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TITLE:

Evaluation of Cerebral Blood Flow Autoregulation in the Rat Using Laser Doppler Flowmetry

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cerebral blood flow, hemorrhage, laser Doppler flowmetry, autoregulation, microcirculation, blood flow

SUMMARY:

This article demonstrates the use of laser Doppler flowmetry to evaluate the ability of the cerebral circulation to autoregulate its blood flow during reductions in arterial blood pressure.

ABSTRACT:

When investigating the body's mechanisms for regulating cerebral blood flow, a relative measurement of microcirculatory blood flow can be obtained using laser Doppler flowmetry (LDF). This paper demonstrates a closed skull preparation that allows cerebral blood flow to be assessed without penetrating the skull or installing a chamber or cerebral window. To evaluate autoregulatory mechanisms, a model of controlled blood pressure reduction via graded hemorrhage can be utilized while simultaneously employing LDF. This enables the real time tracking of the relative changes in the blood flow in response to reductions in arterial blood pressure produced by the withdrawal of circulating blood volume. This paradigm is a valuable approach to study cerebral blood flow autoregulation during reductions in arterial blood pressure and, with minor modifications in the protocol, is also valuable as an experimental model of hemorrhagic shock. In addition to evaluating autoregulatory responses, LDF can be used to monitor the cortical blood flow when investigating metabolic, myogenic, endothelial, humoral, or neural mechanisms that regulate cerebral blood flow and the impact of various experimental interventions and pathological conditions on cerebral blood flow.

INTRODUCTION:

Autoregulatory mechanisms in the cerebral circulation play a crucial role in maintaining

homeostasis and normal function in the brain. Autoregulation of the cerebral blood flow is affected by multiple factors including heart rate, blood velocity, perfusion pressure, the diameter of the cerebral resistance arteries, and the microcirculatory resistance, all of which play a role in maintaining the total cerebral blood flow constant in the brain over the physiological range of systemic blood pressures. When arterial pressure increases, these mechanisms constrict arterioles and resistance arteries to prevent dangerous increases in intracranial pressure. When arterial blood pressure decreases, local control mechanisms dilate the arterioles to maintain tissue perfusion and O_2 delivery. Various pathological conditions such as hypercapnia, traumatic or global hypoxic brain injury, and diabetic microangiopathy[1–6] may disrupt the brain's ability to autoregulate its blood flow. For example, chronic hypertension shifts the effective autoregulatory range toward higher pressures[7–9], and a high salt (HS) diet not only interferes with normal endothelium-dependent dilation in the cerebral microcirculation[10], but also impairs the ability of autoregulatory mechanisms in the cerebral circulation to dilate and maintain tissue perfusion when arterial pressure is reduced[11]. Cerebral autoregulation is also impaired in Dahl salt-sensitive rats when they are fed a HS diet[12].

During reductions in arterial pressure, dilation of the cerebral resistance arteries and arterioles initially returns cerebral blood flow to control values despite the reduced perfusion pressure. As arterial pressure is reduced further, cerebral blood flow remains constant at the lower pressure (plateau phase of the autoregulatory response) until the vasculature can no longer dilate to maintain blood flow at the lower pressure. The lowest pressure at which an organ can maintain normal blood flow is termed the lower limit of autoregulation (LLA). At pressures below the LLA, cerebral blood flow decreases significantly from resting values and decreases in a linear fashion with each reduction in arterial perfusion pressure[13,14]. An upward shift in the LLA, as observed in hypertension[7–9], may increase the risk and severity of ischemic injury during conditions where the arterial perfusion pressure is reduced (e.g., myocardial infarction, ischemic stroke, or circulatory shock).

LDF has proven to be an extremely valuable approach to evaluate the blood flow in the microcirculation under a variety of circumstances, including autoregulation of the blood flow in the cerebral circulation[11,14,15]. In addition to evaluating autoregulatory responses, LDF can be used to monitor the cortical blood flow when investigating metabolic, myogenic, endothelial, humoral, or neural mechanisms that regulate the cerebral blood flow and the impact of various experimental interventions and pathological conditions on cerebral blood flow[10,16–21].

LDF measures the shift in reflected laser light in response to the number and velocity of moving particles--in this case, red blood cells (RBC). For studies of cerebral vascular autoregulation, arterial blood pressure is changed either by the infusion of an alpha-adrenergic agonist to increase arterial pressure (because the cerebral circulation itself is insensitive to alpha-adrenergic vasoconstrictor agonists)[12,15] or via controlled blood volume withdrawal to reduce arterial pressure[11,14]. In the present study, LDF is utilized to demonstrate the effects of graded reductions in blood pressure on cerebral autoregulation in a healthy rat. Although open and closed skull methods have been described in the literature[22–25], the present paper demonstrates a closed skull preparation, allowing cerebral blood flow to be assessed without penetrating the

skull or installing a chamber or cerebral window.

PROTOCOL:

The Medical College of Wisconsin Institutional Animal Care and Use Committee (IACUC) approved all protocols described in this paper and all procedures are in compliance with the National Institutes of Health (NIH) Office of Laboratory Animal Welfare (OLAW) regulations.

1. Experimental animals and preparation for recording

1.1. Use 8–12-week-old male Sprague-Dawley rats weighing 250–300 g. For these experiments, feed rats a standard diet consisting of 0.4% NaCl, 200 g/kg casein, 3 g/kg DL-methionine, 497.77 g/kg sucrose, 150 g/kg cornstarch, 50 g/kg corn oil, 50 g/kg cellulose, 2 g/kg choline bitartrate, 35 g/kg mineral mix, and 10 g/kg vitamin mix.

