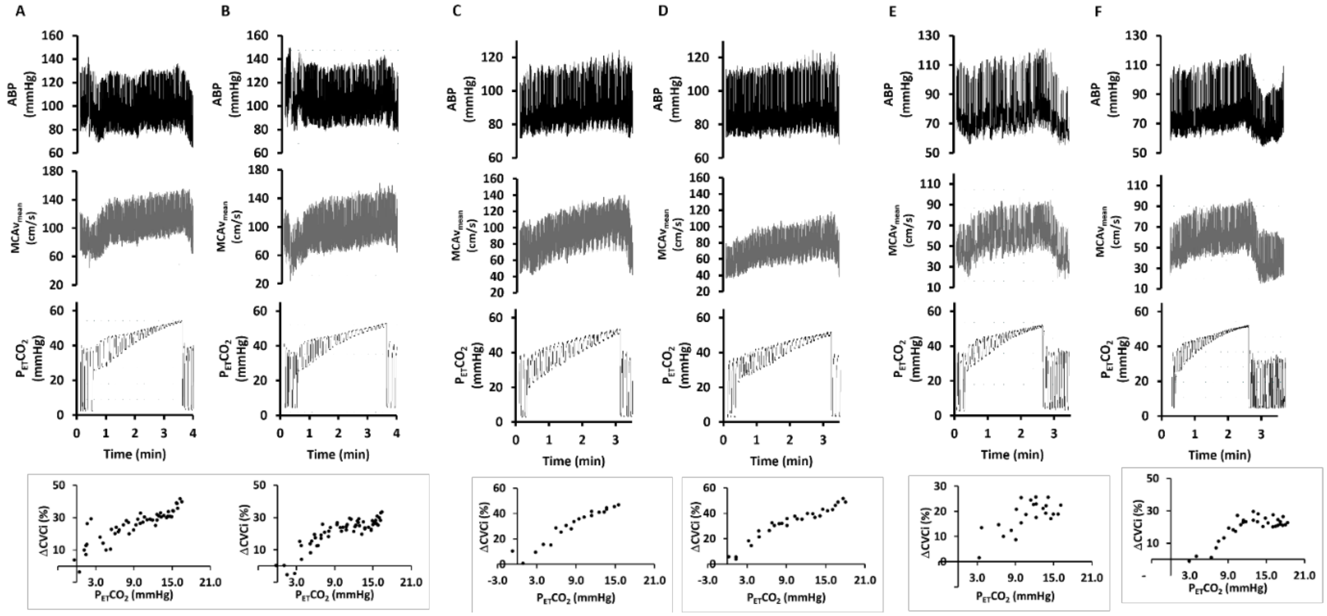
A
B
C
D
E
F
ABP (mmHg)
MCAv$_{mean}$ (cm/s)
$P_{ET}CO_2$ (mmHg)
Time (min)
ΔCVCi (%)
$P_{ET}CO_2$ (mmHg)
-3.0
3.0
9.0
15.0
21.0

