

Summer Student Project Report 2024

Student

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Title of Project

Investigating the impact of microvascular obstruction on the local cellular environment using optical imaging



Supervisor

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Lay Summary

Cerebral microinfarcts are discrete lesions of ischemic origin, which result from micro-occlusions in the vasculature. These 'silent' mini strokes are a prevalent clinical characteristic of the ageing brain and are particularly common in cases of cognitive decline and dementia[1]. In ischaemic tissue, vessels are damaged and become dysfunctional, leading to the build-up of microthrombi, which exacerbate pathological processes. This project therefore investigated the consequences of microvascular occlusions on the local cellular environment and the composition of these occlusive thrombi, as this has important therapeutic implications. Micro-thrombotic occlusions in ischaemic tissue primarily consists of platelets, red blood cells and fibrin and understanding the specific composition of these clots is important for informing treatment strategies. For fibrin dominated clots, thrombolytic agents such as tissue Plasminogen Activator (tPA), which targets surface fibrin to dissolve the clots, has been approved by the FDA. However, these drugs are less effective when targeting platelet-rich occlusions, so further research on strategies to accelerate the removal of platelet-rich thrombi is required.

The body has several endogenous mechanisms for micro-occlusion removal, primarily through activation of the coagulation cascade, coupled with increased blood pressure to aid in clot washout. However, if micro emboli are not removed via these processes, angiophagy, or Thromboangioplasty (TAP) may occur locally to remove these clots. Thromboangioplasty is a process by which the endothelial (EC) and smooth muscle cells (SMC) actively remodel around a thrombus, leading to total engulfment and extrusion of a clot from the blocked vessel [3]. This project therefore also aimed to explore rates of angiophagy in platelet versus fibrin-rich clots and examine the ultimate health of the surrounding tissue in the weeks after these occlusions.

Project Aim

The overall aim of this project was to explore the effect of cerebral microvascular occlusions on local tissue health. Following injection of microbeads to induce ischaemic in a murine model of microinfarction, we monitored the effects of these emboli on vascular plasticity, myelin maintenance, microglial reactivity and neuronal survival over time.

Methods

Embolisation and intravital imaging

Micro-occlusions were induced in mice by 1) Injection of 2500 15 μ m microspheres or fluorescent fibrin-rich emboli into the middle cerebral artery or 2) application of ferric chloride to an arteriole to induce platelet-rich thrombi. In vivo repeated imaging of the pial vasculature and thrombi was performed using a Leica SP8 confocal microscope with a 20X water immersion objective (1.0 NA, Leica).

Tissue processing and immunohistochemistry

For fixed tissue analysis, mice were perfused with 4% paraformaldehyde followed by a 24-h immersion postfix at 24 hours, 7 days, 14 days and 30 days post-surgery. Immunohistochemistry was performed on 60- μ m coronal free-floating sections. Antigen retrieval was performed prior to staining using sodium citrate solution (pH 6) at 90°C for 20mins. Tissue was treated with primary antibodies and the corresponding species-specific secondary antibodies. The following antibodies were used: neurons (NeuN), myelin (CNP), vascular endothelial cells (CD31), blood vessels (lectin), microglia (Iba1), platelets (CD41), fibrin (fibrinogen).

Image analysis

Endothelial envelopment of thrombi was quantified using ImageJ. A maximum intensity z-projection was generated, and the degree of envelopment of thrombi was quantified by tracing the GFP signal of the endothelium around the clot. The percent coverage of the thrombus was calculated as EC-GFP/ thrombus perimeter x100. To quantify platelet/fibrin thrombi, images were thresholded using ImageJ and area of thrombi calculated and counted.

Results

Platelet and fibrin accumulation is associated with ischaemic injury

The no-reflow phenomenon is a key feature of ischaemic injury and is characterised by the accumulation of obstructive microthrombi in vascular [5]. These microthrombi are composed of platelets, fibrin, red blood cells and leukocytes and the composition of the thrombi can have consequences on the surrounding microenvironment. To examine the ratio of platelet to fibrin microvascular occlusions, we performed immunohistochemistry on ischaemic brain tissue. We found an accumulation of both platelet and fibrin emboli within ischemic territories in the ipsilateral hemisphere, however, there were higher numbers of fibrin thrombi. Interestingly, there was no significant difference in the total area of fibrin microthrombi versus platelets, indicating equal coverage of fibrin and platelet occlusions in ischaemic regions.

