Evaluation of the peri-infarct vasculature in two different mouse models of ischemic stroke: photo-thrombotic (PT) and middle cerebral artery occlusion (MCAo)

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ABSTRACT

As the leading cause of adult disability in the US, stroke requires extensive preclinical research models to develop modes of treatment. With post-stroke angiogenesis shown to potentially play a crucial role in improved clinical outcomes, pro-angiogenic therapy is currently being widely studied as a post-stroke recovery therapy. However, a lack of literature exists on preclinical animal stroke models in their effect on angiogenesis. In this study, we investigate peri-infarct vasculature in the two widely used mouse models, photo-thrombotic (PT) and middle cerebral artery occlusion (MCAo). The first finding showed that the peri-infarct vascularization in the MCAo model was significantly greater than the PT model at both timepoints. Furthermore, no significant difference was found between timepoints when looking independently at each stroke model. This may suggest stability and remodeling was attained before the 2 weeks timepoint after stroke. The second finding compared the evolution of the peri-infarct vasculature over time between the two stroke models. Results showed that the peri-infarct vascularization in the PT model has a greater increase (faster process of vascularization) between 2 and 4 weeks compared to the MCAo model, suggesting that the remodeling in the PT model is still in progress. Thus, using the PT model to study pro-angiogenic therapies could require to sacrifice mice at a later time point, as the angiogenic process is slower in this model. Future studies should compare angiogenesis in human stroke recovery and investigate the process at different timepoints to better identify the most appropriate animal stroke model for experimental studies.

1. Introduction

Stroke is the leading cause of adult disability in the US and the third cause of death worldwide.[1] A majority of strokes are ischemic strokes. Ischemic stroke results from an occlusion of a vascular structure within the brain and unsuccessful re-establishment of reperfusion.[2] Restricted to the territory of the artery, this occlusion leads to a reduction in blood flow to the rest of the brain, resulting in neural cell death, as depicted in Figure 1.

[1]Go AS, Mozaffarian D, Roger VL, Benjamin EJ, Berry JD, Blaha MJ, Dai S, Ford ES, Fox CS, Franco S, et al.: Heart disease and stroke statistics--2014 update: a report from the American Heart Association. Circulation 2014, 129:e28-e292.

[2]Martin RL, Lloyd HG, Cowan AI: The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neurosci 1994, 17:251-257.

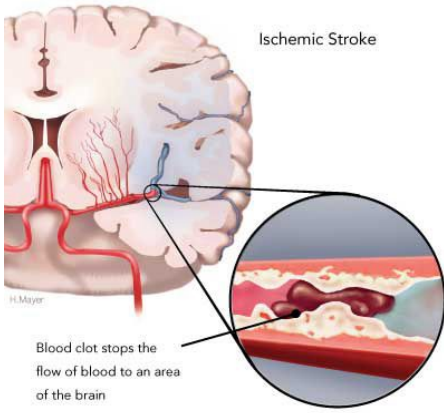

Fig. 1. Illustration of ischemic stroke showing occlusion and obstruction in blood flow[3]

Researchers continue to investigate methods in promoting recovery after stroke, though no therapies currently exist. Recent data suggests that strategies to enhance neurogenesis and angiogenesis following stroke injuries may provide opportunities for improved clinical outcomes and brain functional recovery. New neuronal connections (neurogenesis) and formation of new vessels (angiogenesis) have shown to be critical in tissue regeneration after stroke.[4] This idea on the importance of angiogenesis is represented in Figure 2, where an increase in the percentage of microvessels is associated with increased survival. Krupinski et al. demonstrated that angiogenesis activated in the peri-infarct area is associated with survival and can play a role in neurogenesis and behavioral recovery.