Figure 2

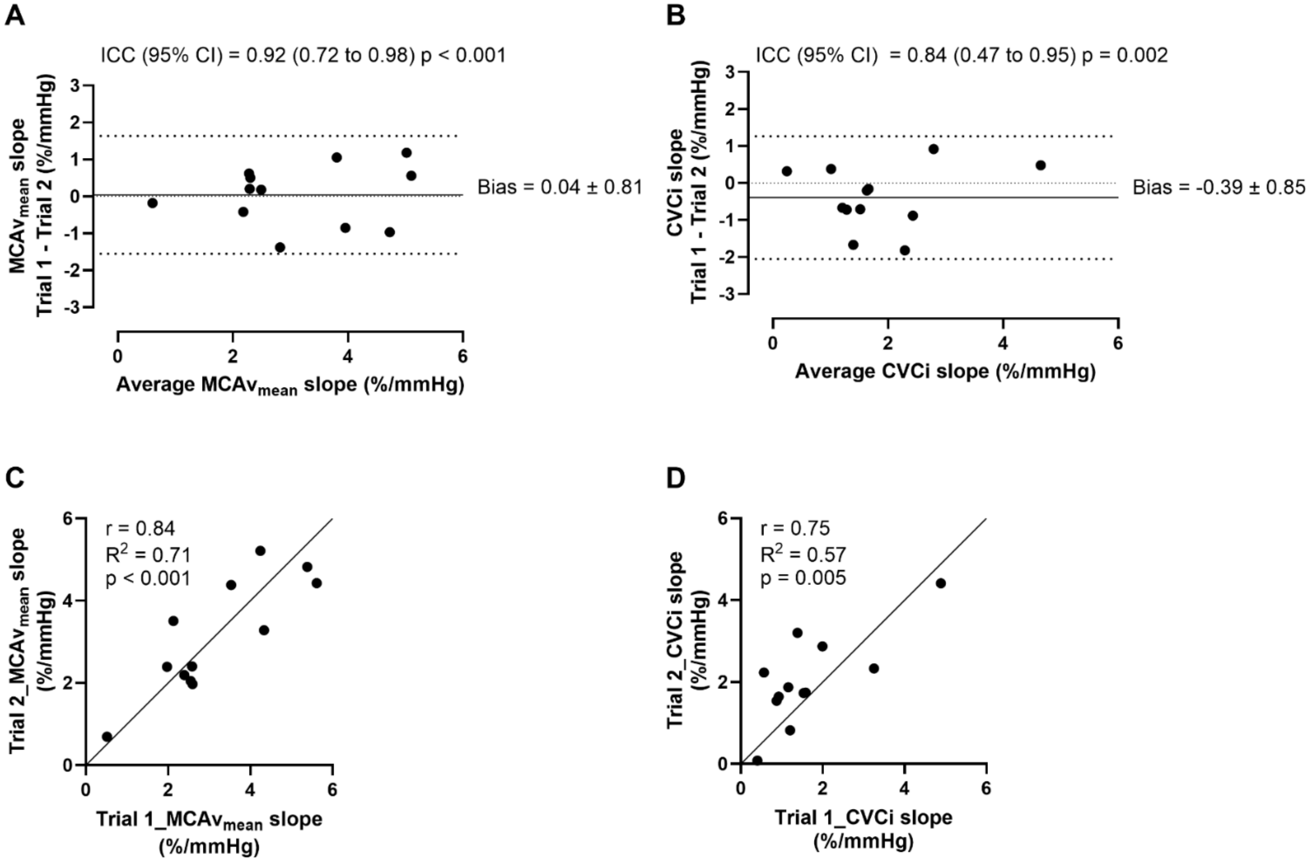
A
ICC (95% CI) = 0.92 (0.72 to 0.98) p < 0.001
MCAv$_{mean}$ slope
Trial 1 - Trial 2 (%/mmHg)
3
2
1
0
-1
-2
-3
Bias = 0.04 ± 0.81
0
2
4
6
Average MCAv$_{mean}$ slope (%/mmHg)
B
ICC (95% CI) = 0.84 (0.47 to 0.95) p = 0.002
CVCi slope
Trial 1 - Trial 2 (%/mmHg)
3
2
1
0
-1
-2
-3
Bias = -0.39 ± 0.85
0
2
4
6
Average CVCi slope (%/mmHg)
C
r = 0.84
R$^2$ = 0.71
p < 0.001
Trial 2_MCAv$_{mean}$ slope
(%/mmHg)
6
4
2
0
0
2
4
6
Trial 1_MCAv$_{mean}$ slope
(%/mmHg)
D
r = 0.75
R$^2$ = 0.57
p = 0.005
Trial 2_CVCi slope
(%/mmHg)
6
4
2
0
0
2
4
6
Trial 1_CVCi slope
(%/mmHg)

