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TITLE:

Quantification of cerebral perfusion using laser speckle imaging and infarct volume using MRI in a pre-clinical model of posterior circulation stroke

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KEYWORDS:

canine stroke; basilar artery occlusion; digital subtraction arteriogram; laser speckle imaging; diffusion weighted imaging

SUMMARY:

A canine model of LVO stroke was utilized to develop laser speckle imaging to monitor cerebral perfusion in real-time. Diffusion-weighted MRI was optimized to image infarct volume utilizing a high b-value, enabling ADC and MRA, correlated with DSA at the time of stroke. Finally, ADC reconstructions correlated with histological findings.

ABSTRACT:

Basilar artery occlusion (BAO) is a subset of posterior circulation stroke that carries a mortality as high as 90%. The current clinical standard to diagnose ischemic stroke include computerized tomography (CT), CT angiography and perfusion and magnetic resonance imaging (MRI). Large animal pre-clinical models to accurately reflect the clinical disease as well as methods to assess stroke burden and evaluate treatments are lacking. We describe a canine model of large vessel occlusion (LVO) stroke in the posterior circulation, and developed a laser speckle imaging (LSI) protocol to monitor perfusion changes in real time. We then utilized high b-value DWI ($b=1800 s/mm^2$) MRI to increase detection sensitivity. We also evaluated the ability of magnetic resonance angiography (MRA) to assess arterial occlusion and correlate with DSA. Finally, we

verified infarct size from apparent diffusion coefficient (ADC) mapping with histology.

Administration of thromboembolism occluded the basilar artery as tracked by DSA (n=7). LSI correlated with DSA, demonstrating a reduction in perfusion after stroke onset that persisted throughout the experiment, allowing us to monitor perfusion in real time. DWI with an optimized b-value for dogs illustrated the stroke volume and allowed us to derive ADC and magnetic resonance angiography (MRA) images. The MRA performed at the end of the experiment correlated with DSA performed after occlusion. Finally, stroke burden on MRI correlated with histology. The studies demonstrate real time perfusion imaging using LSI of a canine thromboembolic LVO model of posterior circulation stroke, which utilizes multimodal imaging important in the diagnosis and treatment of ischemic stroke.

INTRODUCTION:

The prevalence of stroke worldwide is almost 25.7 million, the majority of which are ischemic[1]. Posterior circulation stroke accounts for 20% of all strokes of which basilar artery occlusion is the most severe, approaching 90% mortality[1,2]. In 1995, recombinant tissue plasminogen activator (rtPA) was the first acute therapy developed for ischemic stroke in patients who presented within 3 hours from stroke onset[3]. More recently, mechanical thrombectomy has demonstrated benefit in treating acute ischemic stroke in patients who present with large vessel occlusion (LVO), which includes the intracranial portion of the internal carotid artery or the first segment of the anterior and middle cerebral arteries[4]. None of the recent clinical trials included posterior circulation stroke and its outcomes remain dismal despite utilizing mechanical thrombectomy for basilar artery occlusion[5,6].

Advances in assessment techniques in stroke patients have an impact on predicting the chance of functional recovery and survival[7]. Pre-clinical models of posterior circulation stroke have been previously described[8-10]; however assessing stroke burden and revascularization remain suboptimal. Smaller species such as rodents offer several advantages including ease of genetic manipulation, inexpensive animal purchase, and low per diem housing costs[11,12]. However, small animal experiments sometimes do not fully represent large animal and human vasculature, physiological conditions, or related inflammatory responses[7]. Large animals more closely mimic human stroke[2,7,13,14]. Moreover, serial blood sampling can be performed for blood analysis of thrombotic and inflammatory markers.

In this study, we describe a canine model of basilar artery occlusion verified by digital subtraction angiography (DSA) from the onset of stroke. We utilize laser speckle perfusion imaging (LSI) to monitor perfusion in real time. We then utilize a novel microvascular enhancement algorithm based on laser speckle perfusion imaging (LSI) acquisition as well as a high b-value magnetic resonance imaging (MRI) technique to optimize infarct imaging[15]. These techniques allow us to monitor and quantify local and global ischemia. Finally, we correlate these imaging findings to histology. Understanding prognosis and the need to study posterior circulation stroke in pre-clinical models is critical in order to improve therapies.

PROTOCOL:

All procedures were performed in compliance with the Animal Welfare Act and the Guide for Care and Use of Laboratory Animals (NRC 2011), as approved by the Ohio State University's Institutional Animal Care and Use Committee (IACUC).

1. Animal preparation and surgery

NOTE: A canine model of basilar artery occlusion (BAO) stroke was used as previously described[9,10].

1.1. Fast adult beagles (8-13 kg, 14-21 months old) overnight with free access to water.

1.2. Inject, pre-anesthetic, an intramuscular administration of acepromazine (0.2 mg/kg)

1.3. Introduce a 20 G catheter into a cephalic vein.

1.4. Induce anesthesia with intravenous administration of ketamine (10 mg/kg) and midazolam (0.025 mg/kg).

1.5. Following anesthetic induction, intubate dogs and mechanically ventilate using constant inhaled anesthesia (2-3% isoflurane).

1.6. Create a 1 cm^2 craniotomy window for laser speckle imaging.

1.7. Introduce a 7 F arterial sheath into the right femoral artery for access and blood pressure measurement.

1.8. Introduce a 16 gauge angiocatheter into the right femoral vein for blood draws.

1.9. Prepare a thromboembolus (blood clot) as previously described[16]. Briefly, draw and mix 5 mL of canine whole blood with 0.5 g of barium sulfate (Ba_2SO_4) in a plastic serum blood collection tube while rolling for 30 s. Rest the mixture undisturbed for 60 min at room temperature before catheter administration.

1.10. Begin recording baseline digital subtraction angiography (DSA) prior to accessing the middle basilar artery. Advance a 4 F guiding catheter under fluoroscopic guidance, using a retrograde trans-aortic approach, into the 7 F arterial sheath previously placed into the right femoral artery through a vertebral artery to the base of the basilar artery. Inject 2 mL of contrast agent with normal saline to identify the basilar artery.

1.11. Using a surgical scalpel, resect the clot into small pieces with both fibrin-rich and erythrocyte-rich layers [16] to load into a 3 mL syringe and inject through the microcatheter into the middle of basilar artery. Allow the clot to stabilize for 10 minutes.

1.11.1. Perform a follow up angiogram to verify the desired clot location. Arterial occlusion can be verified by DSA and decreasing cerebral perfusion by laser speckle imaging (LSI).

2. Laser speckle imaging

2.1. Focus the laser speckle perfusion imaging (LSI) camera on the cranial window. Configure the high resolution laser speckle imaging (LSI) camera system as previously described[15].

2.2. Record perfusion with interruptions during performance of angiogram at desired time points. Acquire data from a 1.5 cm x 1.5 cm field of view using a 785 nm wavelength and 80 mW lasers with a sampling rate of 60 Hz at a working distance of 10 cm in this canine model.

2.3. From the real-time perfusion graphs, choose the time-of-interest (TOI) to include lower peaks only to exclude the respiratory motion related artifacts.

2.3.1. Average relative perfusion units over a 10 s sampling period using PimSoft v1.4 software. Perform laser speckle contrast analysis (LASCA) as previously described[15].