1.2. Record arterial blood pressure and LDF readings using data acquisition software or any comparable recording method.

1.3. Attach the arterial pressure transducer to one channel of the recording system and the LDF probe to the other channel on the recording system.

1.4. Prior to the measurement, calibrate the laser Doppler probe to set a motility standard and ensure that the laser Doppler flowmeter is providing a steady output.

1.5. Prepare additional equipment needed for the preparatory surgery and for the experiment: a dissecting microscope, a rodent ventilator, an end tidal CO_2 monitor, a stereotaxic instrument to fix the rat's head in position, and a micromanipulator to locate the LDF probe over the pial microcirculation and maintain it in a steady position.

2. Surgical preparation

2.1. Weigh the rat and anesthetize the animal in an induction chamber with 4–5% isoflurane and 30% O_2 supplement.

2.2. Remove the animal from the induction chamber and substitute an anesthetic mask delivering 1.5–3% isoflurane with a 30% O_2 supplement.

2.3. Place the rat on a circulating water blanket maintained at 37 ˚C and check reflexes with a toe pinch to ensure that there is a withdrawal reflex. Apply sterile ophthalmic ointment to both eyes to prevent corneal desiccation.

2.4. Shave the top of the cranium, ventral neck area, and femoral triangles. Remove any loose hair from those areas and clean with rubbing alcohol.

2.5. Place the rat in a supine position on a heating pad with a circulating warm water pump to maintain the animal's body temperature at 37 °C and temporarily secure it to the pad using medical tape.

2.6. Install a tracheal cannula (PE240 polyethylene tubing) through a ventral incision in the neck as described elsewhere[26].

2.7. Attach the tracheal cannula to an end tidal CO_2 monitor and the ventilator delivering 2.5–3.0% isoflurane (depending on the size of the animal) and a 30% O_2 inhalation supplement. Make sure the respiratory rate, inspiratory time, and minute ventilatory volume are set and monitored to ensure an expired end tidal CO_2 of approximately 35 mmHg throughout the experiment.

NOTE: This is generally achieved with a respiratory rate of approximately 48–60 breaths/min, a tidal volume of 1.70–2.30 mL, and an inspiration time of 0.50–0.60 s for a 250–300 g rat.

2.8. Fill two PE50 polyethylene cannulas with 1 U/mL heparin in isotonic NaCl solution to prevent clotting and to maintain patency of the catheters. After filling, seal one end of each cannula with heat and bevel the opposite end with surgical scissors to facilitate insertion into the arteries.

2.9. Cannulate the right and left femoral arteries as described elsewhere[27] to allow continuous monitoring of arterial pressure in one catheter and blood withdrawal from the other catheter.

2.9.1. After carefully separating the arteries from the surrounding tissue under a dissecting microscope, ligate the distal end of the artery and place two additional sutures around the middle and proximal ends of the artery without tightening the knots.

2.9.2. Use the proximal suture as a lifting ligature to prevent bleeding from the artery after the incision for cannula insertion (step 2.11).

2.10. Insert a V-shaped wire fashioned from a paper clip under the artery in order to occlude the vessel until the cannula is secured.

2.11. Under a dissecting microscope make a small incision in the femoral artery near the distal ligation using Vannas scissors. Insert the beveled end of the cannula into the incision and advance it into the femoral artery. Tighten the knot on the middle ligature to secure the cannula in place so it is not dislodged by arterial pressure when the lifting ligature or paper clip is removed.

2.12. After the middle ligature is tightened, release the tension on the lifting ligature and/or remove the paper clip, and tighten the proximal ligature.

2.13. Close the incision with fine sutures (3–0 silk) or a surgical staple. Alternatively, place a moist gauze over the incision site, depending on the size of the incision.

3. Skull thinning for LDF measurements

3.1. Immediately after the cannulas are in place, place the animal in a sternal position and secure the head in a stereotaxic device, being careful not to dislodge the catheters or tracheal tube.

3.2. Use surgical scissors to make an elliptical incision in the skin covering the cranium. Use a cotton swab to remove any connective tissue, ensuring that the cranium is clean and dry. Place a small elongated and rolled piece of tissue paper around the incision on the scalp to stop any bleeding.

3.3. Under the dissecting microscope, use a Dremel tool or a dental drill with a 2.15 mm drill bit to thin a small area of bone (approximately 0.5–1 cm depending on the size of the rat) in the parietal area over the left or right somatosensory cortex.

CAUTION: Thin the bone slowly and carefully to avoid penetrating the skull. While performing this step, saline solution should be applied liberally to prevent the area from overheating.

3.4. Once the skull has been thinned and the area has a pink appearance and/or blood vessels are visualized, cover the area with mineral oil and use a micromanipulator to position the laser Doppler probe over the exposed cerebral microcirculation so that the tip of the probe is just touching the top of the pool of mineral oil (**Figure 1**).

NOTE: It is essential to take LDF measurements in an area where there are no external vibrations that would interfere with the laser Doppler readings and that the probe is securely fixed over the same target area throughout the experiment.

4. Assessing cerebral vascular autoregulation

4.1. Once the LDF probe is fixed in position, allow a 30–45 min equilibration period before beginning the experiment. After the equilibration period, measure the mean arterial pressure (MAP) and laser cerebral blood flow (LCBF) every 30 s for 2 min and average the values to obtain the baseline values for the prehemorrhage blood pressure and LCBF.