Figure 3

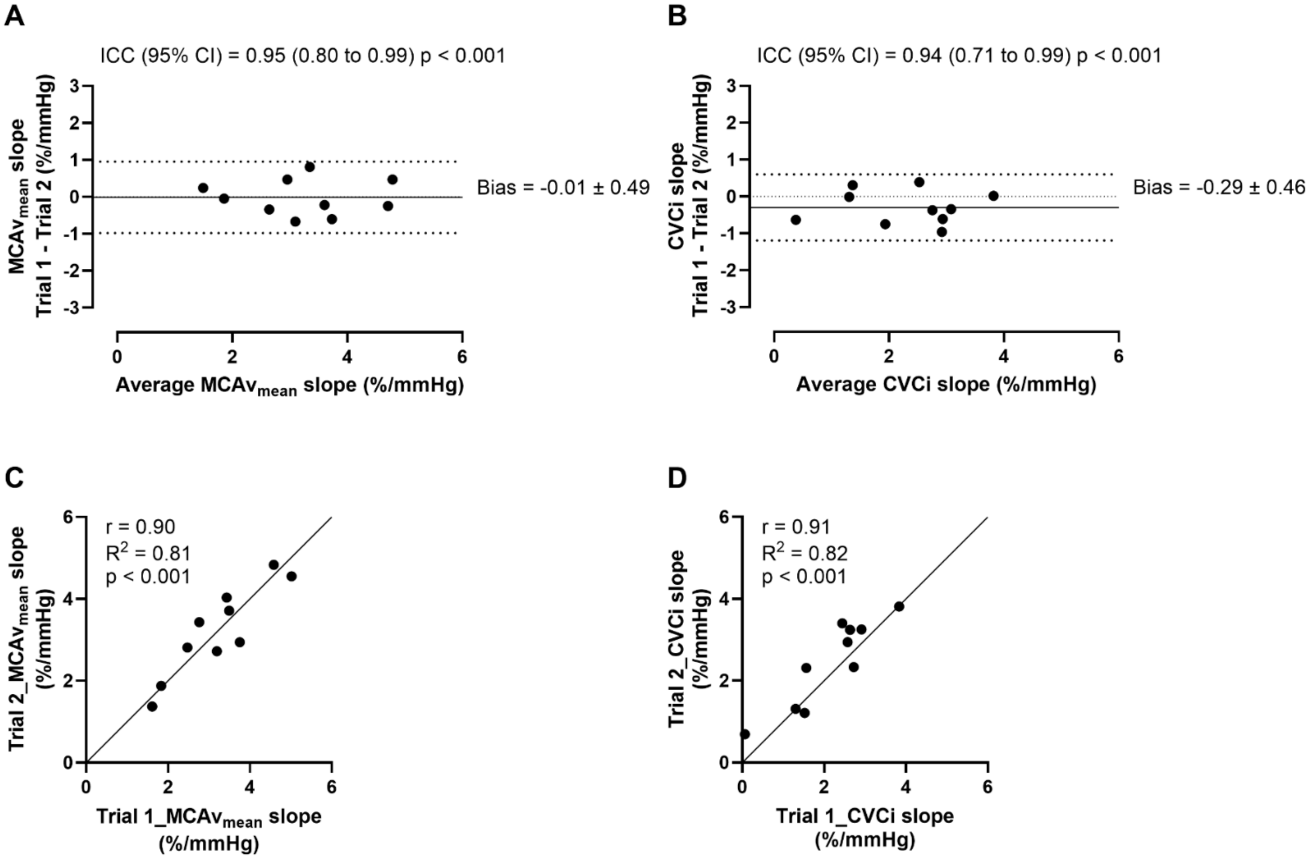
A
ICC (95% CI) = 0.95 (0.80 to 0.99) p < 0.001
MCAv$_{mean}$ slope
Trial 1 - Trial 2 (%/mmHg)
3
2
1
0
-1
-2
-3
Bias = -0.01 ± 0.49
0
2
4
6
Average MCAv$_{mean}$ slope (%/mmHg)
B
ICC (95% CI) = 0.94 (0.71 to 0.99) p < 0.001
CVCi slope
Trial 1 - Trial 2 (%/mmHg)
3
2
1
0
-1
-2
-3
Bias = -0.29 ± 0.46
0
2
4
6
Average CVCi slope (%/mmHg)
C
r = 0.90
R$^2$ = 0.81
p < 0.001
Trial 2_MCAv$_{mean}$ slope
(%/mmHg)
6
4
2
0
0
2
4
6
Trial 1_MCAv$_{mean}$ slope
(%/mmHg)
D
r = 0.91
R$^2$ = 0.82
p < 0.001
Trial 2_CVCi slope
(%/mmHg)
6
4
2
0
0
2
4
6
Trial 1_CVCi slope
(%/mmHg)