2.4. To optimize the quantification of brain microvasculature in this canine model, record images at 15 frames per second and perform intensity and variance calculations with spatiotemporal averaging over a 5 × 5 pixel area with 5 frames. The overall frame rate for the intensity and variance data was 3 frames per second.

2.4.1. Choose the median value of perfusion for each pixel to reduce the effects on the mean of large sudden changes in perfusion readings due to motion from canine respiration. Convert raw data into binary files and process the data into meaningful imaging of the vasculature.

2.4.2. Utilize the program LASCA algorithm (rt-LASCA) to use the variance of the contrast data over time to determine the locations of vasculature as previously described [15].

3. Magnetic resonance imaging (MRI) and magnetic resonance angiography

3.1. Perform MRI the day before surgery for comparison if desired, then repeat to confirm BAO and again before sacrifice if a therapeutic is to be evaluated.

3.2. Place continuously anesthetized canines head-first in a supine position as previously described in a 3 Tesla field strength and 60 cm-diameter bore MRI scanner including a 32 channel head coil as a receiver with enhanced parallel imaging performance to obtain brain images[17].

3.3. Perform localizer scans to acquire pilot images of each canine brain before the anatomical imaging begins. The system utilized to obtain the presented data has an integrated imaging system which allows faster scanning in optimal spatial and temporal resolutions. The 80 mT/m gradients generate high-quality T2-weighted, diffusion-weighted images and MR angiograms. Diffusion-weighted imaging (DWI) is sensitive enough and can show more anatomic sub-structure

than by conventional structural MRI methods such as T2-weighted images. In this study, MRI was performed 4 h after BAO.

3.4. After proper localization, perform T2-weighted gradient echo imaging (Parameters: FOV = 130 mm, Matrix size = 320 x 320, pixel size = 0.3 x 0.3 mm, Slice thickness =3 mm, TR= 4s, FA= 180 degrees, BW =255 Hz/pixel, NEX= 2, TE=75ms, Resolution= 2.4615 pixels per mm) followed by a flow attenuated inversion recovery (FLAIR) imaging to visualize the structure of the brain anatomy.

3.5. Perform magnetic resonance angiography (MRA) to visualize the vascular anatomy and blood circulation measurement. Acquire MRA of the brain covering the head and neck with a time-of-flight-3D (TOF) sequence in transverse view (Parameters: FOV = 129x129 mm, Matrix size = 768 x 768, pixel size = 0.3 x 0.3 mm, slice thickness = 81.59 mm, TR= 25 ms, FA= 18 degrees, BW =185 Hz/pixel, NEX= 1, TE=4.22ms, Resolution = 5.91 pixels per mm).

3.5.1. Perform maximum intensity projection (MIP) with 3D color-coded visualization to maximize the signal intensity in the blood vessels. Post-process acquired DICOM images to visualize the blood vessels and to confirm that the basilar artery was occluded.

4. Diffusion weighted imaging and stroke volume calculation

4.1. Perform diffusion weighted imaging sequence to detect acute ischemic strokes (Parameters: FOV = 149mm ×149 mm, Matrix size = 132 ×0×0× 100, pixel size = 0.30mm × 0.30 mm, slice thickness = 4 mm, TR = 4.6s, FA = 90 degrees, BW = 255 Hz/pixel, NEX= 1, TE = 86ms, Resolution= 0.93 pixels per mm). Transfer DICOM images for post-processing.

4.2. Generate apparent diffusion maps (ADC) from DWI images and calculate infarct volumes using OsiriX MD v.5.0 software.

4.3. Trace the whole brain and infarct areas per slice and multiply by slice thickness to acquire infarct volumes.

4.4. Convert the absolute whole volume to 100 units to calculate the percent stroke volume of each canine.

5. Hematoxylin and Eosin staining brain histology

5.1. At time of sacrifice in anesthetized canine, harvest the brain and cut two medial sections 4 mm thick with a sharp scalpel, one section will be used for TTC staining below.

5.2. Fix the 4 mm section in 10% formalin for a minimum of 7 days to allow infiltration throughout the entire section.

5.3. Embed the fixed brain section in paraffin[17].

5.4. Trim and level each paraffin block (multiple blocks can be stored and processed at the same time).

5.5. Section each paraffin block at 4 µm and place the cut tissue on a 2" x 3" inch slide.

5.6. Process each slide in Hematoxylin 560 for 8 min, differentiate with 1% acid alcohol for 1 s three times with rinsing in tap water.

5.7. Blue each slide with 1% ammonium hydroxide for 1 s and rinse for 2 s with tap water.

5.8. Dehydrate in 70% ethanol for 1 s twelve times, counterstained in eosin for 1 min.

5.9. Dehydrate in 95% for 1 s twelve times followed by 100% ethanol.

5.10. Clear in xylene and apply a 2" × 3" inch coverslip with mounting media, removing air bubbles.

6. 2% 2,3,5-triphenyltetrazolium chloride brain staining

6.1. Place the second 4 mm section which was harvested beside the H&E section into a previously prepared solution containing with 100 mL of 2% 2,3,5-triphenyl-2H-tetrazolium chloride (TTC) in pH 7.4 PBS warmed to 37 °C in the dark.

6.2. Incubate in the dark at 37 °C for at least 20 min, flipping the brain section over gently every 5 minutes.

6.3. When the section turns cherry red on both sides, remove the TTC solution and replace with 4% paraformaldehyde in PBS, pH 7.4, to optimize the contrast overnight.

6.4. When the contrast is optimal between white and red staining in the brain (1-3 days), place between clear plastic sheets, dry excess fluid, and scan at high resolution.

6.5. Trace the ischemic regions and whole brain slide to obtain percent infarction in each section as previously described[17].

7. Image fusion overlay

7.1. Utilize the Matlab code previously written to fuse an image on anatomical images described[15]. The code function reads both background and foreground images.

7.2. Threshold both background and foreground images to avoid excessive signal intensity.

7.3. On the displayed output image, adjust the foreground image transparency to visualize the

background image preserving the effect of the foreground image. This code features the functional image overlay and transparency adjustment for future studies.

REPRESENTATIVE RESULTS

Laser Speckle Perfusion Recording and Imaging: Perfusion recording was performed continuously until the animal was transported to the MRI, and again at sacrifice (**Figure 1A**). Data showed that cerebral perfusion decreased by ~15% to 83 $\pm$ 10% at the time point before basilar artery occlusion (pre-BAO). This nominal decline is likely the result of a microcatheter insertion in the distal vertebral artery. After injecting the prepare thromboembolus, the post-BAO perfusion dropped to an average of 33 $\pm$ 2.6%, representing a ~67% decline from baseline (**Figure 1B**). Angiographic observation showed that blood circulation in the basilar artery was patent prior to BAO (**Figure 2A**) and fully occluded after administration of the autologous clot (**Figure 2B**). In agreement with the angiograms, laser speckle perfusion imaging (LSI) observed through the cranial window depicted unrestricted perfusion (**Figure 2C**) in contrast to restricted perfusion after occlusion (**Figure 2D**). The efficacy of embolization in the BAO model was then verified. For this purpose, the vasculature was visualized and quantified using the rt-LASCA technique[15]. The brain vasculatures at baseline were clearly observed **(Figure 2E**) while vasculature was not visualized during occlusion (**Figure 2F**). The mean real time perfusion (average of 10 s recording) was 191.71±20.61 pu at baseline measurement and dropped to 64.71±11.35 pu (only 34% residual flow in a representative dog) at BAO. After injection of an autologous thromboembolus, the cerebellum region was immediately occluded and its territories were no longer visible during DSA analysis.