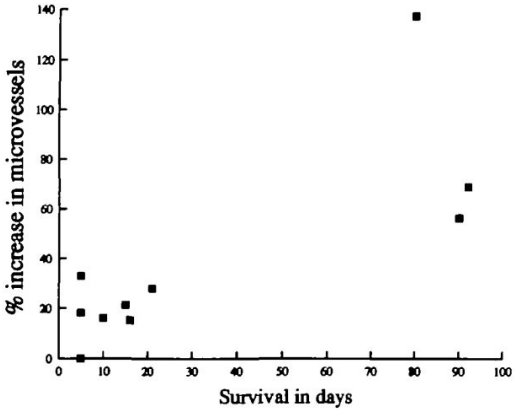

Fig. 2. Graph showing percentage of increase in microvessel density (infarcted compared with contralateral hemisphere) versus survival in patients suffering from stroke[5]

[3]Types of Stroke. (2017, April 5). Retrieved June 13, 2017, from University of Florida Health https://com-neurology-stroke.sites.medinfo.ufl.edu/for-patients/types-of-stroke/.
[4]Ergul A, Alhusban A, Fagan SC. Angiogenesis: A harmonized target for recovery after stroke. *Stroke*. 2012;43:2270-2274
[5]Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM. Role of angiogenesis in patients with cerebral ischemic stroke. Stroke. 1994;25(9):1794-8.

Thus, though the full mechanism behind angiogenesis in stroke is still unknown, pro-angiogenic therapy treatments may prove beneficial in promoting brain recovery. Though the phenomenon of angiogenesis has been highly studied in stroke, there is a lack of literature describing how different animal stroke models affect this process. As angiogenesis plays a critical component in stroke research, how different animal stroke models impact angiogenesis, untreated from therapy, is important. Thus, the rationale of this study is to investigate the most appropriate model for experimental studies. Specifically, this project investigates angiogenesis and vascular surface of the peri-infarct area surrounding the core in two most prevalent animal stroke models: photo-thrombotic (PT) and middle cerebral artery occlusion (MCAo). The experiments will solely focus on stroke mice, with no hydrogel transplantation. So far, in research literature, if the PT method was used for cell therapy experiments, the MCAo was preferably used for targeted pro-angiogenic therapies, with no reported showing of why. Differences in angiogenesis between the two stroke models will determine whether PT could be used for pro-angiogenic hydrogel experiments.

We will evaluate this through 2 specific aims, stated below.

Aim 1: Compare brain vascular status *in vivo* in PT and MCAo models at Day 14 post-stroke. For this, adult mice will be subjected to a stroke (Day 0) and sacrificed by PFA perfusion on Day 14. A total number of 6 mice per group and per stroke model will be used.

Aim 2: Compare brain vascular status *in vivo* in PT and MCAo stroke models at 1 month post-stroke. For this, adult mice will be subjected to a stroke (Day 0) and sacrificed by perfusion on Day 30 after stroke. A total number of 6 mice per group and per stroke model will be used.

2. Materials and methods

2.1 Stroke induction

Two stroke models, MCAo and PT strokes, were performed on adult mice at Day 0. MCAo was done with a drilling device to expose the target artery that was blocked through cauterization, inducing a stroke, depicted in Figure 3 and 4. PT was done using an injection of Rosebengal solution (IP) and a light apparatus at 1.55 lateral left of the bregma (located through a needle apparatus and visualization) to induce a stroke, depicted in Figure 5.

A B

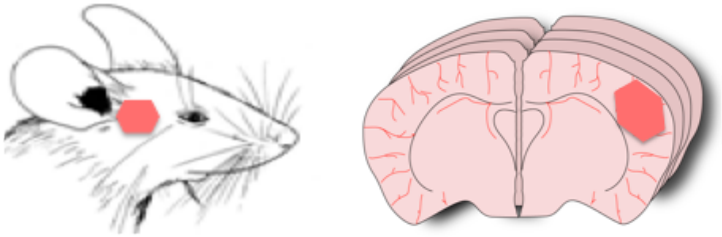

Fig. 3. Schematic illustrations of cortico-temporal damage area in MCAo model[6]