Figure 4

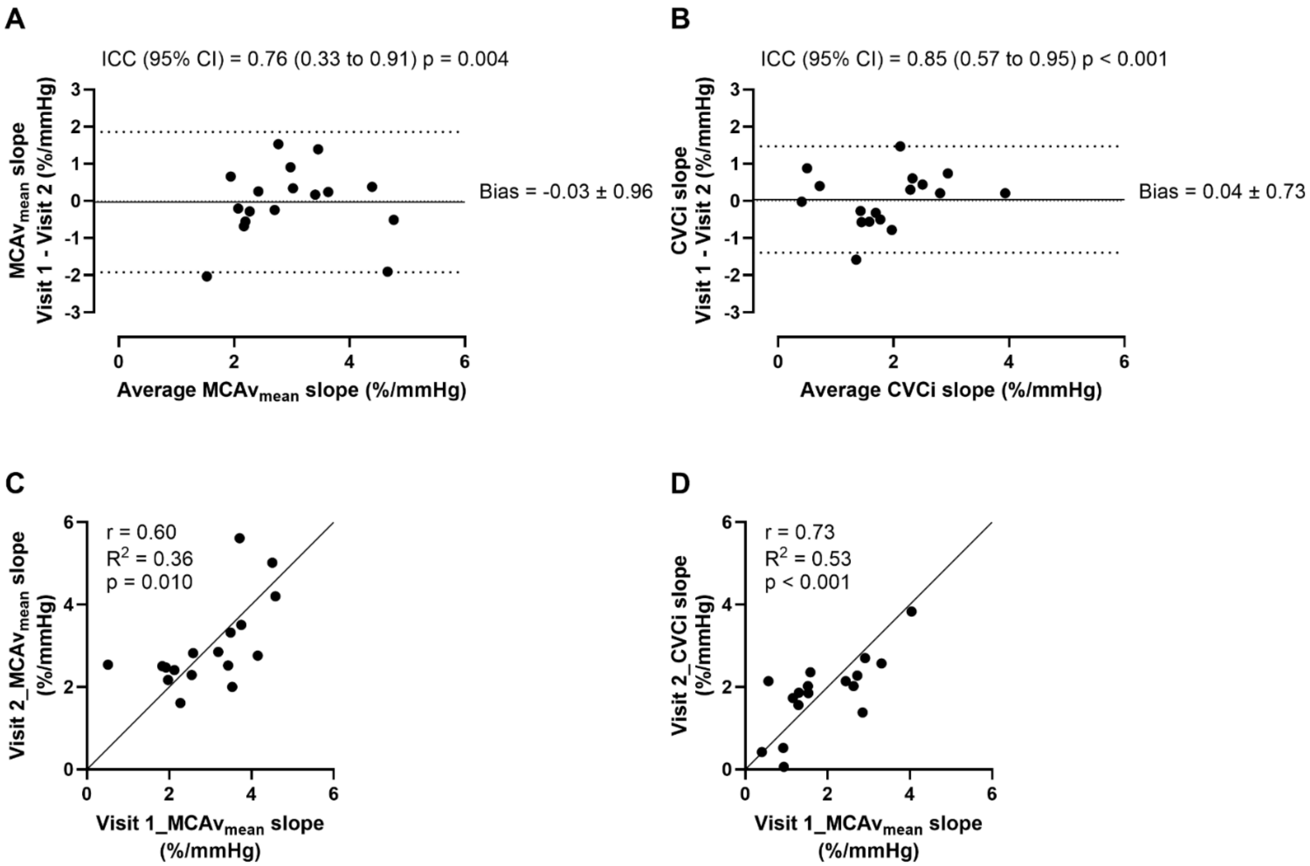
A
ICC (95% CI) = 0.76 (0.33 to 0.91) p = 0.004
MCAv$_{mean}$ slope
Visit 1 - Visit 2 (%/mmHg)
Bias = -0.03 ± 0.96
Average MCAv$_{mean}$ slope (%/mmHg)
B
ICC (95% CI) = 0.85 (0.57 to 0.95) p < 0.001
CVCi slope
Visit 1 - Visit 2 (%/mmHg)
Bias = 0.04 ± 0.73
Average CVCi slope (%/mmHg)
C
r = 0.60
R$^2$ = 0.36
p = 0.010
Visit 2_MCAv$_{mean}$ slope (%/mmHg)
Visit 1_MCAv$_{mean}$ slope (%/mmHg)
D
r = 0.73
R$^2$ = 0.53
p < 0.001
Visit 2_CVCi slope (%/mmHg)
Visit 1_MCAv$_{mean}$ slope (%/mmHg)
3
2
1
0
-1
-2
-3
0
2
4
6