4.2. To evaluate the cerebral vascular autoregulation in response to arterial pressure reduction, measure the LCBF and MAP following successive withdrawals of 1.5 mL of blood from the femoral artery[11]. To keep the catheter patent, ensure that a volume of heparin solution (100 U/mL in isotonic saline) approximately equal to the catheter volume is infused after each blood draw.

NOTE: When infusing the heparin solution to maintain catheter patency, it is important to match the volume of the heparin solution to the volume of the catheter as closely as possible to prevent the animal from receiving too much heparin, which could cause unwanted bleeding.

4.3. After each blood volume withdrawal, allow the rat to equilibrate for 2 min, after which the MAP and LCBF are recorded every 30 s for 2 min. Repeat the blood volume withdrawals until the animal reaches a MAP of approximately 20 mmHg.

4.4. Determine the effective autoregulatory range by identifying the range of blood pressures from the prehemorrhage MAP to the LLA (steps 4.5 and 5.3, below).

4.5. Determine the LLA by identifying the lowest pressure at which LCBF still returns to within 20% of the prehemorrhage control value following blood volume withdrawal, as previously described[11,28] or by identifying the intersection point of the regression lines determined during the plateau phase of autoregulation and below the LLA, where LCBF decreases with each successive blood withdrawal (step 5.3, below).

NOTE: The criteria for defining the LLA and autoregulatory plateau may differ between laboratories (e.g., Takada et al.[28] vs. Jones et al.[29]) as well as procedures for reducing arterial blood pressure (e.g., withdrawal of a specific volume of blood vs. controlled hemorrhage to reach specific arterial pressure levels)[11].

4.6. At the end of the experiment, euthanize the animal by creating a bilateral pneumothorax while under a surgical plane of anesthesia, as approved by the IACUC.

4.7. LDF values obtained in the tissue after the animal is euthanized will provide the zero baseline flow value for the experimental setup.

5. Statistical analysis

5.1 Perform linear regression analysis to evaluate the correlation between the LDF values and their corresponding arterial pressure. Use the baseline LDF readings obtained after the animal is euthanized to ensure that there was no nonspecific LDF signal affecting the measured flow rates.

5.2 Calculate the LLA using the intersection between the regression lines above and below the autoregulatory plateau. To calculate the LLA using this method, combine the two regression equations and solve the resulting equation for arterial pressure.

5.3. When comparing different experimental groups, use linear regression analysis to calculate the slopes of the LDF vs. arterial pressure relationship above and below the LLA for each animal and summarize them as mean ± SEM for the animals in that experimental group.

REPRESENTATIVE RESULTS:

Figure 2 summarizes the results of experiments conducted in 10 male Sprague-Dawley rats fed standard laboratory chow. In those experiments, mean LCBF was maintained within 20% of the prehemorrhage value following the first three blood volume withdrawals, until the mean arterial pressure reached the LLA. Subsequent blood volume withdrawals at pressures below the LLA caused a progressive reduction of LCBF, showing that the cerebral circulation was no longer able to produce a sufficient level of vasodilation to maintain cerebral blood flow constant at the lower perfusion pressures.

Figure 3 summarizes the relationship between mean arterial pressure and LCBF in the plateau phase (MAP $\geq$65 mmHg) and the decompensatory phase (MAP <65 mmHg) of CBF autoregulation. At pressures at or above the LLA, there was no significant correlation between LCBF and arterial pressure (r^2 = 0.0246; p = 0.3534), showing that the LCBF was independent of arterial pressure in the plateau range of the autoregulatory curve. Below the LLA, the LCBF/arterial pressure relationship had a negative slope and LCBF was significantly correlated with arterial pressure (r^2 = 0.7907; p = 8.7 x 10^{-25}).

FIGURE AND TABLE LEGENDS:

Figure 1: Placement of laser Doppler probe over the thinned skull of an anesthetized rat. Rat in stereotaxic apparatus with an LDF probe positioned over a thinned area of the skull and held in place with a micromanipulator.

Figure 2: Autoregulation of the cerebral blood flow in response to hemorrhage-induced reductions in arterial blood pressure. Summarized relationship between blood volume withdrawal and (**A**) mean arterial pressure (MAP) and (**B**) laser cerebral blood flow (LCBF) in rats fed a standard diet and subjected to sequential blood volume withdrawals. Data shown as mean ± SEM for n = 6–10 after each blood volume withdrawal.

Figure 3: Relationship between the mean arterial pressure and laser cerebral blood flow. Relationship during the plateau phase of the autoregulatory response (n = 37 observations) and in the decompensatory phase of the response (n = 70 observations) are shown, where arterial pressures fell below the LLA (~65 mmHg). LCBF was highly correlated with MAP in the decompensatory phase of autoregulation (r^2 = 0.7907; p = 8.7 x 10^{-25}) but not during the plateau phase of autoregulation (r^2 = 0.0246; p = 0.3534).

DISCUSSION:

Evaluation of Tissue Blood Flow Responses with Laser Doppler Flowmetry (LDF). As noted above, the LDF signal is proportional to the number and velocity of moving particles, in this case RBC, in the microcirculation. LDF readings in different organs are well correlated with whole organ blood flow assessed by established methods such as electromagnetic flow meters and radioactive microspheres[30] and are generally consistent with studies evaluating the regulation of active tone in cannulated artery preparations[10,31–34] and in situ microcirculatory preparations[35,36].

