Quantification of Neurovasculature Changes in a Post-Hemorrhagic Stroke Animal Model

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INTRODUCTION

- Hemorrhagic stroke is associated with high mortality, morbidity and disability - with a 1-year mortality rate of up to 58% (*Smajlović*).
- Changes in microvascular branching contribute to the etiology of hemorrhagic stroke.
- Foundational studies to further define changes in cerebrovascular microcirculation, including volume and branching, associated with hemorrhagic stroke are needed.
- Micro-Computed Tomography (micro-CT), coupled with bundled SkyScan 1176 software (Bruker), can produce 3D reconstructions of the vascular network at a resolution sufficient to resolve small blood vessels, while leaving the tissue intact for subsequent analysis.

Figure 1: SkyScan 1176 based in PORL, University of Alberta.

Soft tissues, such as cerebral blood vessels, are not easily detected by X rays using micro-CT under normal conditions - however, when filled with a radiopaque contrast dye, such as Vascupaint™ silicone bismuth (Medi- Lumine™, Montreal, QC) the vessels can be visualized by micro-CT.

- The imaging power of this technique can be advanced even further when coupled with a fluorescent imager and a fluorescent dye, such as Evan's Blue.

OBJECTIVE

Establish a methodology to define changes in neurovascular network parameters, such as volume and connectivity, in rodent brains before and after stroke using high-resolution imaging technology and a novel perfusion casting agent.

HYPOTHESIS

Post-stroke rats will have less intact vasculature surrounding the site of hemmorhage and less neurovascular branching, as compared to pre-stroke rats.

MATERIALS AND METHODS

This study utilizes female and male spontaneously hypertensive stroke-prone rats (SHRsp) in which the incidence of stroke is approximately 80%. Uncontrolled hypertension is the leading controllable factor contributing to hemorrhagic stroke in humans (Wajngarten), thus this animal model is translatable.

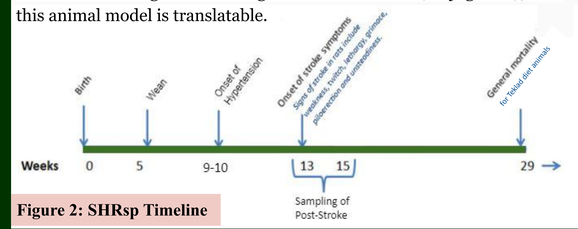

Figure 2: SHRsp Timeline

MATERIALS AND METHODS

All experimental procedures and animal breeding activities were in compliance with guidelines set forth by the Animal Care ethics committee (Protocol #20-30-ND)

- Rats anaesthetized using 50mg:10mg/kg ketamine/xylazine
- Evan's Blue (EB, 30mg/kg) infused through the femoral artery and allowed to circulate for 20 minutes
- Thoracic chest opened; abdominal aorta and inferior vena cava clamped
- Warm heparinized 1% phosphate-buffered saline (PBS) perfused transcardially at a rate of 1.2ml/min using a perfusion pump (MasterFlex L/S Standard Drive) and winged infusion set
- 80ml 4% paraformaldehyde (PFA) in PBS perfused at 0.9ml/min to fix the anterior half of the body
- Vascupaint™ (VP) prepared using 5ml bismuth component/6ml diluent/0.25ml catalyst and perfused through the aortic arch at rate of 1.5ml/min until first sign of Vascupaint™ observed in key indicator regions, approximately 30 seconds (see Figure 3). The rate then reduced to 0.5ml/min.

Figure 3: Key Indicators of VP Perfusion to Brain. Conjunctival/episcleral vessels (left) and exterior nares/sublingual vasculature (right)

- Backflow from the right atrium observed, confirming completion of perfusion circuit
- Carcass hung for 48h in refrigeration prior to sampling
- Sample preserved in 10% buffered formalin phosphate at 4℃ until micro-CT performed

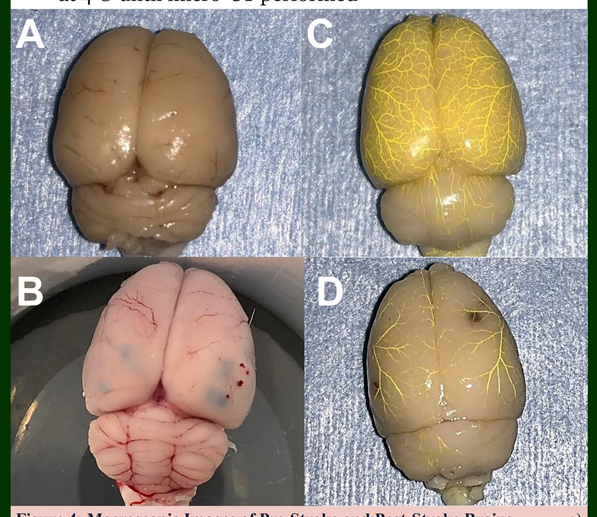

Figure 4: Macroscopic Images of Pre-Stroke and Post-Stroke Brains a) pre-stroke brain, EB b) post-stroke brain, EB c) pre-stroke brain, VP d) post-stroke brain, VP