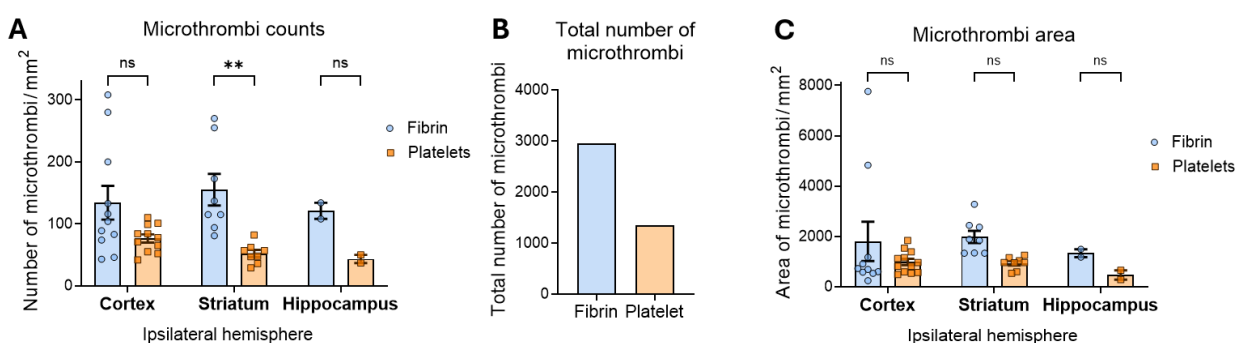
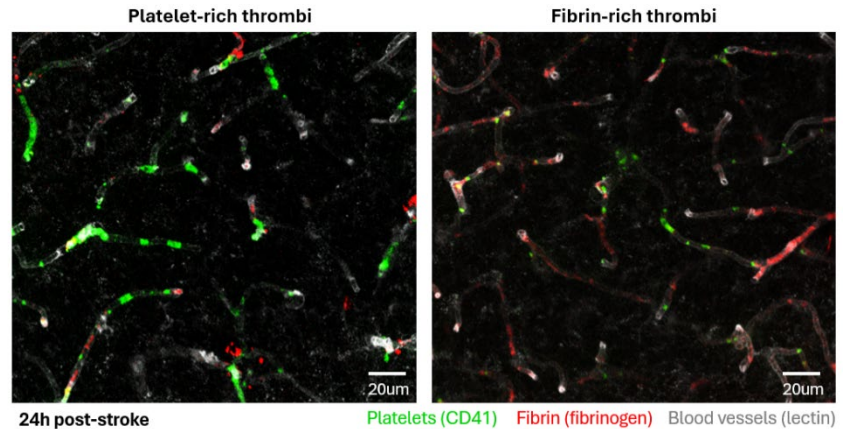


Figure 1: Platelet and fibrin composition of microthrombi in ischaemic brain tissue. A) Number of platelet and fibrin microthrombi quantified in the cortex, striatum and hippocampus of ischaemic brain tissue. B) Total numbers of platelet and fibrin microthrombi. C) Total area of platelet and fibrin thrombi in ischaemic brain tissue. ** $P=0.0039$. 2-way ANOVA followed by Sidak's multiple comparisons test. Data were expressed as mean \pm SEM.

Figure 2: Representative image of platelet and fibrin rich microthrombi in ischaemic cortical brain tissue



Platelet-rich occlusions induce accelerated microvascular remodelling

Removal of microthrombi can occur through activation of the coagulation cascade, blood pressure washout or thromboangioplasty (TAP) – a novel vascular remodelling process where emboli are engulfed and extruded [3]. To assess the efficacy of thromboangioplasty, we injected fibrin emboli (Fig 3A) and generated platelet rich emboli (Fig 3B and D). Intriguingly, the speed of TAP was accelerated in platelet rich emboli (Fig 3A-C). This suggests that platelets may play a key role in triggering or accelerating TAP. Understanding the mechanisms through which platelets facilitate this process could provide valuable insights into therapeutic strategies aimed at enhancing clot clearance, potentially leading to faster restoration of blood flow in ischaemic events.