T2-weighted and FLAIR imaging: A T2-weighted MRI is inadequate to detect early acute strokes (**Figure 3A**)[18,19]. This was also the case with FLAIR images (**Figure 3B**). T2-weighted imaging was performed to confirm whether these scans are required in the therapeutic treatment in both pre-clinical and clinical evaluations.

Diffusion weighted imaging (DWI) and stroke volume calculation: Representative results demonstrated that diffusion weighted scanning is sensitive enough to detect an early acute stroke (<24 h). The bright signal in DWI represents the stroke affected zone (**Figure 4A**) corresponding to the color enhanced image (**Figure 4C**). The stroke burden in this model was calculated as a percentage of the whole brain because the posterior communicating arteries carry thromboembolus to the anterior circulation. Further processing was done by converting DWI into apparent diffusion coefficient (ADC) maps (**Figure 4B**) and color enhanced image (**Figure 4D**). In obtaining ADC maps, two sets of images, one with b = 0 s/mm^2 and the other with b = 1800 s/mm^2, were used to mathematically create the exponential image to create an ADC map. By selecting a range of b-values during image acquisition, we determined the optimal b-value as b=1800 s/mm^2. From the DICOM images while post-processing, by delineating a border on the enhanced ADC images using OsiriX MD v.5.0 software, infarct volume was calculated as detailed in the method section which confirmed the infarct region. The calculated average infarct size in 7 canines which we completed BAO on was 55.2% $\pm$ 11.5% of the whole brain (**Figure 4E**).

Digital Subtraction Angiography and Magnetic Resonance Angiography: Magnetic Resonance

Angiography (MRA) was performed using a 3D-time-of-flight (TOF) sequence for high spatial resolution. DICOM image series were reconstructed and maximum intensity projection (MIP) was applied during post processing to visualize the blood vessels from the head and neck to confirm BAO. DSA demonstrated vascular occlusion post-BAO (**Figure 5A**). MRA images clearly demonstrated the restricted flow through the basilar artery as shown in representative color scale vascular image (**Figure 5B**).

Histological Correlation with MRI: We traced representative canine brain TTC images (**Figure 6A**) and MRI images (**Figure 6B**) to correlate infarct size and revealed a significant Pearson's Correlation Coefficient of r=0.77 (**Figure 6C**)**.**

Physiological parameters: Physiological parameters including heart rate (HR), blood pressures (BP) and temperature were monitored throughout the procedure and are critical to ensure physiological surgical procedures and proper interpretation of therapeutic and device testing (**Supplementary Table 1**). Representative electrocardiograms at baseline (**Supplementary Figure S1A**) and post-BAO before sacrifice (**Supplementary Figure S1B**) are shown. Laboratories who perform this model as presented should expect a significant decrease in systolic blood pressure in an acute time frame.

FIGURE AND TABLE LEGENDS

Figure 1. Quantification of laser speckle perfusion data shows restricted blood flow during and after BAO. Laser speckle perfusion recording during the experimental process performed on all seven dogs. Perfusion recording was performed for the time points: baseline, before BAO, clot placement, during onset of occlusion, throughout saline infusion (duration of stroke) after MRI exams. Data were acquired from a cranial window, providing a 1.5 cm × 1.5 cm field of view. The perfusion data were normalized as a percentage of baseline. (A) Perfusion data are shown with color line graphs for all seven dogs which showed that there is an individual difference in perfusion. (B) Mean perfusion fold change over time are shown. Data = mean ± SEM, n = 7. Arrows indicate the time points of interest. LSI data during MRI could not be collected. This part of graph is shown with dotted lines with continuation sign (»).

Figure 2. Cerebral perfusion measurement through cranial window correlates with changes in DSA. (**A**) Baseline (pre-BAO) digital subtraction angiogram (DSA) of the basilar artery and Circle of Willis. Basilar artery indicated by arrows. (**B**) DSA at post-BAO. (**C**) Laser speckle perfusion imaging (LSI) at baseline (pre-BAO) and (**D**) during occlusion after delivery of autologous clot. Post-processed analysis for the detection and enhancement of microvasculature using retooled laser speckle contrast analysis (rt-LASCA) technique at (**E**) baseline and (**F**) post-BAO. Color bar code: blue to red = low to high perfusion. Cranial window shown by dotted rectangles.

Figure 3. T2-weighted magnetic resonance imaging after stroke. T2-weighted MRI using rapid acquisition of relaxation enhancement (RARE) sequence is inadequately sensitive to detect early acute strokes (**A**) which was also corroborated on flow attenuated inversion recovery (FLAIR) sequence images (**B**).

Figure 4. High b-value diffusion weighted (DWI) and apparent diffusion coefficient (ADC) mapping and quantification of acute ischemic stroke volume. (**A**) DWI images showing region of restricted diffusion (high signal intensity area, dotted outline) acquired after occlusion of the basilar artery. (**B**) ADC maps were obtained after processing of two DWI images using a range of b-values. Optimized images to measure stroke volumes were obtained with b=1800 s/mm^2. (**C**) Color enhanced diffusion weighted image. (**D**) Color enhanced ADC map. Quantification of stroke volume in canine model was performed by tracing the stroke borders to calculate area and multiplied by the slice thickness. DICOM image post-processing was done by delineating a border on the enhanced ADC images. Whole brain and infarct volumes were calculated from the measured values. (**E**) Whole brain volume as 100 percent, the infarct volume percent was compared. Data = mean ± SEM.

Figure 5. Digital subtraction angiography and magnetic resonance angiography of brain help visualize thromboembolic basilar artery occlusion. (**A**) Representative DSA showing basilar artery occlusion. (**B**) Representative canine brain MRA using 3D-time-of-flight sequence. Maximum intensity projection (MIP) volume rendering technique, leveling, filtering, and surface display was performed to create the MR angiograms from the DICOM image series. Color coding using color look-it-up table. Representative image shows restricted flow in basilar artery (arrows).

Figure 6. Pre-clinical model provides opportunity to identify anatomical locations of stroke lesions using histochemical methods. Representative TTC stained medial section (**A**). Tissue harvested at the time of sacrifice. MRI-ADC map (**B**), taken to represent functional image, were overlaid on TTC stained image (**C**). H&E stained section immediately proximal to TTC section (**D**). Same ADC map (**E**) was also overlaid on H&E image to match the stroke site (**F**).

Supplementary Figure S1. Physiological data before BAO and at time of sacrifice. Baseline data were compared with post-BAO in all seven dogs. Ages range from 14-21 months and body weights range from 8-13 kilograms. Mean, systole, and diastole blood pressures (BP) are shown. Representative electrocardiographs (ECGs) at baseline (**A**) and just before sacrifice are shown (**B**).