[6]Lina R. Nih

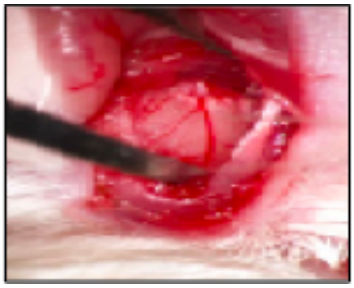

Fig. 4. Image of craniotomy to expose the middle cerebral artery, which is then cauterized

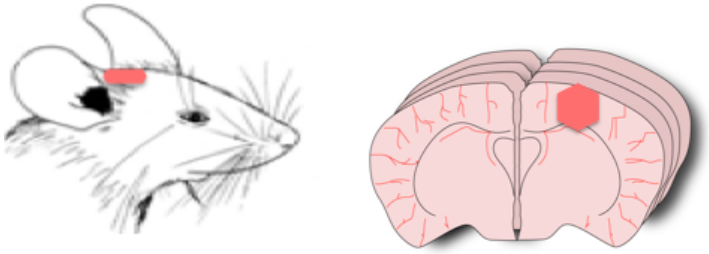

Fig. 5. Schematic illustrations of cortico-dorsal damage area in PT model[7]

2.2 Sacrifice by perfusion and incubation

Intracardiac perfusion to fixate the brain was performed at Day 14 and 1 month post-stroke for the two timepoints. Perfusion was done using PFA (20 mL) after PBS (50 mL). After perfusion, brains were kept in PFA for 4 hours and then transferred into sucrose for 2-3 days before sectioning.

2.3 Cryostat sectioning

Brain tissue sectioning was conducted using a cryostat with width 30 μm. After sectioning, slides were kept at -80 degrees before staining.

2.4 Fluorescent staining

Fluorescent staining was done for both timepoints per stroke model with DAPI to visualize the cell nucleus, and the Iary antibody Glut-1 (Rabbit) and the IIary antibody Alexa-Fluor-555 (Donkey anti Rabbit) to visualize vessels. For the second staining, referred to only in the discussion, the Iary antibodies Iba-1 and GFAP with IIary antibodies Alexa-Fluor-555 (Donkey anti Rabbit) and Alexa-Fluor-488 (Donkey anti Rat) were used. The first slide (with 6 sections) for each mouse was chosen for staining. Next was drying, dehydration to take away water and fat, and cover-slip placement.

[7]Lina R. Nih

2.5 Confocal microscopy imaging and image analysis

Imaging was done of vessels in the peri-infarct area in 20x using a Nikon confocal microscope. 3 images were taken per brain. Images were then analyzed using Image J, a scientific image processing program. 4 region of interests (ROIs) were placed for each image in the peri-infarct area (8 ROIs for contralateral side). Each ROI was a square of 80 x 80 μm^2. Then, measured % area in each ROI and averaged per image, per brain, then per timepoint and stroke model. This gives an average vascular area per group. This information was used in plotting and quantification analysis, done using Prism, a scientific graphing and statistics software. A one-way ANOVA test among groups was performed for statistical significance in Prism.

3. Results

First, the peri-infarct vasculature was analyzed using image analysis of fluorescent images of both timepoints and stroke models. Figure 6 shows the fluorescent images of the glut-1 staining to image vessels. Qualitative differences of peri-infarct vasculature can be seen between stroke models and timepoints.