One consideration when conducting studies of cerebral autoregulation, and possibly autoregulation in other vascular beds, is the potential effect of anesthesia on autoregulatory responses. Although cerebral autoregulation was present in the current study and in an earlier study by our group[11] and consistent with the known effects of a HS diet on the vasodilator responses of the cerebral resistance arteries[31,32,37], the rat pial arterioles[35] and the in situ arterioles of the hamster cheek pouch[36], isoflurane anesthesia has been reported to have a strong vasodilator effect[38] and to cause cardiovascular suppression[39]. Isoflurane has also been reported to cause a loss of cerebral vascular autoregulation in mice[40,41], so some investigators have used alpha-chloralose anesthesia either alone[41] or in combination with urethane[42] to study cerebral autoregulation instead.

The numbers and velocities of RBC vary within a microcirculatory bed, between individuals, and within an individual subject over time. Thus, LDF does not provide an absolute value of blood flow within an organ or its microcirculation, between different organs, or in different regions of the microcirculation. Therefore, it is essential to firmly secure the LDF probe so that it remains in the same position and is not subjected to any vibration throughout the experiment. To accurately assess changes in the cerebral blood flow, the rat's head is positioned in a stereotaxic instrument and the LDF probe is held in a micromanipulator over a thinned area of the skull to prevent movement artifacts and to maintain the probe's position relative to the region being studied (**Figure 1**). Any movement of the probe away from its initial site will produce a signal determined by blood flow in a different area of the tissue, impeding comparisons. Although LDF does not provide a measurement of absolute blood flow, when performed properly it is still a convenient and valuable approach to evaluate the regulation of blood flow at the level of the whole vascular bed[30], and the magnitude of the relative increases or decreases in LDF flow relative to a control value can be compared statistically.

Autoregulation of Cerebral Blood Flow. The cerebral circulation can normally tolerate large changes in arterial blood pressure that cause vasoconstriction when arterial pressure is elevated and vasodilation when arterial pressure is reduced via autoregulatory mechanisms. These mechanisms are crucially important to prevent dangerous increases in intracranial pressure when systemic blood pressure increases and to maintain adequate tissue perfusion and oxygen supply when arterial pressure decreases. The present experiments focused on the ability of autoregulatory mechanisms to maintain cerebral blood flow constant as arterial pressure is reduced (rather than the ability of the cerebral circulation to maintain constant blood flow as MAP is increased), although LDF is very valuable and extensively used for the latter studies as well. Another valuable application of this experimental design is to study microvascular blood flow during hemorrhage and in various forms of circulatory shock[43–46].

Autoregulation of LCBF during hemorrhage-induced reductions in arterial pressure is assessed by comparing the LDF flow and MAP measured 2 min after each blood withdrawal with the prehemorrhage control MAP and LCBF measured immediately prior to blood volume withdrawal. At this point, the autoregulatory mechanisms will have acted to dilate the microvasculature to maintain blood flow at the lower perfusion pressure. The LLA is identified as the lowest MAP where autoregulatory mechanisms can still restore blood flow despite the reduction in perfusion pressure. At arterial pressures below the LLA, autoregulatory mechanisms have reached their limit and can no longer dilate the cerebral vasculature enough to prevent further reductions in cerebral blood flow. After the LLA is passed, there is a significant and progressive reduction in LCBF from the prehemorrhage value following each withdrawal of blood to reach the new pressure[11]. The effectiveness of cerebral vascular autoregulation in response to reductions in arterial blood pressure is evaluated by comparing the slope of the LCBF vs. the arterial pressure relationship before and after the LLA and the width of the plateau phase of autoregulation, defined as the arterial pressure range between prehemorrhage MAP and the LLA. For example, a recent study evaluating the effect of a HS diet on cerebral autoregulation[11] found that cerebral blood flow was maintained at a constant level in rats fed with a low salt (LS; 0.4% NaCl) diet

during sustained reductions in arterial pressure to values as low as 40–50 mmHg. This finding is consistent with previous estimations of the LLA in healthy rats[16,47]. However, the plateau phase of cerebral blood flow autoregulation in normotensive Sprague-Dawley rats fed a short-term (3 days) and chronic (4 weeks) high salt (HS; 4% NaCl) diet decreased progressively. This led to successive reductions in arterial pressure. With this finding, one can conclude that a HS diet eliminates the plateau phase of blood flow regulation that is normally present in healthy normotensive rats and adversely affects the ability of the cerebral circulation to maintain tissue perfusion in the face of reductions in blood pressure[11]. The finding that autoregulation of cerebral blood flow in response to reduced blood pressure is impaired in rats fed a HS diet is consistent with the results of studies showing that increases in dietary salt impair the relaxation of resistance arteries[31–34,37] and arterioles[35,36] of normotensive rats and hamsters.

In addition to providing valuable insights regarding the ability of the microcirculation to autoregulate its blood flow, LDF measurements can be employed in a wide range of applications that provide a dynamic estimation of blood flow control that is unavailable with conventional methods, such as microspheres and electromagnetic flow probes. For example, LDF measurements are extremely valuable in evaluating the response of the microcirculation to vasoactive stimuli such as ACh infusion and administration of other vasoactive agents[31–34,37], elevated arterial pCO_2[10], hypoxia[17,48], neurovascular coupling in response to sensory stimuli[21,49], functional hyperemia in the brain[20], and evaluating tissue responses to hemorrhagic hypotensive stress and various types of circulatory shock[43–46].

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DISCLOSURES:

The authors have nothing to disclose.