Analysis via Micro-CT and SkyScan 1176 Software

- Using an exposure time of 300ms, a 180° scan of the entire brain was performed with a rotation step of 0.5 degrees (3 projections averaged every half a degree)
- 9 µm resolution produced 400 image projections that were reconstructed using bundled NRecon program to create over 2500 axial (coronal) slices of each brain
- Brains were rotated in DataViewer to standardize the orientation between scans
- Region of Interest was analyzed using CT-Analyze in proximity of the middle cerebral artery (MCA), as this is the most common location for hemorrhagic stroke

RESULTS

Rat n=53 total; of these, n=8 pre-stroke and n=6 post-stroke were included in final analysis. The remaining n=39 were used to establish an infusion Standard Operating Procedure (SOP). To optimize the technique, variations in casting agent volume/dilution, perfusion flow rate, site of infusion and parameters for analysis were considered. Post-stroke animals exhibited EB extravasation and decreased VP perfusion macroscopically. The 3D-rendering of brains upon micro-CT imaging indicated decreased perfusion intracerebrally post-stroke. Branching, connectivity and percent vascular volume analyses (considered to be affected during hemorrhage) were developed and are currently being optimized. Our study established a standardized method for the preparation, imaging and measurement of rat cerebral blood supply through use of radiopaque contrast dye.

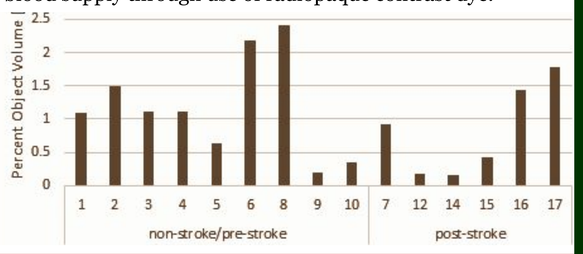

Figure 5: Percent (%) Object Volume: % of the total region of interest (ROI) that is vasculature

Pre-stroke average (n= 8) = **1.175%** (SD 0.714)
Post-stroke (n=6) = **0.813%** (SD 0.659)

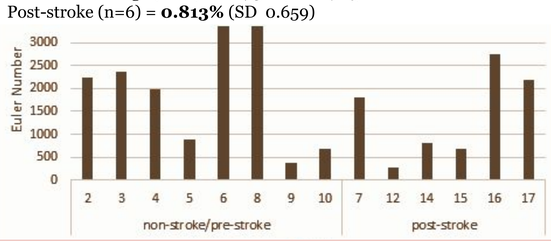

Figure 6: Euler Number (excluding Rat 1): connectedness of a 3D structure

Pre-stroke (n=8) = **1904.75** (SD 1169)
Post-stroke (n=6) = **1412.5** (SD 979)

Table 1: Comparison of Parameters Before and After Stroke

	Structure Model Index	Structure Thickness (um)	% Object Volume	Fractal Dimension	Euler Number	Connectivity Value
Rat 6 (pre-stroke)	3.08525	104.30371	1.57015%	1.97235	1304	218
Rat 12 (post-stroke)	3.2017	104.29618	0.16623%	1.95451	176	26

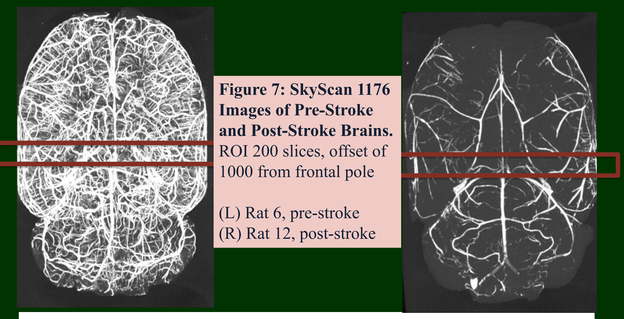

Figure 7: SkyScan 1176 Images of Pre-Stroke and Post-Stroke Brains. ROI 200 slices, offset of 1000 from frontal pole

(L) Rat 6, pre-stroke
(R) Rat 12, post-stroke

FUTURE DIRECTIONS

The method developed serves as foundation for in-depth quantitative analysis.

- Ex vivo sampling will continue in both female and male strokers, along with age-matched pre-stroke controls
- In-vivo imaging of pre and post stroke neurovasculature using SkyScan 1176 is a critical next step
- Immunohistochemistry to detect relevant markers of inflammation contributing to hemorrhage pathophysiology
- ART Optix FLUOR imaging to quantitatively define association between extravasation of EB & destruction of surrounding vasculature

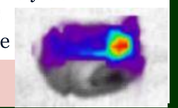

Figure 8: Fluorescence Imaging to Detect EB. Dorsal view of Rat 14 post-stroke brain using a 670nm laser

CONCLUSIONS

Our study used a novel casting compound and micro-CT technology in a unique stroke-prone animal model for the quantification of cerebrovascular anatomy. Advances in visualization of vasculature is critical for the potential development of new treatment strategies, enabling accurate quantification of cerebrovascular supply. Early results support our hypothesis that there will be less intact neurovasculature post-stroke, as compared to pre-stroke. Micro-CT images acquired demonstrate that arteries, veins and capillaries can be perfused successfully to detect changes in neurovasculature post-hemorrhagic stroke. Further work to standardize and validate the quantification technique is necessary.

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