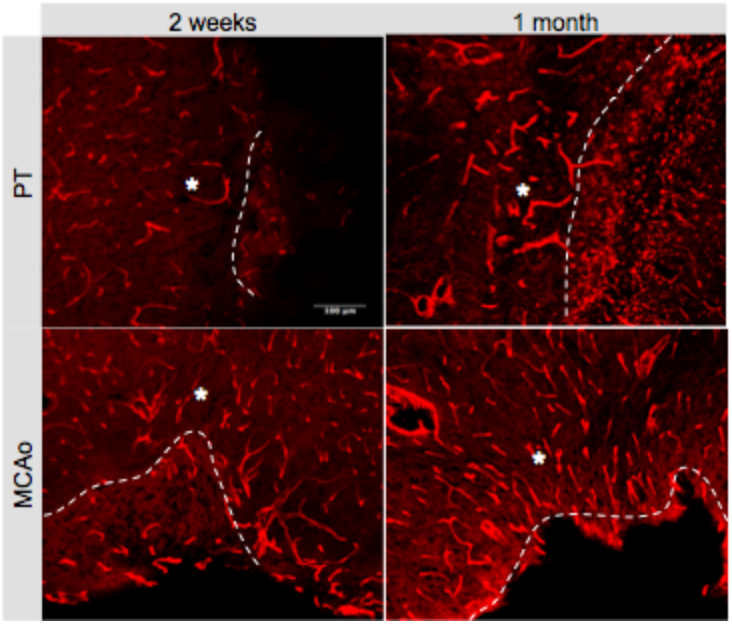

Fig. 6. Fluorescent brain section images of peri-infarct area (*) and boundaries of stroke (---) of vessels (glut-1 with Alexa Fluor 555) at 2 weeks and 1 month after stroke implementation. Scale bar: 100 μm.

Figure 7 gives a quantitative understanding of the differences, showing that peri-infarct vascularization in the MCAo model was significantly greater than the PT model at both timepoints. This finding aligns with the images in Figure 6 where comparing MCAo to PT for both timepoints, the MCAo model shows more vascularization. This finding also helps to understand both stroke models effect on angiogenesis, which can now be compared to human stroke patient angiogenesis studies to find the most similar model. Furthermore, no significant difference was found between timepoints when looking independently at each stroke model. This

may suggest stability and remodeling was attained before the 2 weeks timepoint after stroke. This relates to literature findings of peak angiogenesis occurring 7 days after stroke.

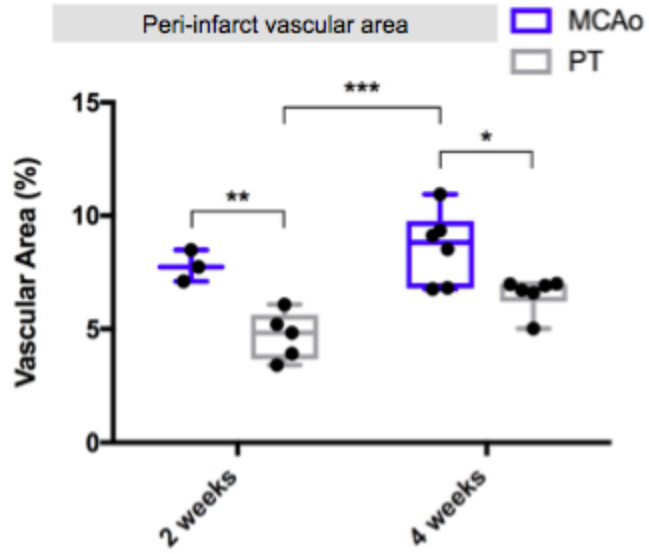

Fig. 7. Post-stroke peri-infarct vascular area quantification. Data represents the average ± SEM (n=3,5,6,6). p values were determined by one-way ANOVA, Tukey's post-hoc test, with *, **, and *** indicating $p< 0.05$, $p < 0.01$, and $p <0.001$ respectively. All significant comparisons labeled.

Secondly, further analysis was done to understand the evolution of the peri-infarct vasculature over time. Figure 8 displays the results and shows the comparison with the contralateral value (averaged among 3 different brains with 3 different images/brain for each timepoint per stroke model group). Results found that the average increase between week 2 and 4 weeks was 0.81 in the MCAo model, while the average increase between week 2 and 4 weeks was 1.85 in the PT model.

