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Reproducibility of measuring cerebral blood flow by laser-Doppler flowmetry in mice

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1. ABSTRACT

Laser-Doppler flowmetry has been widely used to trace hemodynamic changes in experimental stroke research. The purpose of the present study was to evaluate the day-to-day test-retest reproducibility of measuring cerebral blood flow by LDF in awake mice. The flux indicating cerebral blood flow (CBF), red blood cell (RBC) velocity, and RBC concentration were measured with LDF via cranial windows for the bilateral somatosensory cortex in awake mice. LDF measurements were performed three times, at baseline, 1 hour after, and 7 days after the baseline measurement. Moreover, breathing rate (BR) and partial pressure of transcutaneous CO_2 (P_tCO_2) were measured simultaneously with LDF measurement. Intraclass correlation coefficient (ICC) and within-subject coefficient of variation (CVw) were calculated. CBF, RBC velocity, and RBC concentration showed good day-to-day test-retest reproducibility (ICC: 0.61 - 0.95, CVw: 8.3% - 15.4%). BR and P_tCO_2 in awake mice were stable during the course of the experiments. The evaluation of cerebral microcirculation using LDF appears to be applicable to long-term studies.

2. INTRODUCTION

Laser-Doppler flowmetry (LDF) allows for real time, non-invasive and continuous data acquisition of cerebral microcirculation (1-3). The LDF method has been widely used to trace hemodynamic changes in superficial and relatively deep brain structures in experimental stroke research (2). However, measurement of cerebral blood flow (CBF) by LDF is very sensitive to alterations in position between the LDF probe and the head of animals (4, 5). Thus, continuous anesthesia and mechanical ventilation are generally used in LDF measurement (1-3, 6-10). Since anesthesia significantly affects the physiological state including the regulation of cerebral circulation throughout the brain (6-10), a system for an awake animal experiment of cerebral microcirculation has been proposed. Sato *et al.* attempted to record local cortical CBF in conscious rats (11). Takuwa *et al.* established a system for the measurement of CBF in an awake mouse model to explore the long-term behavior of CBF and animal locomotion repeatedly while maintaining a relatively natural environment (12). Although the day-to-day reproducibility of measuring CBF by LDF should be confirmed especially

for longitudinal studies in awake animals, it had not yet been evaluated. We improved the system for measuring CBF utilizing a polyvinyl chloride tube to stabilize the probe attachment for obtaining correct results over longer observation periods.

The purpose of the present study was to evaluate the day-to-day test-retest reproducibility of measuring CBF by LDF in awake mice. To evaluate measurement error and effects due to changes in respiratory status, we performed simultaneous recordings of flux indicating CBF, red blood cell (RBC) velocity, and RBC concentration using LDF at three time points, at baseline, 1 hour after, and 7 days after the baseline measurement. We then investigated the reproducibility and variance of each of the parameters.

3. MATERIAL AND METHODS

3.1. Animal preparation

All experiments were performed in accordance with the institutional guidelines on humane care and use of laboratory animals and were approved by the Institutional Committee for Animal Experimentation. A total of 30 male C57BL/6 J mice (20–30 g, 7–11 weeks; Japan SLC, Inc., Hamamatsu, Japan) were used in this study. The animals were housed in a 12-hour dark and 12-hour light-cycle room at a temperature of 25°C with water and feed ad libitum.

For the surgical procedure, mice (N=10) were anesthetized with a mixture of air, oxygen, and isoflurane (3-5% for induction and 2% for surgery) via facemask. The animals were fixed in a stereotactic frame and the rectal temperature was maintained at 37°C by heating pad (ATC-210, Unique Medical Co. Ltd., Tokyo, Japan). The methods for preparing the chronic cranial window have been reported in detail previously (13, 14). A midline incision (10 mm) was made to expose the skull. A craniotomy was performed in two places of the skull over each somatosensory cortex, keeping the dura intact (3-mm hole, centered at 1.8 mm caudal and 2.5 mm lateral from bregma). The two holes in the brain surface were sealed with a 3-mm diameter coverslip using dental cement (Ionosit, DMG, Hamburg, Germany) to make the preparation waterproof. A custom-made plastic U-shaped plate was affixed to the front of the skull. After completion of the surgery, the animals were allowed to recover from anesthesia and housed for at least one week before initiation of the experiments.