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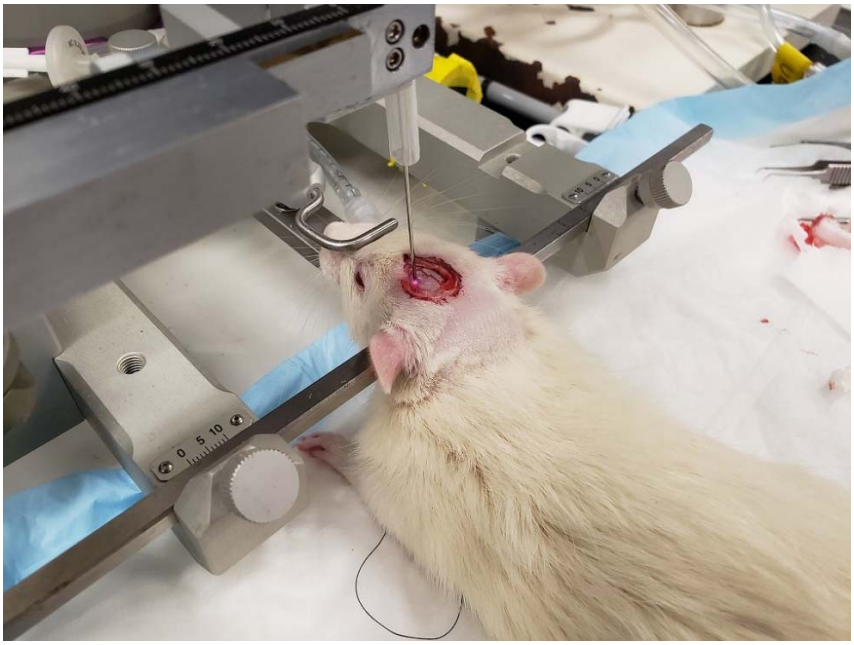