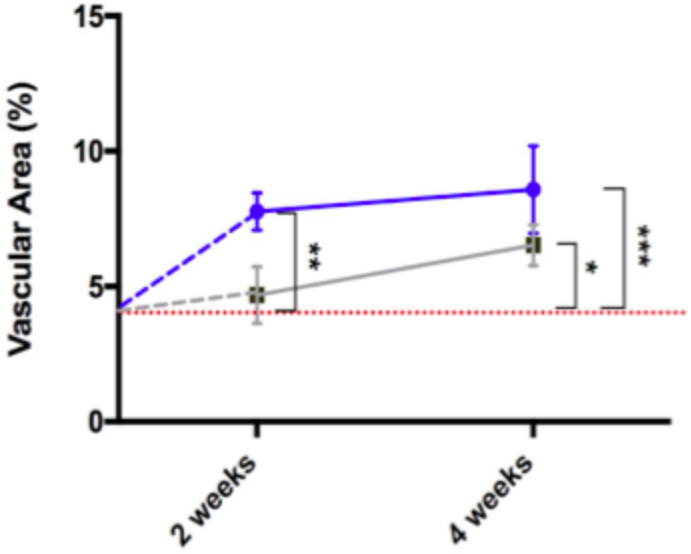

Fig. 8. Post-stroke peri-infarct vascular area over time quantification. Data represents the average ± SEM (n=3,5,6,6). p values were determined by one-way ANOVA, Tukey's post-hoc test, with *, **, and *** indicating $p< 0.05$, $p < 0.01$, and $p <0.001$ respectively. All significant comparisons labeled.

As shown, the peri-infarct vascularization in the PT model has a greater increase (faster process of vascularization) between 2 and 4 weeks compared to the MCAo model, suggesting that the remodeling in the PT model is still in progress.

4. Discussion

The difference between the contralateral side and the peri-infarct vascular area suggests that the vascular remodelling after stroke happens earlier in the MCAo model. The findings suggest that the endogenous capacity of the brain to create vessels after stroke is naturally greater in MCAo than in PT. Looking specifically at the peri-infarct vasculature process, both models show stabilization from 2-4 weeks, indicating the most increase in angiogenesis occurs before the 2 weeks timepoint. However, with further analysis, it is clear that both models are increasing, with PT model increasing at a faster rate. The difference between week 2 and 4 suggests that despite the non-significance, the vascular area could continue increasing, at a slower rate than before week 2. Additionally, this finding suggests that the values obtained in PT could reach the MCAo values at a later time point. Using the PT model to study pro-angiogenic therapies could require to sacrifice mice at a later time point, as the angiogenic process is slower in this model. The difference obtained between the 2 models could be explained by the location and the occlusion mode (local for MCAo vs multi-vascular in PT).

To investigate deeper the reason the PT model might have a slower vascularization process, a second staining was done. Sections were stained with GFAP and Iba-1 to stain for the astrocytic scar and microglial cells respectively. Figures 9 (astrocytes) and 10 (microglial cells) give a qualitative possibility to the reason for the difference in vascularization between the two models. As a representative of inflammation, these stainings show stark qualitative differences in the peri-infarct and stroke area that may lead to vascularization effects.

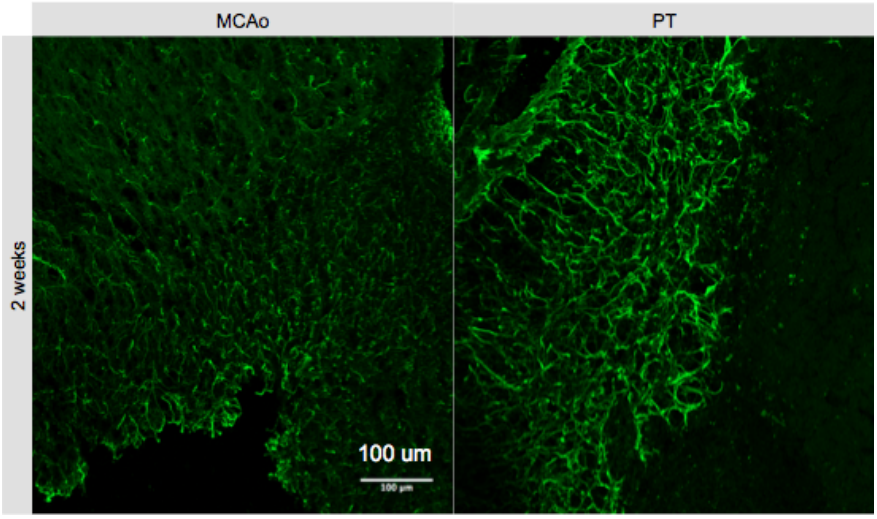

Fig. 9. Fluorescent brain section images of peri-infarct area (GFAP with Alexa Fluor 488) at 2 weeks and 1 month after stroke implementation. Scale bar: 100 μm.