3.2. CBF, RBC velocity and RBC concentration measurements

CBF was measured with an LDF system (laser wavelength 780 nm; FLO-C1, OMEGAWAVE, Tokyo, Japan) using an LDF probe with a 1.0 mm outside diameter (Type NS, OMEGAWAVE). A polyvinyl chloride guide tube (inner diameter 1.1 mm, length 20 mm) for the LDF probe was affixed perpendicularly to each cranial window above the barrel cortex in the somatosensory cortex, avoiding areas with large blood vessels using dental cement (Luxaflow, DMG) above the each cranial window (15). At the time of the LDF measurement, the probe was attached to the cranial window through the guide tube. Our LDF system simultaneously provided three parameters: flux indicating CBF, RBC velocity and RBC concentration, where RBC velocity = CBF / RBC concentration (16). The volume of LDF measurement was approximately 1 mm^3 (17). A sampling rate of 0.1 sec was used for measuring all LDF signals (CBF, RBC velocity and RBC concentration).

During the experiments, LDF data were recorded using a polygraph data acquisition system (MP150; BIOPAC Systems, Inc., Goleta, CA, USA) and analyzed offline. At first, baseline values of all three parameters (CBF, RBC velocity and RBC concentration) were obtained bilaterally. The values of all three parameters were presented as averaged values for 30-min periods. Further, all three parameters were measured 1 hour (1 hour) and 7 days (day 7) after the first measurement (baseline). Next we calculated the left-to-right ratios of all three parameters at each time point.

3.3 Breathing rate measurements

Breathing rate (BR) was measured with a custom-made piezoelectric transducer. A pressure sensor (FSR402, Interlink Electronics Inc., Camarillo, CA, USA) was placed under a lower part of the abdomen of the mice (Figure 1). During the experiments, the piezoelectric transducer detected changes in pressure caused by abdominal movement resulting from breathing. Digitalized signals from the piezoelectric transducer were sent to a polygraph data acquisition system (MP150, BIOPAC Systems) and were analyzed offline (Fig.1). BR measurements were performed simultaneously with LDF measurements (day 0, 1 hour and day 7).

3.4. Partial pressure of transcutaneous CO_2 (P_tCO_2) measurements

We used a transcutaneous blood gas monitor and calibrator (TCM4, RADIOMETER, Tokyo, Japan). Before each experiment the probe was subjected to a two-point calibration using 5% and 10% CO_2 gas standards. Calibration and measurements were performed using a constant probe temperature of 42°C. Mice were anesthetized with a mixture of air, oxygen, and isoflurane (3-5% for induction and 2% for surgery) via facemask and then hair was removed from the abdomen using depilatory cream. The probe was fixed to an adhesive ring that was attached to the abdomen skin. Recording was started approximately 30 min after the cessation of anesthesia to stabilize the experimental conditions. The signals recorded by TCM4 were sent to a polygraph data acquisition system (MP150, BIOPAC Systems). P_tCO_2 measurements were performed simultaneously with LDF measurements (day 0, 1 hour and day 7).

3.5. Partial pressure of arterial CO_2 (P_aCO_2) measurements

Arterial blood samples (0.3-0.5 mL) were collected via intracardiac puncture aimed at the left ventricle of anesthetized mice (3% for induction and 2.0% during surgery) using heparinized syringes (N=20). Body temperature was monitored with a rectal probe and maintained at approximately 37.0°C by heating pad (ATC-