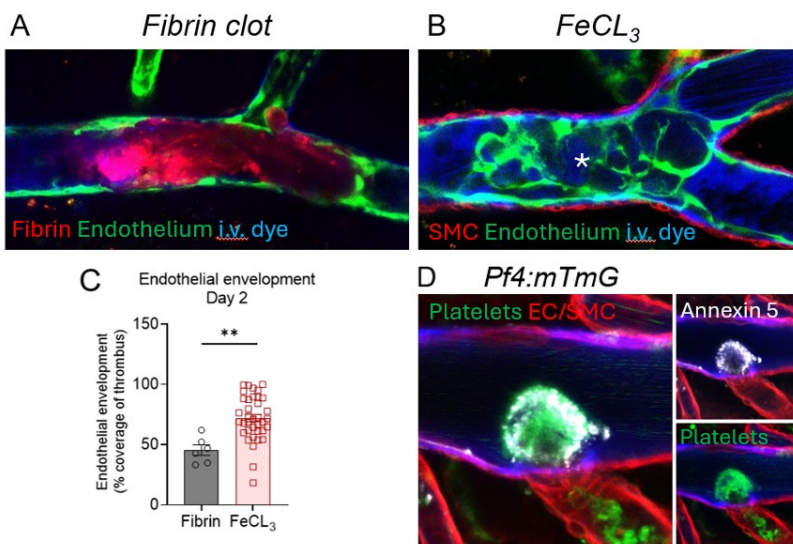


Figure 3: Speed of thromboangioplasty is dependent on platelets. Intravital imaging showed A) Fibrin (red) lodgement in a Tie2GFP mouse (endothelium, green). B) Ferric chloride induced endogenous thrombus (asterisk) lodged in a SMAMCherry:Tie2GFP mouse (endothelium, green; smooth muscle cell, red). C) Quantification of endothelial engulfment of fibrin rich and platelet rich thrombi. D) Ferric chloride induced endogenous thrombi are induced in Pf4-mTmG mice (platelets, green; EC/SMC, red; i.v. dye, blue; Annexin A5, white).

Consequences of microvascular occlusions on tissue health

To investigate tissue outcomes following microvascular occlusion, ischaemic tissue was stained with a range of markers (CNP, NeuN, Iba1). Myelin maintenance was observed over time (Fig 4), along with microglial reactivity and neuronal survival (Fig 5). Regions of myelin degradation were visualised at 24 hours post stroke with microglia engulfing the damaged myelin (Fig 4A). However, 15 µm microvascular

beads did not have a significant impact on the surrounding myelin (Fig 4B). This may imply that there is a stroke intensity threshold for oligodendrocyte death and myelin breakdown, which was not reached by the 15 μ m microspheres. The literature also supports that oligodendrocytes can withstand substantial injury, taking up to 45 days to die [4] which exceeds the duration of this experiment. As illustrated in Fig 5, microglia appear to attack and attempt to engulf the occlusive microspheres. This suggests that microglia are heavily involved in the immune response to microvascular occlusions.

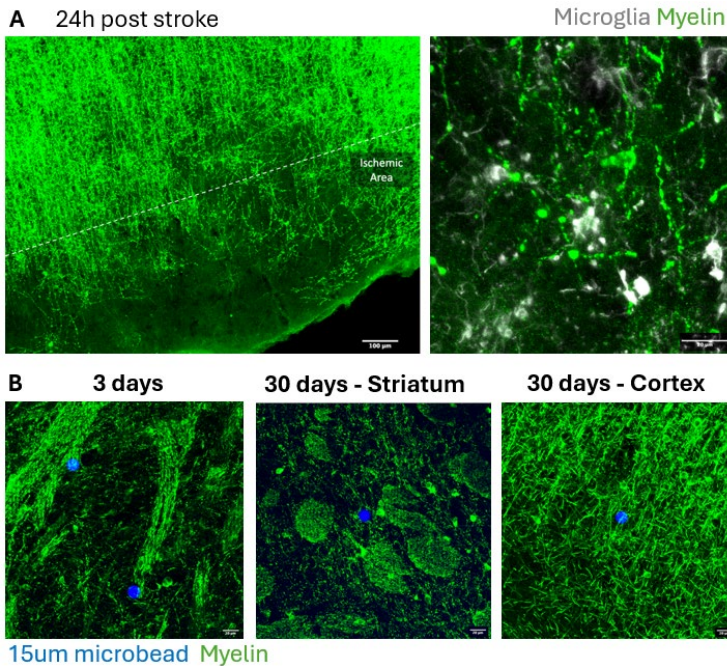
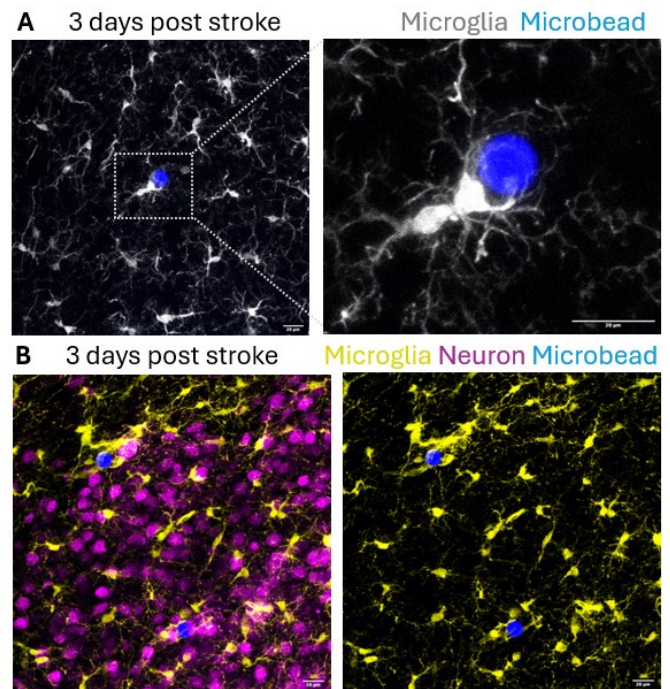