DISCUSSION:

The most common causes of posterior circulation stroke include embolism, large-artery atherosclerosis, and small artery disease[5]. Basilar arterial occlusion (BAO) represents a subset of posterior circulation strokes, carrying significant morbidity and mortality[13]. In this context, a canine model of acute posterior stroke was utilized and we developed an LSI protocol to monitor perfusion of the occluded region in real time. Laser speckle perfusion imaging was performed through a small cranial window, providing real-time information about a defined region of interest. Perfusion decreased after stroke onset and persisted to the end of the experiment. This window can provide real time information about changes in the perfusion after therapy with thrombolytic drugs or thrombectomy devices. After performing LSI through the cranial window, the perfusion data was further processed to visualize vascular anatomy of the stroke affected region using our rt-LASCA algorithm[15]. In this study, the posterior circulation occlusion recorded by laser speckle perfusion correlated with intra-operative angiography. The cranial window,

although it takes less than 5 minutes to complete, can be modified for the interests of the investigation but the actual size is only slightly larger than the field that will be recorded. Therefore, making the window larger will only incur greater injury to the surrounding tissue and more bleeding, the recording field cannot be expanded by the equipment. Having said that, this method can be used to access thrombolytics, prophylactics, or any drug which may alter cerebral flow and therefore can be moved to interrogate any region where the area can reasonably be accessed with the surgical setup.

Cerebral infarction in pre-clinical studies has been generally diagnosed on the basis of post-mortem histological staining. Clinically, stroke burden is assessed by MRI[20], as it is more sensitive than a non-contrast head CT[21]. MR diffusion weighted imaging (DWI) sequence paired with an apparent diffusion coefficient (ADC) map is the most sensitive sequence for acute strokes[22]. On the other hand, the gold standard for vascular imaging for LVO stroke is a cerebral angiogram and should be considered when initial, noninvasive imaging is non-diagnostic or conflicting[13]. Successful recanalization of LVO is an important predictor of a better outcome after stroke[23]. The optimized b-value DWI for the dog was able to detect the acute ischemic lesion within 4 hours post BAO. An optimized b-value is essential to achieve ideal sensitivity and is different depending on what is being studied. The ideal b-value in adult humans is b=1000 s/mm^2, in neonates it is b=600 s/mm^2. In this canine model, we found ideal DWI at b=1800 s/mm^2. ADC maps corresponding to high b-value are more sensitive for hyper-acute ischemic stroke as early as 30 min after stroke onset[21]. Furthermore, the ADC maps enabled better discrimination of infarct and normal tissue thereby increasing the accuracy of tracing lesion boarder for accurate infarct volume calculation[24].

A 3D-time-of-flight (TOF) non-contrast-enhanced method is based on flow related vessel enhancement and has increased signal-to-noise ratio, better spatial resolution, faster acquisition times, and better resistance to intrascan motion artifacts[25,26]. In fact, better spatial resolution enables more accurate analysis of changes in the vessel patency, particularly with vessel branches in close proximity, and in the pathological settings of stenosis and thrombosis[20]. In this study, the MRA technique successfully illustrated BAO. In addition, we have developed a Matlab-based program to fuse ADC reconstruction on histology.

Results of the present study in dogs indicated that the use of multimodal imaging platform using DSA, LSI, MRI, MRA and histologically staining techniques become powerful tools to evaluate acute ischemic stroke and provide preclinical assessment of drug and device therapies.

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DISCLOSURES:
The authors have nothing to disclose

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Figure 1

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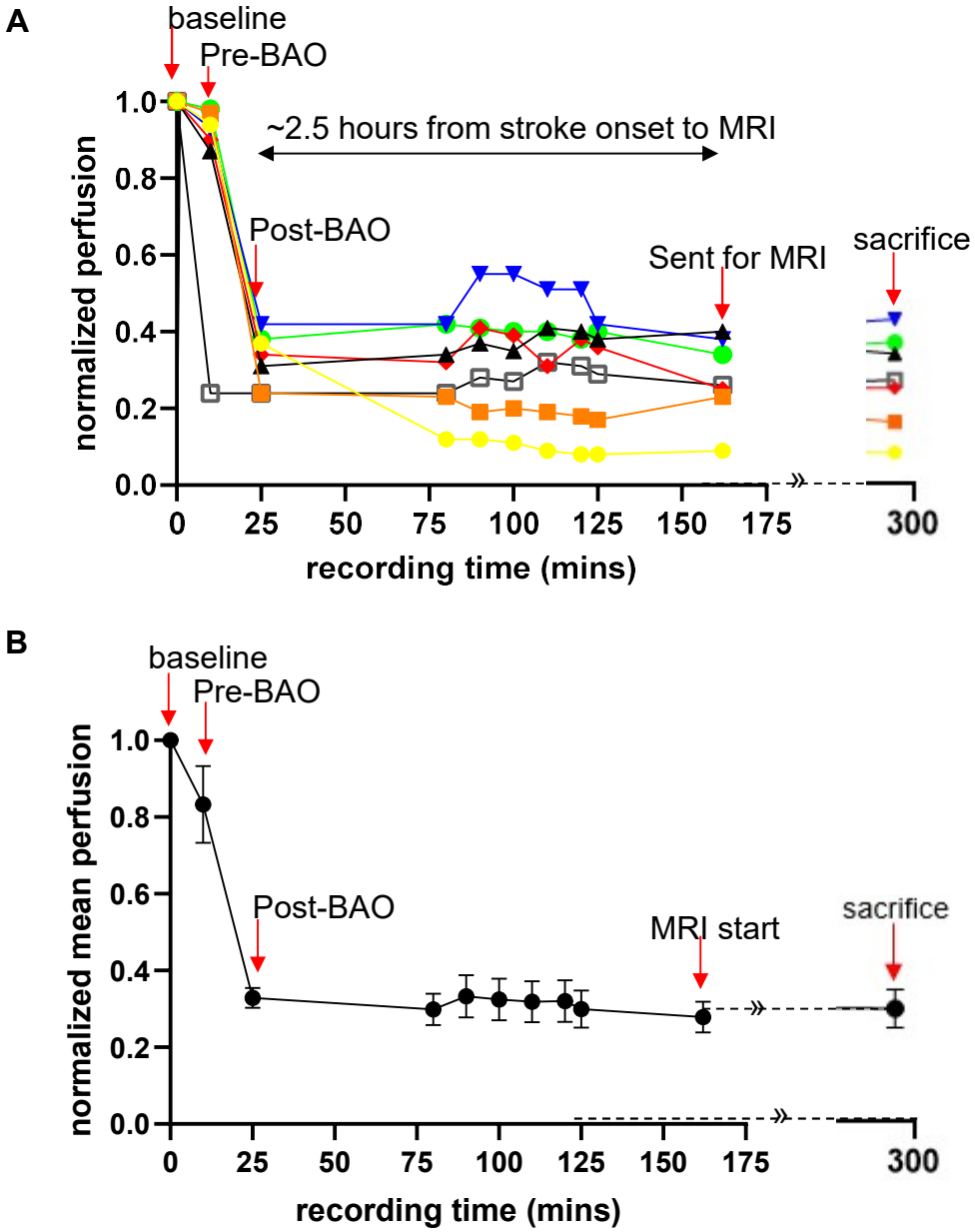