Figure 1

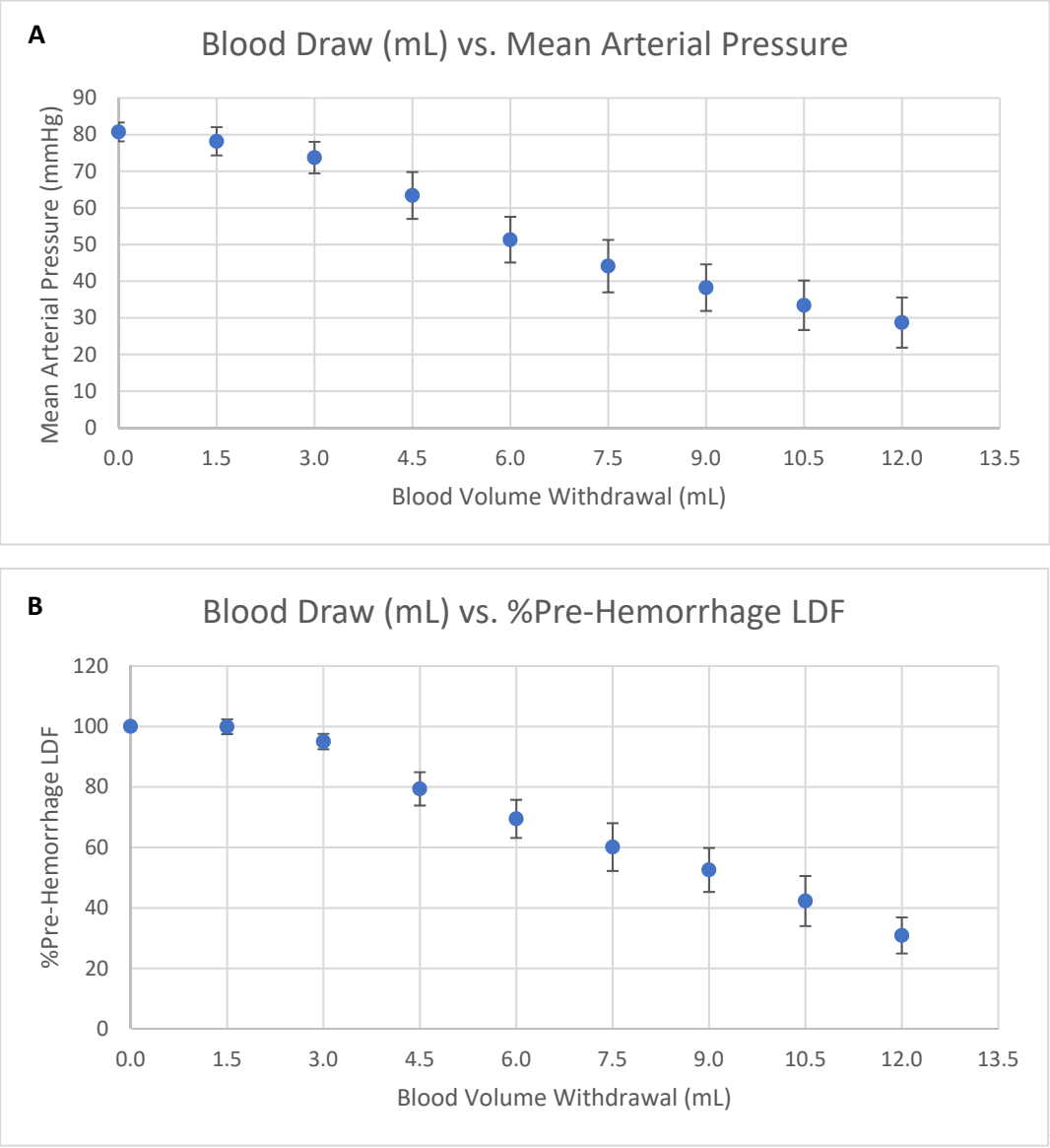

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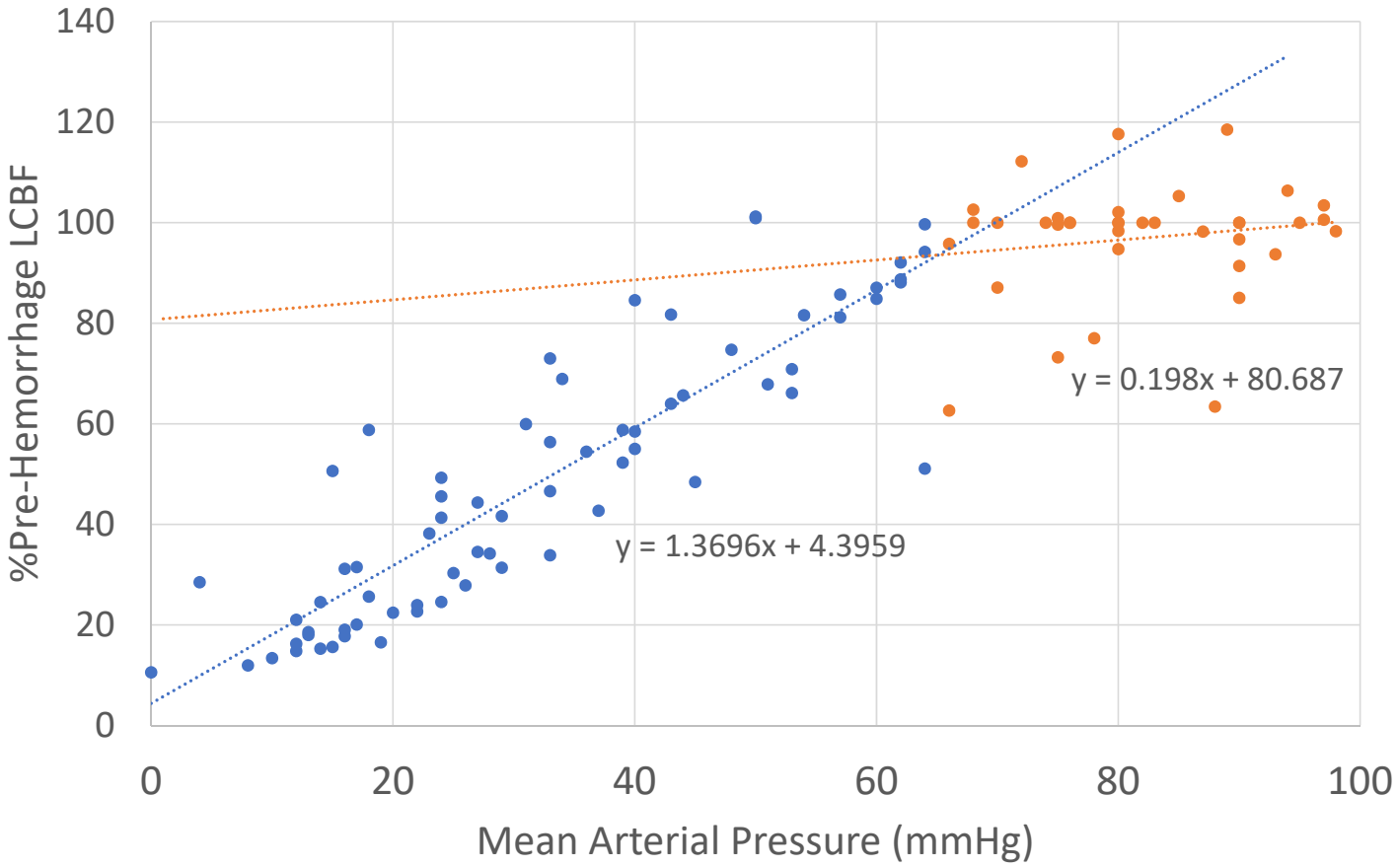
Figure 2.

Figure 3



Name of Material/ Equipment	Company
3-0 braided black silk suture	Midwest Vet
Arterial Pressure Transducer	Merit Medical
Automated Data Acquisition Systems (WINDAQ & BIOPAC system)	DATAQ Instruments
Blood Pressure Display Unit	Stoelting
Circulating warm water pump	Gaymar Industries
End-tidal CO2 monitor	Stoelting
Heparin Sodium	Midwest Vet
Kimwipe	Fisher Scientific
Laser Doppler Flow Meter	Perimed
Laser Doppler Refill Motility Standard	Perimed
Polyethylene Tubing (PE240) (for trachea cannula)	VWR
Polyethylene Tubing (PE50) (for femoral catheters)	VWR
Rodent Ventilator	Cwe/Stoelting
Saline	Midwest Vet
Sprague-Dawley Outbred Rats	Variable
Standard Rat Chow	Dyets, Inc.
Stereotaxic Instrument	Cwe/Stoelting

Catalog Number	Comments/Description
193.73000.2	
041516504A	
50115	
T-pump	
Capstar-100	
191.46720.3	
06-666A	
PeriFlux 5000 LDPM	
PF1001	
63018-828	
63019-048	
SAR-830/P	
193.74504.3	
N/A	Rats were ordered from various companies
113755	
Clasic Lab Standard	

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Author(s): L.A. Allen, M. Terashvili, Alison Gifford, J.H. Lumbard

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CORRESPONDING AUTHOR

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Institution:	Medical College of Wisconsin, Milwaukee, WI 53226
Title:	
Signature:	JHLombard
Date:	July 8, 2019

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Your manuscript, JoVE60540 "Evaluation of Cerebral Blood Flow Autoregulation Using Laser-Doppler Flowmetry," has been editorially and peer reviewed, and the following comments need to be addressed. Note that editorial comments address both requirements for video production and formatting of the article for publication. Please track the changes within the manuscript to identify all of the edits.