Table 1. Resting cardiorespiratory and cerebrovascular measures

| | HR, beats/minute | MAP, mmHg | RR, Breaths/minute | MCAv$_{mean}$, cm/s | CVCi, cm/s/mmHg | PETCO$_2$, mmHg |
|---|---|---|---|---|---|---|
| Within-day short interval (n = 12) | | | | | | |
| Trial 1 | 68 ± 9 | 84 ± 7 | 13 ± 5 | 77 ± 16 | 0.93 ± 0.22 | 44 ± 4 |
| Trial 2 | 67 ± 11 | 86 ± 6 | 14 ± 5 | 79 ± 17 | 0.92 ± 0.21 | 44 ± 4 |
| Paired t-test p (effect size) | 0.568 (0.2) | 0.111 (0.5) | 0.119 (0.5) | 0.555 (0.3) | 0.712 (0.1) | 0.838 (0.0) |
| ICC (95% CI) | 0.92 (0.71 to 0.98)* | 0.91 (0.67 to 0.97)* | 0.96 (0.85 to 0.99)* | 0.97 (0.88 to 0.99)* | 0.96 (0.88 to 0.99)* | 0.96 (0.88 to 0.99)* |
| CV% (95% CI) | 5.7 (2.6 to 7.7) | 3.1 (1.7 to 4.1) | 11.9 (7.1 to 15.2) | 5.4 (3.8 to 6.5) | 5.8 (4.1 to 7.2) | 2.1 (1.3 to 2.6) |
| TEM (%TEM) | 4.2 (6.2) | 2.5 (2.9) | 1.3 (10.1) | 4.4 (5.6) | 0.1 (6.4) | 1.0 (2.2) |
| Within-day long interval (n = 10) | | | | | | |
| Trial 1 | 58 ± 5 | 83 ± 5 | 12 ± 3 | 68 ± 7 | 0.82 ± 0.08 | 41 ± 2 |
| Trial 2 | 58 ± 8 | 84 ± 6 | 12 ± 4 | 66 ± 8 | 0.79 ± 0.10 | 40 ± 2 |
| Paired t-test p effect size | 0.779 (0.0) | 0.446 (0.3) | 0.932 (0.0) | 0.257 (0.5) | 0.103 (0.5) | 0.137 (0.6) |
| ICC (95% CI) | 0.88 (0.48 to 0.97)* | 0.87 (0.48 to 0.97)* | 0.69 (-0.39 to 0.93) | 0.92 (0.71 to 0.98)* | 0.87 (0.47 to 0.97)* | 0.74 (0.09 to 0.93)* |
| CV% (95% CI) | 4.8 (1.6 to 6.7) | 3.2 (1.4 to 4.3) | 23.8 (0.0 to 37.8) | 4.5 (0.0 to 7.4) | 5.7 (0.0 to 9.2) | 2.9 (1.5 to 3.9) |
| TEM (%TEM) | 3.1 (5.3) | 2.8 (3.3) | 2.5 (20.8) | 2.8 (4.1) | 0.04 (5.1) | 1.1 (2.7) |
| Between day (n = 17) | | | | | | |
| Visit 1 | 64 ± 9 | 83 ± 6 | 13 ± 5 | 75 ± 14 | 0.90 ± 0.19 | 42 ± 3 |
| Visit 2 | 64 ± 11 | 82 ± 5 | 14 ± 5 | 73 ± 11 | 0.89 ± 0.16 | 42 ± 2 |
| Paired t-test p effect size | 0.771 (0.0) | 0.276 (0.3) | 0.229 (0.2) | 0.270 (0.3) | 0.484 (0.1) | 0.399 (0.0) |
| ICC (95% CI) | 0.94 (0.82 to 0.98)* | 0.87 (0.65 to 0.95)* | 0.84 (0.57 to 0.94)* | 0.91 (0.75 to 0.97)* | 0.95 (0.87 to 0.98)* | 0.79 (0.44 to 0.93)* |
| CV% (95% CI) | 5.2 (2.9 to 6.8) | 3.1 (2.2 to 3.9) | 20.4 (7.0 to 28.0) | 6.8 (3.1 to 9.1) | 5.9 (3.8 to 7.5) | 3.6 (1.2 to 5.0) |
| TEM (%TEM) | 3.5 (5.4) | 2.6 (3.1) | 2.3 (17.2) | 5.1 (6.9) | 0.1 (5.9) | 1.6 (3.7) |