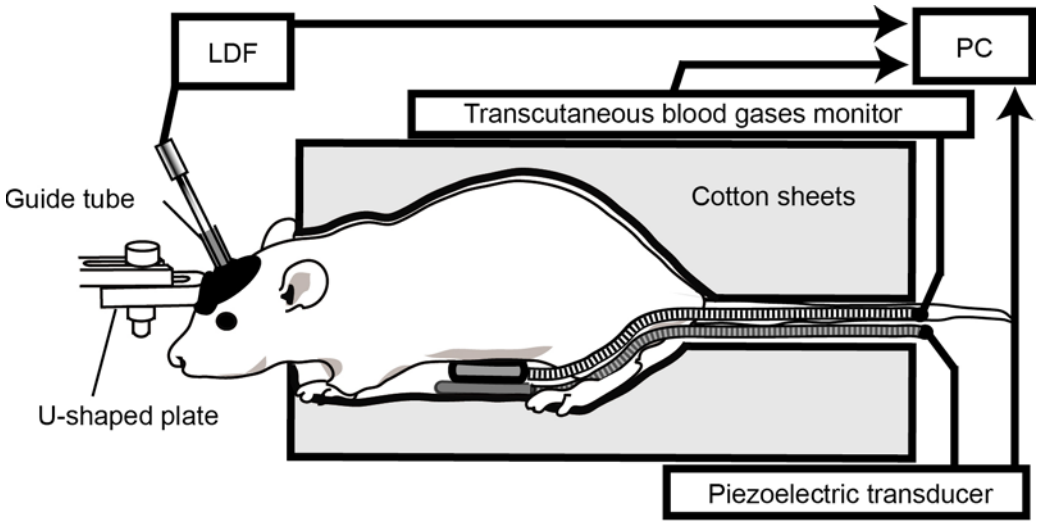

Figure 1. Experimental arrangement. A custom-made plastic U-shaped plate was attached to the animal skull and the head was fixed to a rod of a stereotactic instrument. The animal was placed in a box that was padded with cotton sheets. The piezoelectric transducer was placed under the abdomen to measure the breathing rate while detecting abdominal movement caused by breathing. A transcutaneous blood gas sensor was attached to the abdomen. Laser-Doppler flowmetry (LDF) was performed to measure CBF, RBC velocity and RBC concentration in the somatosensory barrel cortex through the polyvinyl chloride tube. Analog outputs converted from the piezoelectric transducer and LDF signals were recorded with a polygraph system connected to a personal computer (PC). Transcutaneous CO_2 pressure was recorded with a transcutaneous blood gas monitor, and the signals were sent to a PC.

210; Unique Medical). Blood samples were analyzed by blood analyzer (i-STAT; Abbott, IL, USA). Analyzer performance was verified daily and before each sample analysis. To confirm the correlation between P_tCO_2 and P_aCO_2, mice (N=20) were given different concentrations of inspired CO_2 randomly (normocapnia, 3% CO_2, 5% CO_2). All mice received 1.5% isoflurane in 2 L/min of 20% O_2. After each mouse achieved a stable P_tCO_2 baseline value, blood samples were collected.

3.6. Statistical analysis

Statistical analysis was done using the Statistical Package for the Social Sciences (SPSS, version 21). At first, the Shapiro-Wilk W test was used to analyze the normal distribution of all data. This was always followed by the application of parametric statistics. BR, P_tCO_2, all three parameters measured by LDF, and their left-to-right ratios were compared among groups by one-way repeated analysis of variance (ANOVA).

Reproducibility of the measurements of CBF, RBC velocity and RBC concentration by LDF was assessed by calculating the within-subject standard deviation (Sw), the within-subject coefficient of variation (CVw = 100x Sw / overall mean) (18), and the intraclass correlation coefficient (ICC). Sw is the common standard deviation of the repeated measurements. The within-subject standard deviation can be estimated by:

$$Sw=\sqrt{\frac{\sum_i S_i^2}{n}},$$

where S_i is the standard deviation in the value of interest on repeated testing for subject i, and n denotes the total number of subjects in the sample. The within-subject standard deviation represents the average variability of an individual's values on repeated testing (19). CVw is the ratio of the standard deviation over the overall mean. The "overall mean" referred to here is the mean of all three measurements of all ten subjects. The ICC value is used as a measure of test-retest reliability. ICC is the ratio of the inter-subject component of variance to the total variance (20). Landis and Koch suggest that agreement values are slight or poor if less than or equal to 0.20, 0.21 to 0.40 is fair, 0.41 to 0.60 is moderate, 0.61 to 0.80 is substantial, and almost perfect is greater than 0.80 (21). For calculating ICC, we used one-way repeated ANOVA. The relationships between P_tCO_2 and P_aCO_2 and between the percentage CBF of baseline in the bilateral cerebral hemisphere and changes in P_tCO_2 from baseline were examined by linear regression. A value of $p < 0.05$ was considered significant.

4. RESULTS

4.1. Respiratory status during the course of the experiments and correlation between P_tCO_2 and P_aCO_2

Table 1 shows the long-term measurement of BR and P_tCO_2 over one week (N=10). There were no significant differences in BR and P_tCO_2 between measurements. Figure 2 shows the relationship between P_tCO_2 and P_aCO_2 (N=20). Good correlation was observed ($Y = 1.23X + 3.5$, Y: P_tCO_2, X: P_aCO_2; $r = 0.94$, $p < 0.001$).