Figure 2

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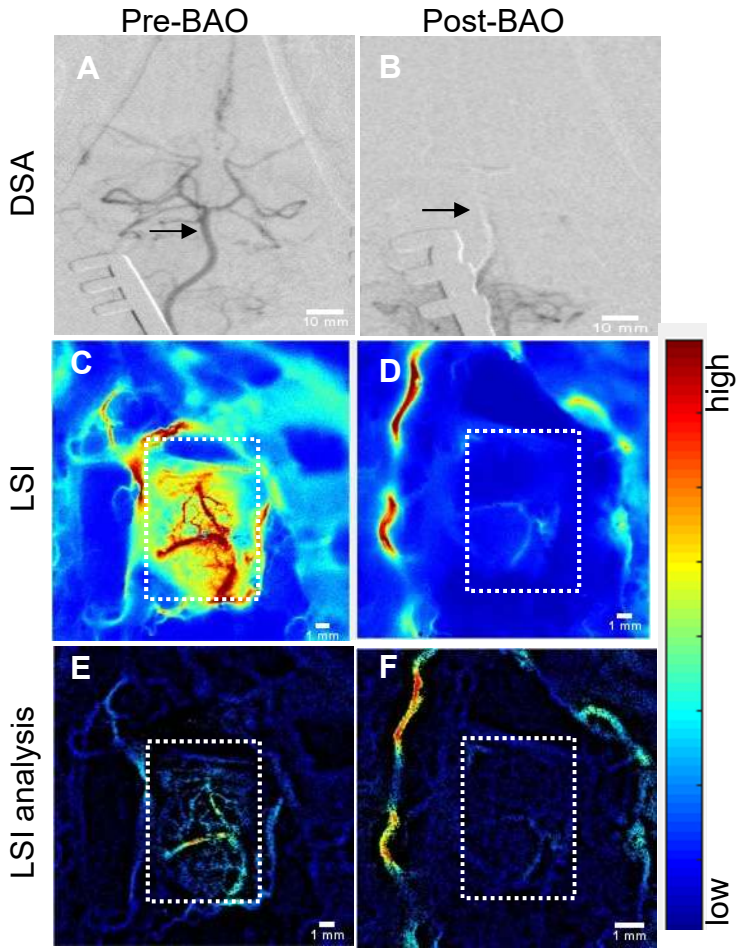