Figure 4: *Myelin dynamics following microvascular occlusion.* A) Degraded myelin (green) observed in ischemic regions 24 hours post-stroke, with microglia (white) actively engulfing damaged myelin. B) Myelin degradation at 3 days and 30 days.

Figure 5: *Neuronal survival and microglial reactivity to occlusion at 3 days and 30 days post-stroke.* A) Microglia (white) engulfing microvascular occlusion (blue 15 μ m microbead) in the corpus collosum at 30 days post-stroke. B) Neuronal survival (magenta) and microglial reactivity (yellow) to occlusion at 3 days post-stroke.



What did you learn from participating in this project?

Throughout this project I developed various key skills that will be invaluable in my future endeavours as a medical scientist. Firstly, this project has taught me the importance of optimising experimental conditions. As several of the protocols we used were not well established, we had to research similar experiments and adapt their methods to fit our specific objectives. This often involved trialling multiple techniques and solution concentrations before determining the most effective protocol, hence teaching me the value of patience and precision in experimental design.

As my experiments were time sensitive, I also had to learn how to schedule my time effectively to maximise efficiency in the lab. Balancing multiple procedures with overlapping tasks, while ensuring to follow the correct timing of each protocol highlighted the value of planning and remaining organised. This experiment also taught me how to work independently and trust my own judgement without seeking constant reassurance from supervisors. I gained confidence in problem-solving, making decisions and taking initiative as required.

Additionally, I acquired various new practical techniques, including immunohistochemistry, animal handling, staining procedures, vibratome and cryostat microtome use, mounting tissue sections onto slides, fluorescence confocal microscopy and solution preparation. These lab skills will help me immensely in my final year undergraduate research project and beyond.

How has this project affected your long-term goals?

This project has inspired me to continue my pursuit of a career in molecular medicine, with a current interest in both neurological disorders and cancer. The passion I found for lab work has led me to seriously consider obtaining a Master's degree or PhD in disease-related research. My supervisor, an exceptional neuroscientist, dedicated PI and incredible mentor, further motivated me to pursue this path. I aspire to achieve her level of success one day.

I was also granted the opportunity to attend weekly lab meetings, where the department would meet to present their work and provide guidance to colleagues. These meetings exposed me to the realities of being a scientist, highlighting that failed experiments are a natural and essential part of the scientific process, leading to the breakthroughs reached by the amazing researchers in the lab. I was also introduced to the level of dedication and discipline required of the PhD students, their work was both fascinating and inspiring, providing me with great insight into the PhD journey that I may be lucky enough to venture on in the near future.

Bibliography:

1. Wang, M., et al., *Cognitive deficits and delayed neuronal loss in a mouse model of multiple microinfarcts*. J Neurosci, 2012. **32**(50): p. 17948-60.
2. Campbell, B.C.V., et al., *Ischaemic stroke*. Nat Rev Dis Primers, 2019. **5**(1): p. 70.
3. Grutzendler, J., et al., *Angiophagy prevents early embolus washout but recanalizes microvessels through embolus extravasation*. Sci Transl Med, 2014. **6**(226): p. 226ra31.
4. Chapman, T.W., et al., *Oligodendrocyte Maturation Alters the Cell Death Mechanisms That Cause Demyelination*. J Neurosci, 2024. **44**(13).
5. Del Zoppo, G. J., et al., *Microvascular changes in cerebral ischemia and reperfusion*. Acta Neurochirurgica (1991) **51**, 201-211.