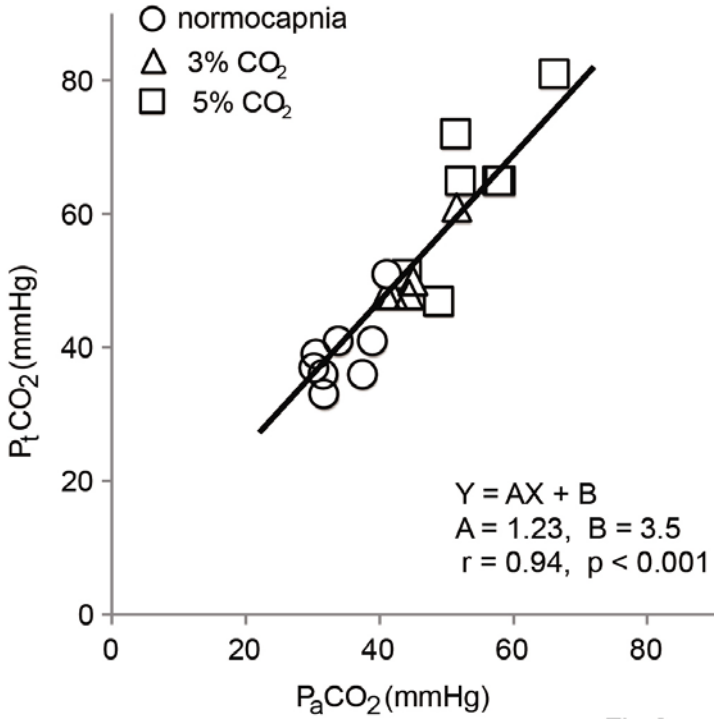

Figure 2. Correlation between partial pressure of arterial CO_2 (P_aCO_2) and partial pressure of transcutaneous CO_2 (P_tCO_2) values (N = 20). Circles, triangles and squares indicate normocapnia, hypercapnia using 3% CO_2 and 5% CO_2 gas, respectively. Arterial blood samples and transcutaneous measurements were obtained simultaneously.

4.2. Reproducibility of LDF measurements

Figure 3 shows CBF, RBC velocity and RBC concentration in the bilateral cerebral hemisphere for each of the experiments (N=10). No significant differences in CBF, RBC velocity and concentration in the bilateral cerebral hemisphere were observed among the measurements. No significant correlation was observed between the percentage CBF of baseline in the bilateral cerebral hemisphere and changes in PtCO2 from baseline at 1 hour and day 7 in all subjects (N=10). Table 2 shows the statistical analysis of the reproducibility of CBF, RBC velocity and RBC concentration. Based on the ICC values, the measurement of CBF, RBC velocity and RBC concentration by LDF showed a substantial to almost perfect level of test-retest reproducibility.

4.3. Reproducibility of left-to-right ratios of LDF measurements

Table 3 shows the left–to-right ratios of CBF, RBC velocity and RBC concentration in each of the experiments (N=10). The mean values of the left-to-right ratios of all three parameters were very close among the measurements, and no significant differences were observed. Table 4 shows the statistical analysis of the left–to-right ratios of CBF, RBC velocity and RBC concentration. Based on the ICC values, the left–to-right ratios of CBF, RBC velocity and RBC concentration measured by using LDF showed an almost perfect level of test-retest reproducibility.

5. DISCUSSION

In the present study, all three parameters, the flux indicating CBF, RBC velocity, and RBC concentration in the bilateral cerebral hemisphere measured by LDF showed a substantial to almost perfect level of test-retest reproducibility in terms of ICC values (0.61 - 0.95). There have been several studies concerning the assessment of the day-to-day reproducibility of CBF measurement using other modalities in humans. Chen *et al.* reported CVw of gray matter CBF from 3.5% to 21.0% and ICC of gray matter CBF from 0.713 to 0.911 using arterial spin labeling (ASL) (22). Henriksen *et al.* reported that CVw of global CBF was 4.8%, 15.1% and 11.9% using ASL, dynamic contrast enhanced perfusion measurement and ^{15}O-labeled water PET, respectively (23). Fiorella *et al.* reported CVw of parenchymal CBF of 20.8% and ICC of parenchymal CBF of 0.87 using dynamic CT perfusion (24). Our results were comparable to those above. This successful recording depends on the attachment of the LDF probe to the cranial window with the assistance of a guide tube fixed to the window. The present method for LDF measurement is expected to prove useful for longitudinal studies evaluating cerebral microcirculation in awake mice. It must be noted that absolute values measured by LDF differ from one subject to the next. There are microregional variations in perfused capillaries and differences in local hematocrit (4). These factors affect the LDF signal by varying degree, and therefore absolute values measured by LDF vary depending