Values are mean ± SD unless otherwise stated. HR, heart rate; MAP, mean arterial blood pressure; RR, respiratory rate; MCAv$_{mean}$, middle cerebral artery mean blood velocity; CVCi, cerebral vascular conductance index; $P_{ET}CO_2$, end-tidal partial pressure of carbon dioxide, ICC, intraclass correlation; CI, confidence interval; CV, coefficient of variation; TEM, typical error of the measurement. *ICC $p < 0.05$.

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Table 2. Cardiorespiratory and cerebrovascular responses at the highest common end-tidal partial pressure of carbon dioxide of the two trials/visits during rebreathing induced hypercapnia

| | $\Delta$MCAv$_{mean}$ (%/mmHg) | $\Delta$CVCi (%/mmHg) | $\Delta$MAP (mmHg) | Highest common $\Delta P_{ET}CO_2$ (mmHg) |
|---|---|---|---|---|
| Within-day short interval (n = 12) | | | | |
| Trial 1 | 49 ± 19 | 29 ± 18 | 13 ± 7 | 15.9 ±1.6 |
| Trial 2 | 43 ± 17 | 31 ± 18 | 8 ± 5 | 15.9 ±1.6 |
| Paired t-test p (effect size) | 0.131 (0.5) | 0.651 (0.2) | 0.026 (0.8) | - |
| ICC (95% CI) | 0.88 (0.59 to 0.96)* | 0.87 (0.55 to 0.96)* | 0.59 (-0.18 to 0.88) | - |
| CV% (95% CI) | 19.5 (10.3 to 25.6) | 39.2 (15.8 to 53.2) | 46.6 (21.6 to 62.2) | - |
| TEM (%TEM) | 7.8 (16.9) | 8.8 (29.3) | 4.1 (38.7) | - |
| Within-day long interval (n = 10) | | | | |
| Trial 1 | 46 ± 17 | 34 ± 13 | 7 ± 6 | 16.6 ± 1.7 |
| Trial 2 | 41 ± 15 | 34 ± 16 | 5 ± 3 | 16.7 ± 1.7 |
| Paired t-test p (effect size) | 0.234 (0.4) | 0.963 (0.0) | 0.260 (0.3) | - |
| ICC (95% CI) | 0.86 (0.47 to 0.96)* | 0.95 (0.78 to 0.99)* | 0.33 (-1.40 to 0.83) | - |
| CV% (95% CI) | 18.5 (8.4 to 24.8) | 16.2 (0.0 to 25.3) | 154.0 (0.0 to 251.7) | - |
| TEM (%TEM) | 7.6 (17.5) | 4.8 (14.0) | 4.5 (77.1) | - |
| Between day (n = 17) | | | | |
| Visit 1 | 44 ± 16 | 30 ± 15 | 9 ± 6 | 16.1 ± 1.8 |
| Visit 2 | 42 ± 12 | 30 ± 9 | 8 ± 5 | 16.2 ± 1.7 |
| Paired t-test p (effect size) | 0.680 (0.1) | 0.892 (0.0) | 0.792 (0.1) | - |
| ICC (95% CI) | 0.72 (0.21 to 0.90)* | 0.86 (0.60 to 0.95)* | 0.33 (-0.99 to 0.76) | - |
| CV% (95% CI) | 21.6 (10.9 to 28.5) | 20.7 (11.7 to 27.0) | 113.6 (0.0 to 187.2) | - |
| TEM (%TEM) | 9.4 (21.7) | 6.2 (20.6) | 5.1 (61.6) | - |