Figure 3

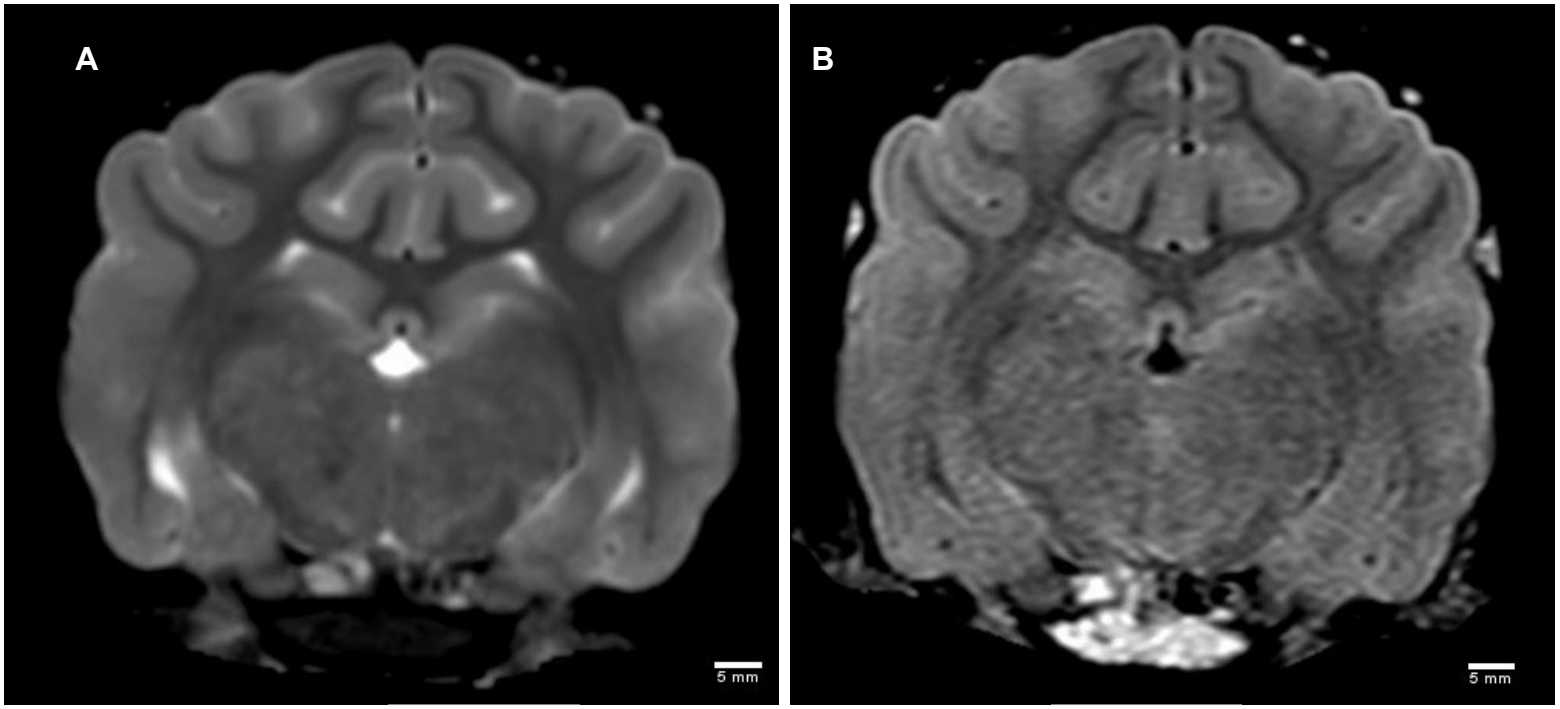


Figure 4

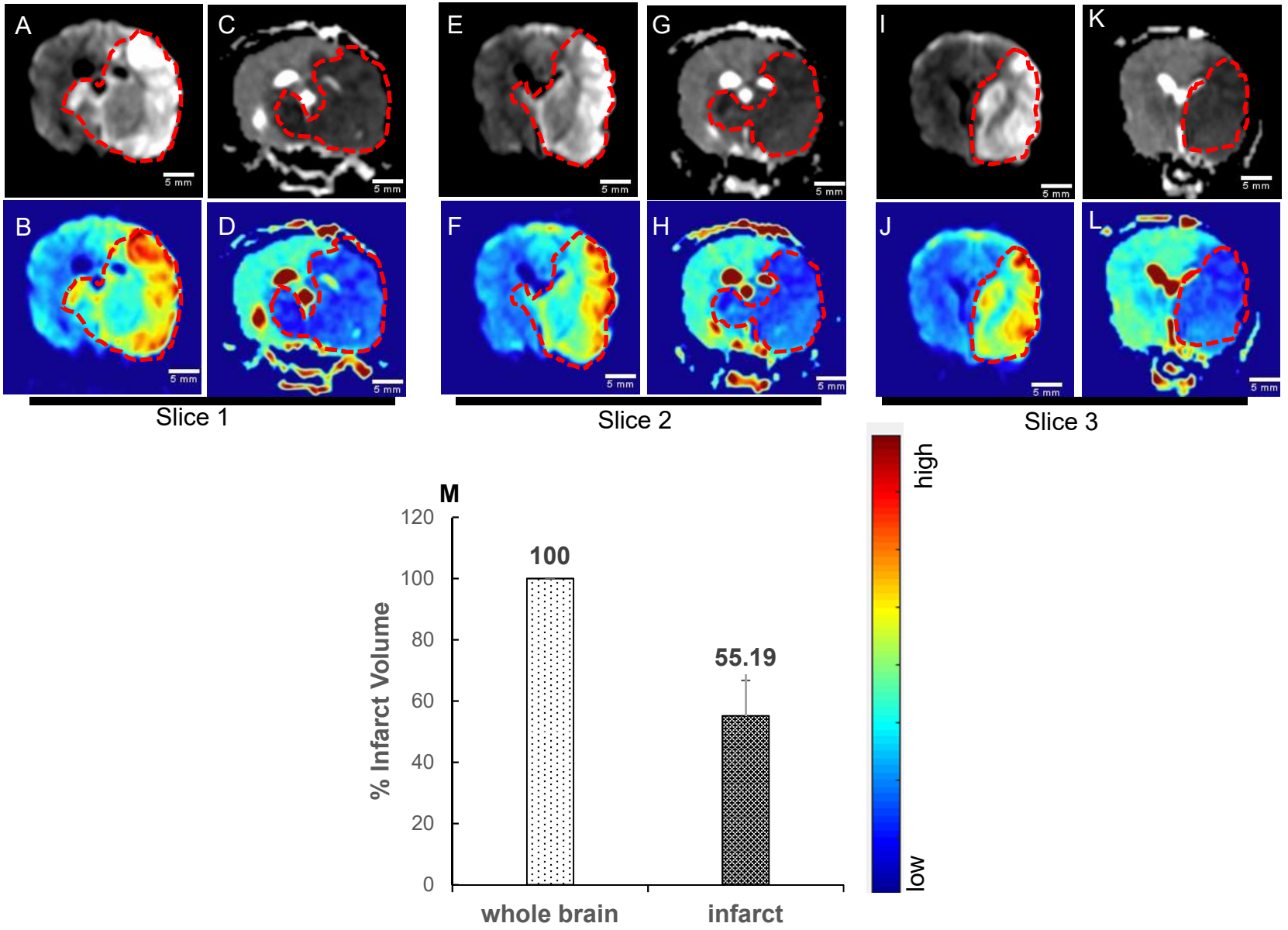
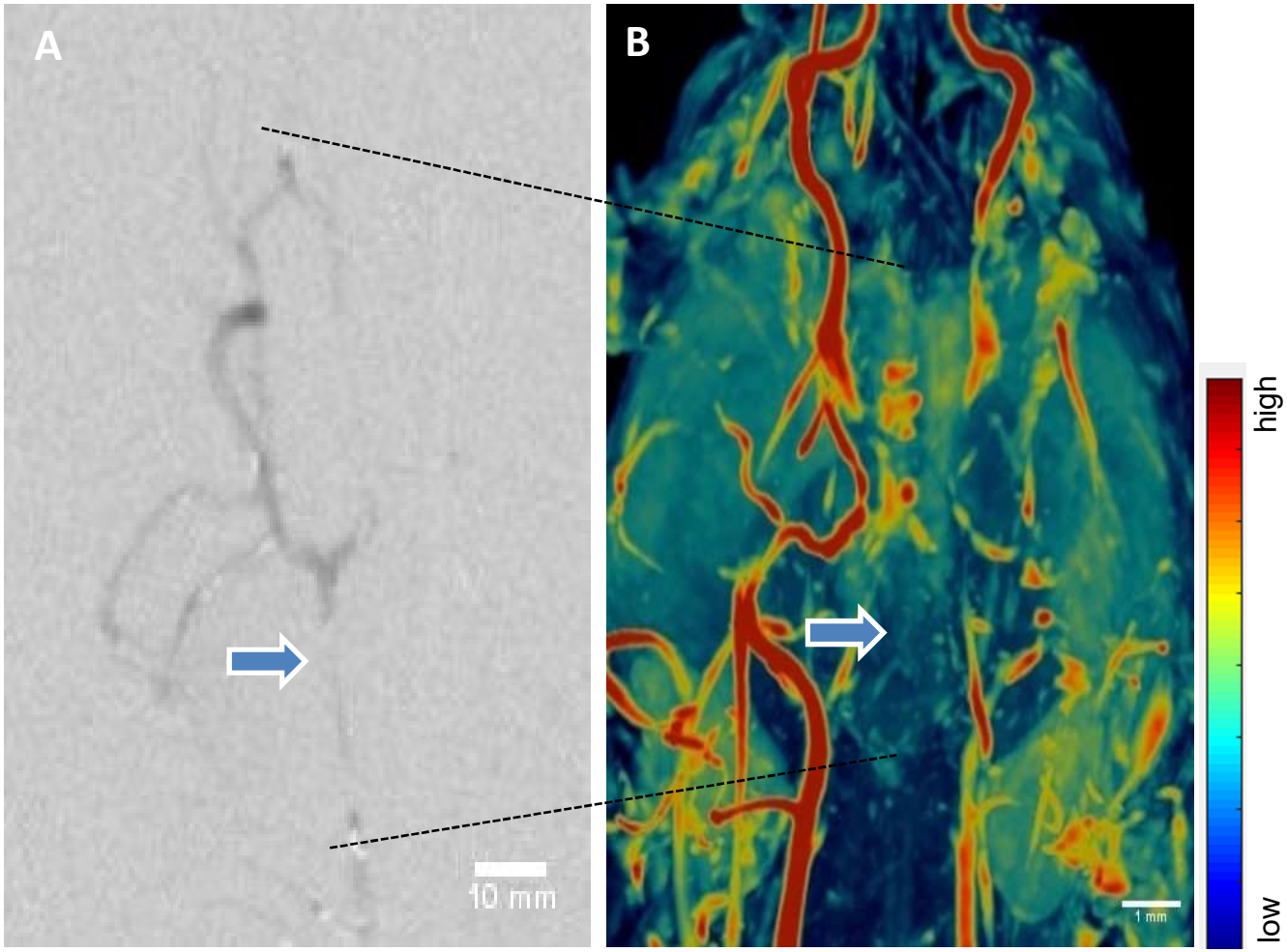
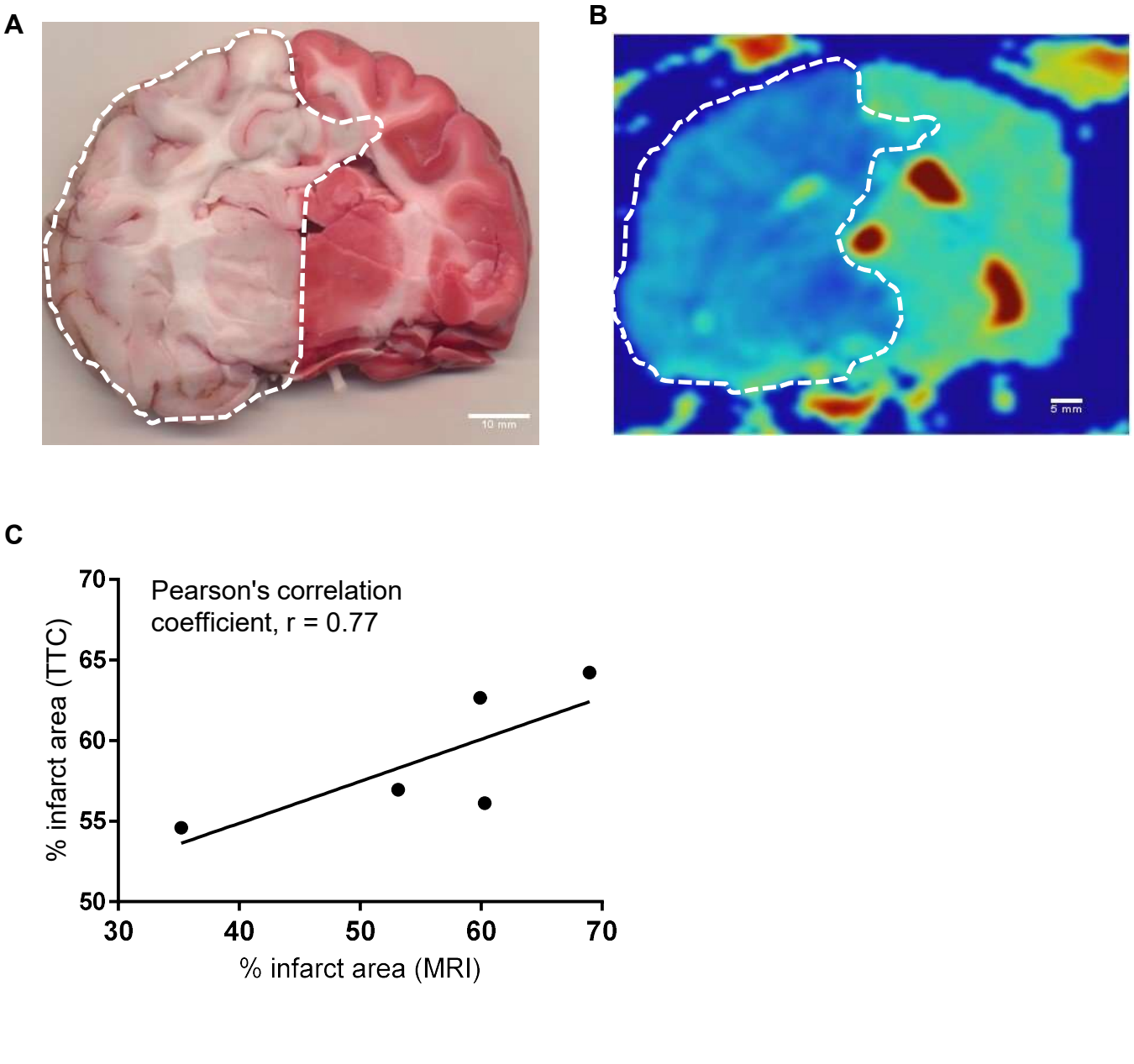