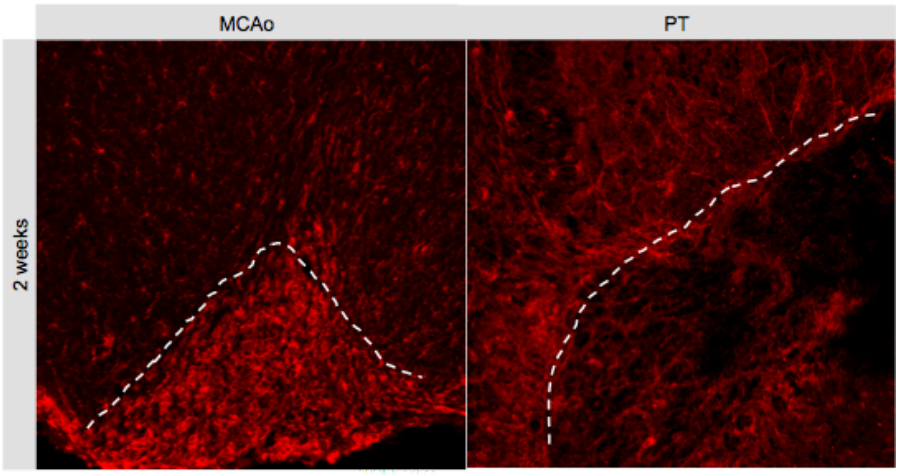

Fig. 10. Fluorescent brain section images of peri-infarct area with --- indicating boundaries of stroke (Iba-1 with Alexa Fluor 555) at 2 weeks and 1 month after stroke implementation. Scale bar: 100 µm.

Figure 9 shows that the PT model has more astrocytes, suggesting more inflammation. Figure 10 shows that the PT model has less concentration of microglial cells within the stroke area compared to the MCAo model, which has a clear dense concentration of microglial cells within the stroke. The PT model, on the other hand, shows a dispersity of microglial cells within the stroke, but also extensively in the peri-infarct area, suggesting more inflammation in the peri-infarct area compared to the MCAo model. This result combined with the previous results align with Nih's studies that have shown anti-inflammatory effects can be associated with more vessels, as the PT model's increased inflammation in the peri-infarct area can be tied to its inhibited vascularization in the peri-infarct area at the 2 weeks and 4 weeks timepoint compared to the MCAo model.[8]

5. Future directions

Results from this study showed that the endogenous capacity of the brain to create vessels after stroke is naturally greater in MCAo than in PT. However, a further analysis needs to be conducted to determine how closely the MCAo model resembles angiogenesis in human post-stroke recovery. Additionally, in analyzing why PT has a slower angiogenic process, quantification of the stainings for astrocytes and microglial cells must be done to give a better representation if inflammation plays a role in the vascularization process.

Additionally, added time points are needed to further refine this study and allow a deeper understanding of the evolution of peri-infarct vasculature over time in both animal stroke models. First, a Day 1 timepoint (where Day 0 is stroke induction) should be added instead of the contralateral side to give a better description of the starting point of post-stroke recovery. Second, a Day 7 timepoint should be added to assess the angiogenesis peak, as described in literature, since this study found non-significance between the 2 weeks and 4 weeks timepoint, suggesting most of the remodeling already took place before the 2 weeks timepoint. Third, a later

[8] Lina R. Nih

timepoint should be added to determine whether the vascular area keeps increasing and whether the PT values eventually attain the MCAo values as suggested by the findings.These future directions, by providing a deeper analysis of angiogenesis in these models, will contribute to identifying an appropriate animal stroke model in experimental studies.

Acknowledgements

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