Values are mean ± SD unless otherwise stated. MCAv$_{mean}$, middle cerebral artery mean blood velocity; CVCi, cerebral vascular conductance index; MAP, mean arterial pressure; $P_{ET}CO_2$, end-tidal partial pressure of carbon dioxide, ICC, intraclass correlation; CI, confidence interval; CV, coefficient of variation; TEM, typical error of the measurement. *ICC $p < 0.05$.

Table 3. Slopes of the absolute changes in cardiorespiratory and cerebrovascular measures during rebreathing induced hypercapnia

| | ΔMCAv$_{mean}$ Slope (cm/s/mmHg) | ΔCVCi slope (cm/s/mmHg$^2$) | ΔMAP slope (mmHg) |
|---|---|---|---|
| Within-day short interval (n = 12) | | | |
| Trial 1 | 2.28 ± 0.84 | 0.012 ± 0.009 | 0.99 ± 0.74 |
| Trial 2 | 2.31 ± 0.95 | 0.017 ± 0.006 | 0.71 ± 0.51 |
| Paired t-test p (effect size) | 0.857 (0.1) | 0.053 (0.7) | 0.151 (0.5) |
| ICC (95% CI) | 0.90 (0.64 to 0.97)* | 0.55 (-0.26 to 0.86) | 0.67 (-0.13 to 0.90)* |
| CV% (95% CI) | 18.3 (10.3 to 23.8) | 53.9 (0.0 to 82.3) | 53.2 (20.4 to 72.4) |
| TEM (%TEM) | 0.4 (17.4) | 0.005 (32.2) | 0.4 (50.7) |
| Within-day long interval (n = 10) | | | |
| Trial 1 | 2.17 ± 0.78 | 0.017 ± 0.009 | 0.68 ± 0.40 |
| Trial 2 | 2.14 ± 0.80 | 0.019 ± 0.007 | 0.54 ± 0.28 |
| Paired t-test p (effect size) | 0.785 (0.1) | 0.168 (0.5) | 0.071 (0.6) |
| ICC (95% CI) | 0.94 (0.75 to 0.96)* | 0.93 (0.72 to 0.98)* | 0.86 (0.42 to 0.97)* |
| CV% (95% CI) | 12.4 (0.0 to 18.5) | 47.1 (0.0 to 82.0) | 26.4 (3.8 to 37.2) |
| TEM (%TEM) | 0.3 (12.9) | 0.003 (16.5) | 0.2 (25.2) |
| Between day (n = 17) | | | |
| Visit 1 | 2.13 ± 0.74 | 0.015 ± 0.009 | 0.69 ± 0.42 |
| Visit 2 | 2.12 ± 0.67 | 0.016 ± 0.009 | 0.73 ± 0.66 |
| Paired t-test p (effect size) | 0.962 (0.0) | 0.718 (0.1) | 0.822 (0.1) |
| ICC (95% CI) | 0.74 (0.25 to 0.91)* | 0.84 (0.56 to 0.94)* | 0.10 (-1.73 to 0.67) |
| CV% (95% CI) | 26.6 (0.0 to 39.7) | 52.7 (0.0 to 79.9) | 63.7 (39.2 to 81.1) |
| TEM (%TEM) | 0.5 (22.0) | 0.004 (27.6) | 0.5 (75.6) |

Values are mean ± SD unless otherwise stated. MCAv$_{mean}$, middle cerebral artery mean blood velocity; CVCi, cerebral vascular conductance index; MAP, mean arterial pressure; $P_{ET}CO_2$, end-tidal partial pressure of carbon dioxide, ICC, intraclass correlation; CI, confidence interval; CV, coefficient of variation; TEM, typical error of the measurement. n = 11 and n = 16 for *within-day short interval* and *between day* CV% respectively as the mean of observations was 0 for one individual in each group. *ICC p< 0.05.