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Table 1. Breathing rate (BR) and partial pressure of transcutaneous CO_2 (P_tCO_2) during the course of the experiments

	Baseline (Mean ± SD)	1 hour (Mean ± SD)	Day 7 (Mean ± SD)
BR (min^{-1})	173 ± 27	174 ± 36	172 ± 25
P_tCO_2 (mmHg)	49.6 ± 5.4	48.6 ± 4.8	48.6 ± 4.2

Table 2. Reproducibility of measuring CBF, RBC velocity and RBC concentration in bilateral cerebral hemisphere

Parameter	side	Sw [1]	CVw [2] %	ICC [3] (95%CI)
CBF (a.u.)	Right	1.0	8.3	0.90 (0.74-0.97)
	Left	1.5	11.8	0.95 (0.86-0.99)
RBC velocity (a.u.)	Right	1.6	15.4	0.61 (0.25-0.87)
	Left	1.2	11.3	0.91 (0.78-0.98)
RBC concentration (a.u.)	Right	1.8	9.8	0.77 (0.49-0.93)
	Left	1.8	10.1	0.92 (0.79-0.98)

Abbreviations: [1]Sw: within-subject standard deviation, [2]CVw: within-subject coefficient of variation, [3]ICC: intraclass correlation coefficient

Table 3. Left–to-right ratio of CBF, red blood cell (RBC) velocity and RBC concentration during the course of the experiments

Parameter	Baseline (Mean ± SD)	1 hour (Mean ± SD)	Day 7 (Mean ± SD)
CBF	1.14 ± 0.73	1.14 ± 0.77	1.19 ± 0.81
RBC velocity	1.04 ± 0.41	1.15 ± 0.47	1.15 ± 0.50
RBC concentration	1.15 ± 0.51	0.99 ± 0.38	1.09 ± 0.53

Table 4. Reproducibility of left–to-right ratios of CBF, RBC velocity and RBC concentration

Parameter	Sw [1]	CVw [2] %	ICC [3] (95%CI)
CBF	0.09	7.5	0.99 (0.97-1.00)
RBC velocity	0.12	11.5	0.92 (0.80-0.98)
RBC concentration	0.21	19.8	0.81 (0.54-0.94)

Abbreviations: [1]Sw: within-subject standard deviation, [2]CVw: within-subject coefficient of variation, [3]ICC: intraclass correlation coefficient