After revising and uploading your submission, please also upload a separate rebuttal document that addresses each of the editorial and peer review comments individually. Please submit each figure as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps., .svg). Please ensure that the image is 1920 x 1080 pixels or 300 dpi. Additionally, please upload tables as .xlsx files.

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Changes to be made by the Author(s):
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Thank you for the reminder. The manuscript has been thoroughly proofread for spelling and grammar.

2. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Windaq Data Acquisition-Data 111 Q Instruments, Akron, Ohio, Catalog #041516504A; Merit Medical, South, Jordan, UT, LDF probe (Model, Periflux 5000; Peri115 Med Instruments,

Ardmore, PA), SAR-830/P Ventilator, Capstar-100 Carbon Dioxide Analyzer, Cwe and 124 distributed by Stoelting, Inc. Wood Dale, IL 60191, Stoelting, Inc, T-Pump, Gaymar Industries, Kimwipe, (WINDAQ & BIOPAC system) (Data Q Instruments, Akron, Ohio), etc.

Commercial language has been removed and generic terms substituted in the body of the manuscript. The commercial products are now referenced in the Table of Materials and Reagents.

3. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns. Once done, please sort the table in alphabetical order.

The table of essential supplies and reagents has been revised as requested.

4. Please reword lines 232-247, 290-293, 304-307, and 337-348 as it matches with previously published literature.

Thanks for catching this oversight. The indicated lines have been re-worded as requested.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

All relevant sections have been changed to imperative tense, and other suggestions are included in "Note" sections.

6. In the JoVE Protocol format, "Notes" should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

Thank you for the information. The information in the steps and notes section has been evaluated and appropriate adjustments made.

7. The Protocol should contain only action items that direct the reader to do something.

The Protocol description has been changed as requested.

8. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections.

Any large paragraphs between steps have been removed.

9. Please ensure you answer the "how" question, i.e., how is the step performed?

Individual steps evaluated and adjusted as necessary.

10. 1: Please use imperative tense throughout. All equipment, materials, reagents can be moved to the table of materials and need not be listed in the protocol.

Any non-imperative tense steps have been revised according to instructions.

11. 2.4: Do you use iodine-based scrub as well?

Iodine-based scrub was not used for these acute experiments.

12. 2.8: This is important for filming, please describe the action in brief.

The description of the femoral artery cannulations **(Steps 2.8-2.12)** has been modified as requested, and the revised manuscript is substantially improved as a result.

13. 2.9: Size of the suture used? Please include post-operative care as well.

3-0 braided black silk suture was used to close the incision. There is no post-operative care because the animal is euthanized after experiment.

14. 3: There is a missing link between 2 and 3. Do you perform this procedure on the same animal? If yes, include the time.

Yes, this is performed on the same animal, approximately 30-45 minutes after starting the catheters. This point is clarified in the revised Protocol description.

15. We cannot film anesthesia and euthanasia experiments. Please remove the highlight for these steps.

Understood—The highlight had been removed from these steps, as requested.

16. 5: **Please include how is this done and use imperative tense throughout**.

The text has been changed as requested.

17. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please ensure that the highlight is 2.75 pages or less of the Protocol (including headings and spacing) and identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Thanks for the guidance. The Protocol and highlighted sections for filmable content have been reviewed and modified as requested.

18. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

Description and discussion revised and improved as requested.

19. Please discuss all the figures in the Representative Results. However, for figures showing the experimental set-up, please reference them in the Protocol.

Text revised and verified as instructed.

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The manuscript does not contain any figures from previous publications.

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a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

Text revised and verified as instructed.

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Reviewers' comments:
Reviewer #1:
Authors described the detailed method of how to evaluate the cerebral autoregulation, which is demonstrated by the relationship between systemic blood pressure and cerebral blood flow, using laser Doppler flowmetry (LDF) in rats. Cerebral autoregulation is one of the crucial and unique phenomena in the cerebral circulation, and the protocol is carefully described to be able to reproduce the study by other researchers. However, several concerns dampen enthusiasm for the manuscript in the present form. Although authors cited their previous publication, they do not describe how to obtain the lower limit of autoregulation (LLA), which is a key-value to evaluate cerebral autoregulation as well as a key step for the data analysis. Further, the reviewer has serious concern for the use of isoflurane as an anesthesia for the study of cerebral autoregulation. Previous publications have demonstrated that isoflurane causes the loss of cerebral autoregulation in mice (Wang Z et al. Exp Brain Res, 2010, Ayata C et al. JCBFM 2004). Authors need to justify the use of isoflurane with scientific foundations. Detailed comments are below.

1. Please include animal species (i.e. rats) in the title.

Thank you for the excellent suggestion! The title has been changed accordingly to indicate that LDF was being used to evaluate cerebral autoregulation in the rat.

2. Introduction; The term "local" autoregulation is not clear (at least for the reviewer) and possibly confuses some readers with the phenomena exhibiting the redistribution of CBF within the brain or the focal change in CBF in a certain part of the brain (e.g. functional hyperemia). The reviewer suggests using just cerebral autoregulation instead of "local" cerebral autoregulation.