Figure 5





Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2% 2,3,5-triphenyltetrazolium chloride (TTC in PBS, pH 7.4)	Sigma Aldrich	T8877	
EDTA K3 vacutainers	Becton Dickinson	BD455036	
Eosin	Surgipath	3801602	
Formalin, neutral buffered, 10%	Richard-Allan Scientific	5701	
Hematoxylin 560	Surgipath	3801570	
HUG-U-VAC positioning system	DRE Veterinary	1320	
LabChart Software	ADInstruments Inc.		
Laser Speckle Imaging camera	Perimed Inc., Jarfalla, Sweden	PeriCam PSI HR System	
Lithium heparin vacutainer, 4.5%	Becton Dickinson	BD 368056	
Matlab	The MathWorks, Inc., Natick, MA		
OsiriX MD v.5.0 software	Pixmeo Inc, Geneva		
Paraformaldehyde 4% in PBS	Alfa Aesar	AAJ61899AP	
PimSoft v1.4 software	Perimed Inc.	software that accompanies LSI equipment	
Prisma Fit 3 tesla (3T) magnet	Siemen's Diagnostics		
Sodium heparin for injection (to coat blood gas syringe)	NovaPlus	402525D	

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• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Mention species in the title. DONE

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1) Split up long steps such as 2.4 into 2 or more steps. DONE

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to all your protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Examples:
1) 1.6: how? Mention all tools used DONE
2) 2.4: Please describe all actions in more detail. Currently this step is too broad and generalized. These are actually only clicks of a mouse to utilize the software which comes with the equipment and are included in the training by the company personnel.

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2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting. DONE

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• **Figures:** Fig 2-6: Please add scale references. DONE

• **References:** Please spell out journal names. DONE

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• **Table of Materials:** Please sort in alphabetical order. The Table was already in alphabetical order but has been updated to include more commercial names

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Reviewer #1:
Manuscript Summary:
This paper illustrates the efficacy of using laser speckle imaging (LSI) to monitor perfusion changes in real time in a canine model of large vessel occlusion. In addition, the authors also present infarct volume changes using diffusion MRI. Overall the LSI technique is quite interesting and I can see how it can be valuable especially in basilar artery occlusion (BAO) cases where nearly half the brain in affected. This technique can be used to monitor treatment and therapeutic effects. However, there are some critical changes that need to be addressed especially in the context of diffusion MRI. Please see comments below.

Major Concerns:
Lines 396-403: The authors address the choice of b-values based on optimization to detect lesions mentioning that the b-value value needs to be a certain value for humans versus neonates versus dogs. However, that is not an effective way to describe the "detection" of lesions. The ischemic region can contain different degrees of water restriction, i.e. the movement of water molecules in the tissue can be dictated by Gaussian or non-Gaussion behavior depending on how much restriction is experienced by the water molecules in the region of ischemia. For e.g., multiple low b-values can emphasize perfusion effects (also called intravoxel incoherent motion - IVIM) while large b-values emphasize the kurtosis effects (non-Gaussian movement of water) due to application of stronger gradients and large echo times. In this experiment with the dogs, the high-b values are emphasizing the non-Gaussian movement of water and that should be clearly explained in the context of water motion rather than just saying that a b-value is optimized for sensitive DWI. The authors need to revise this section significantly to clearly explain the underlying physical principles that lead to the high b-value choice. See second paragraph of discussion.

Line 98: The craniotomy that is performed is localized to a 1 cm^2 window. This seems to be effective only if a significant region of the brain is infarcted, which is the case in BAO. However, if the infarct region is smaller and only isolated to a smaller region of the brain, then how would you decide where to create the craniotomy window? Perhaps some discussion about this issue should be addressed in the Discussion section since the focus of this paper is in the use of LSI as a new way to look at perfusion in the infarcted regions. See first paragraph of discussion.