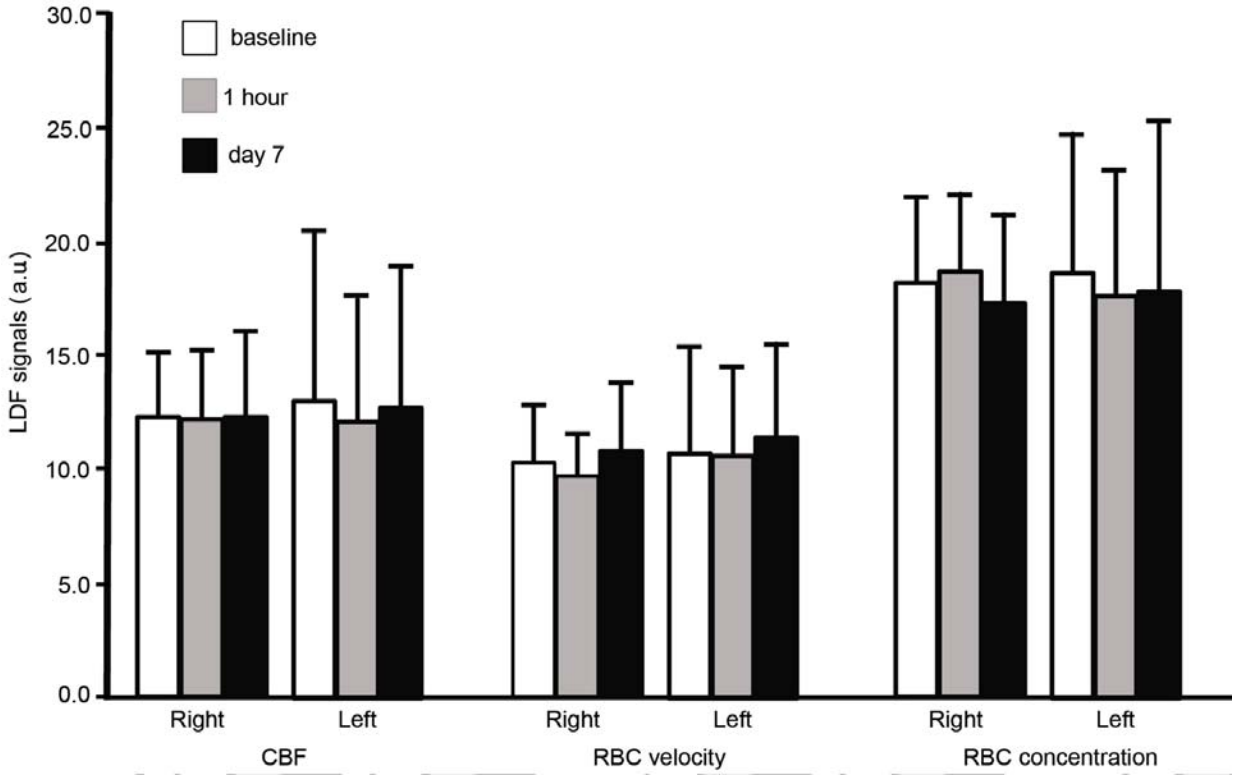

Figure 3. Cerebral blood flow (CBF), red blood cell (RBC) velocity and RBC concentration in the bilateral cerebral hemisphere during the course of the experiments (N = 10). White, gray and black bars indicate baseline, 1 hour and day 7, respectively. The error bars indicate SD.

on the probe position. This highlights the importance of longitudinally comparing the values in the same subject.

In addition, the present study evaluated left–to-right ratios of all three parameters measured by LDF using a dual cranial window model, as they had been used for investigation of cerebral pathophysiology, especially in cases of cerebrovascular disease (25, 26). In patients with acute ischemic stroke, a lesion-to-contralateral ratio of CBF below 0.6 should not be included in a trial of therapeutic reperfusion (26). In a Japanese EC-IC trial, the indication of EC-IC bypass surgery was based on a lesion-to-contralateral ratio of CBF below 0.8 in patients with hemodynamic cerebral ischemia (27). In the present study, the mean values of the left-to-right ratios of all three parameters were not exactly 1, perhaps due to variations in the absolute values measured by LDF with the probe positioning. Nevertheless, an excellent test–retest reproducibility was observed in the left–to-right ratios of the three parameters, especially in CBF (CVw = 7.5%, ICC = 0.99). When asymmetry in CBF is expected, such as in animal experiments using the cerebral ischemic models of unilateral side, the CBF ratio of the left-to-right hemisphere should be useful because of the excellent test–retest reproducibility.

P_tCO_2 can be measured without invasive arterial blood sampling. In the present study, a good correlation between P_tCO_2 and P_aCO_2 was observed, allowing substitution of P_tCO_2 for P_aCO_2. Elevations in P_aCO_2 (hypercapnia) lead to vasodilation of cerebral arterioles and a subsequent increase in CBF, whereas a reduction in P_aCO_2 (hypocapnia) leads to vasoconstriction and a subsequent decrease in CBF (28, 29). Since P_aCO_2 in awake mice was stable during the course of the experiments in the present study, no significant correlation was observed between the percentage CBF of baseline in the bilateral cerebral hemisphere and changes in P_tCO_2 from baseline. Thus, there is no doubt that this fact contributed to the good reproducibility of the recording by LDF.

In conclusion, good day-to-day test-retest reproducibility of the recording by LDF in awake mice was confirmed by the present study. Under unanesthetized condition, the change in P_aCO_2 is not significant between LDF measurements. An evaluation of cerebral microcirculation using LDF in awake mice is useful and appears to be applicable to long-term studies.

6. ACKNOWLEDGMENTS

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Abbreviations: CBF: cerebrl blood flow; LDF: lase-Doppler flowmetry; RBC: red blood cell; BR: breathing rate; P_tCO_2: partial pressure of transcutaneous CO_2; P_aCO_2: partial pressure of arterial CO_2; ICC: intraclass correlation coefficient; CVw: within-subject coefficient of variation; Sw: within-subject standard deviation; ASL: arterial spin labeling

Key Words: Laser-Doppler Flowmetry, Cerebral Blood Flow, Test-retest Reproducibility, Intraclass Correlation Coefficient, Within-subject Coefficient of Variation

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