Thank you for the comment and for the helpful suggestion. We have clarified the text accordingly and are using cerebral autoregulation throughout the manuscript, rather than “local cerebral autoregulation.” The revised version using the more precise terminology is clearly improved from the original version.

3. Introduction; Cerebral autoregulation does not solely rely on the change in cerebral arterial diameter, but also other factors such as heart rate, blood velocity, microcirculatory resistance, and perfusion pressure also play a role in the maintenance of the total cerebral blood flow/volume constant in the brain over the range of physiological systemic blood pressure. Please include this point in the introduction.

Thank you for the inciteful comment. The reviewer is correct. We have clarified this issue in the first paragraph of the revised introduction and believe that the current description is much more accurate and comprehensive. Thank you once again for the helpful suggestion.

4. Protocol 2.2; Isoflurane is a strong vasodilator (Jensen NF et al. Anesthesiology, 1992) and known to cause cardiovascular suppression (Avram MJ et al. Anesthesiology, 2000) and loss of cerebral autoregulation (Wang Z et al. Exp Brain Res, 2010, Ayata C et al. JCBFM 2004). Some researchers

prefer to use alpha-chloralose (Ayata C et al. JCBFM 2004) or the combination of alpha-chloralose and urethane (Niwa et al. Am J Physiol, 2002) for the studies of cerebral autoregulation. Please provide the evidence(s) that isoflurane does not affect cerebral autoregulation in rats or justify why authors chose to use isoflurane for the study.

Historically, we have been using Nembutal for all our experiments. However, several manufacturers dropped out, leaving only one. That company increased their prices to unaffordable levels. Part of the rationale for many investigators at MCW switching to isoflurane from pentobarbital (and other injectables like ketamine/xylazine) has to do with the ability to maintain anesthesia for only as long as needed and the relatively rapid recovery. While cerebral autoregulation was maintained in the present study and in our previous autoregulation paper in rats (and was consistent with known effects of high salt diet on cerebral arteries and arterioles), your point is well taken, and your advice is extremely valuable as we go forward. We have addressed these issues in the revised discussion and have included reference citations addressing the important points that you raised (second paragraph of the revised discussion). Thank you once again for the very helpful and important information.

5. Protocol 3.2; Does silver nitrate, which "can be used to control bleeding", itself have any effects on the mechanisms of cerebral autoregulation (e.g. endothelial function, smooth muscle contractility, and neuronal activity/viability)?

Thanks for catching this. In fact, we used silver nitrate on only a very few occasions. Because the studies are conducted on a thinned, but closed, skull preparation, the silver nitrate did not appear to have any effect on cerebral autoregulation. As a precaution, we have eliminated the statement about silver nitrate in the revised manuscript.

6. Protocol 4.4; Obtaining the lower limit of autoregulation (LLA) is a key step for the data analysis (i.e. as a threshold for two separate liner regression analysis). Authors cited their previous publication and stated as "previously described by our laboratory". However, the reviewer strongly suggests outlining the method of how to obtain the LLA again in this manuscript.

The reviewer is correct. The other reviewer also noted that other methods have been used to evaluate cerebral vascular autoregulation. In the revised manuscript, we have used linear regression techniques to identify the LLA more precisely. We have described these techniques more precisely in the revised manuscript. Thank you for this very helpful suggestion.

7. Discussion "LDF does not provide an absolute value of blood flow within an organ or its microcirculation, between different organs, or in different regions of the microcirculation"; Yes, this is a very important point, and also means that it is difficult to make a comparison between different groups (e.g. disease model) of animals because the absolute value of cerebral blood flow in pre-hemorrhage condition may be different. Please discuss this point.

The reviewer is absolutely correct. Thank you for this very helpful recommendation. We have revised the discussion accordingly (third paragraph of the discussion, last sentence) and believe that the revised version is clearly improved over the original.

8. Figure 3; The x-axis should be displayed in numerical order starting on the left (i.e. the smallest number on the left end). The reviewer also suggests combining the two graphs into one graph (containing two liner-regression analysis and the LLA) to show the whole view of the relationship between CBF and systemic blood pressure.

Thank you for the suggestion. The LDF vs. pressure data have been replotted in our revised **Figure 3.** We believe that the manuscript is substantially improved as a result.

Reviewer #2:
Manuscript Summary:
Manuscript summarizes LDF measurement of cortical CBF through closed but thinned cerebral window

Major Concerns:

Is LDF measured this way compatible with a golden standard CBF monitoring (like thermodilution with Hemedex)?

Thank you for the comment and suggestion. We have addressed this question in the first paragraph of the discussion with an appropriate reference citation (Smits, et al., J. Appl. Physiol. 61:666-672, 1986). Details about absolute volume measurement vs. thermodilution and different parameters.

Minor Concerns:

Figure 3. Can you combine baseline values and ischemia range on one graph and reverse x axis (i.e. from low value to high values of ABP) as one Lassen curve?

Thank you for the suggestion. This suggestion was also made by **Reviewer #1**, and the LDF vs. pressure data have been replotted in our revised **Figure 3.** We believe that the manuscript is substantially improved as a result.

LLA is usually detected as intercept point of linear regression lines (piecewise) above and below autoregulation breakpoint.

The new plot of the LDF vs. pressure data now includes the intercept point of the two regression lines above and below the autoregulation breakpoint, as suggested by the reviewer. The manuscript is greatly improved as a result—Many thanks!

If points on Fig 3 are consecutive point in a single animal measured in time, p value is not correct (measurement points are not independent!)

The points on **Figure 3** reflect multiple animals, rather than a single animal with time. We have clarified this in the revised manuscript. Thanks for catching this.