Line 98: How long does it take to do the craniotomy? This might be pertinent to know since time is of the essence in treating stroke. See first paragraph of discussion

Lines 272-273: Increased signal intensity as a typical sign of infarction would apply to DWI images. That really depends on the type of image you look at. On ADC maps, for e.g., the infarct region is dark. So, it's better to omit that sentence. Omitted

Line 349: Change the figure to add panel E and change y-label to "% infarct volume" to be consistent with the figure legend. "E" added and amended.

Figure 4: I understand that the slice shown has a much larger restricted volume than 55% indicated in the graph, which actually shows the infarct volume over the whole brain. It might be appropriate to show a slice that shows a similar ratio to the one on the graph shown in panel E. Or show multiple slices of the brain so that the quantification is clearer to the reader. Figure and legend amended as suggested.

Lines 300: I don't think it's necessary to overlay the images. It looks confusing at first look. Just showing the TTC stain and ADC map would suffice to see the correlation between the two images. Additional correlation plots showing the volumes from the TTC stains and MRI images should also be added as quantification of the correlation instead of just a subjective visual view. Figure and legend amended as suggested.

Minor Concerns:
Line 95: Change to "… mechanically ventilate using…" CHANGED
Line 162: remove Magnetic Resonance Imaging and mention that in Line 71 with first mention of MRI. CHANGED
Line 171: Change mRA to MRA. CHANGED
Line 182: Correct the matrix size. Looks like a typo. Also, given that this is the methods section, the b-values need to be specified here that were used for the diffusion-weighted imaging.
Line 192: omit "to represent" CHANGED
Line 208: Change to "… and place the cut tissue…" CHANGED
Line 223-224: Change to "…into a previously prepared solution containing 100mL…" CHANGED
Line 256: Change to "…After injecting the prepared thromboembolus…"
Line 324: Change to "…could not be collected. This part of the graph…" CHANGED

Reviewer #2:
Dear authors,

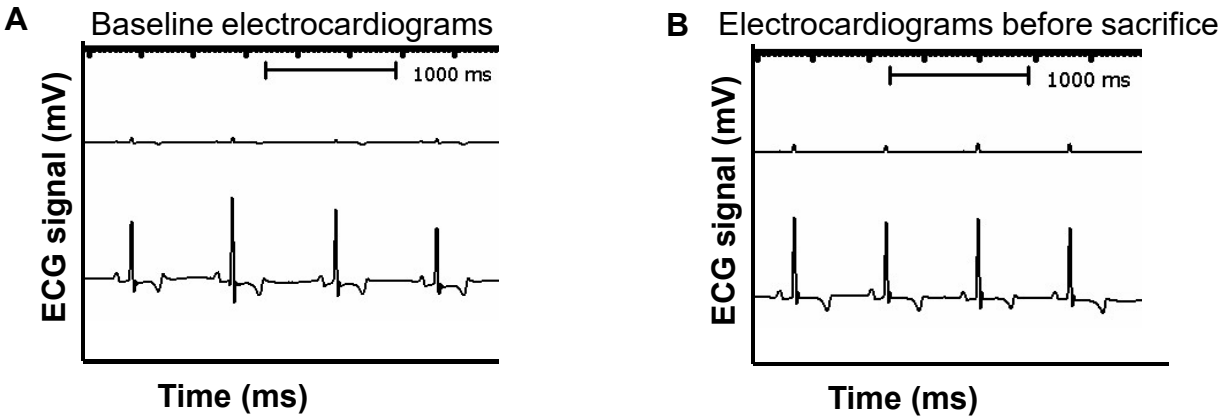
This is an important work as it describes an endovascular model of stroke in canines reproducing a devastating clinical condition - basilar artery occlusion. The authors use MRI and laser speckle to determine blood flow. In addition, the authors use TTC and H&E staining to confirm infarction at the time of sacrifice.

The authors performed the procedure seven times. The paper is a good proof-of-concept paper. Namely, it is feasible to perform a craniotomy, conduct laser speckle imaging, perform an endovascular basilar artery occlusion, then MRI imaging and sacrifice the animal. The model, MRI imaging, and sacrifice have been described before. The addition of the cranial window and laser speckle imaging is, to my knowledge, novel. I have a few areas of concern that temper my enthusiasm for this paper. The basilar artery occlusion in canines could result in "anterior" circulation strokes in the middle cerebral artery distribution from embolization of the clot through the posterior communicating artery. This increases the variability of the study to examine stroke size and neurological impairment as a result of the stroke or its treatment. This issue if it was identified should be discussed and addressed in the discussion. The basilar artery occlusion model can also produce some immediate hemodynamic changes - occasionally heart block occurs and this needs to be addressed as well. Also, the use of a craniotomy has been shown to decrease stroke size so this is a confounder to measurement of stroke. The location of the craniotomy in the canine is also of interest as the basilar artery is in a difficult place to access from the side of the head. The craniotomy location should be precisely described (maybe it was and I did not see it in the paper). Lastly, this is unlikely to be a survival model given the severity of disability with basilar artery occlusion. This will limit the utility of the model. Variability in this model with this procedure was NOT encountered in canines, but was encountered in mice with MCAO. We are currently writing up the stroke paper for both canines and mice and found that 7 animals per group were sufficient to reach significance with the canine model to access thrombolysis. Hemodynamic issues occurred only in the acute period immediately after clot placement, but did not affect the experiment to the extent that all canines were successfully transported, imaged, and returned for sacrifice at the designated experimental time point. In the next manuscript describing thrombolysis with this model, we performed craniotomy on ALL treatment groups and will add to that manuscript that exact stroke volumes are not as accurate as the difference in stroke volumes with treatment. We completely agreed with the reviewer in that BAO is severe, this is evidenced by the high mortality seen in patients when this area is affected. BAO was used because the size of a beagle may allow large animal studies since they reduce the costs of reagents, thrombolytics, per diem, animal purchase, etc but MCAO is not possible unless the size of the canine and all other expenses are increased along with it.

Having provided the above criticisms, I still think it is worthy of publication. I believe the paper would be strengthened if it addresses the aforementioned weaknesses.

Supplementary Table 1: Baseline compared to 4hrs post-BAO in thrombolytic dogs

Parameters	Baseline (mean±SD, n=7)	Post-BAO (mean±SD, n=7)	P value
Age (months)	18.71 ± 2.50	-	-
Body Weight (Kg)	10.86 ± 1.53	-	-
Heart Rate (beats/min)	91.29 ± 13.30	101.14 ± 14.23	0.60
SpO_2(%)	99.00 ± 1.00	98.00 ± 0.82	0.32
Mean Blood Pressure (mmHg)	63.57 ± 9.78	55.14± 6.69	0.35
Systolic Blood Pressure (mmHg)	84.71 ± 12.54	66.71 ± 7.54	0.04
Diastolic Blood Pressure (mmHg)	52.14 ± 8.36	47.86 ± 8.82	0.75



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Title of Article: Quantification of canine cerebral perfusion using laser speckle imaging and infarct volume using MRI in a pre-clinical model of posterior circulation stroke

Author(s): Surya Gnyawali Allyson Huttman Ian Mandybur Jenna Bove Matthew Joseph Debra Wheeler Cole Anderson Catherine Lee Caitlin Hatten Shahid Mujeeb

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