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# Investigating a time course of ischemic stroke-induced heart injury in the left coronary artery

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## Abstract

Besides cell death and loss of function in the brain, ischemic stroke is often associated with poor cardiac outcomes, increasing the risk for atrial fibrillation and myocardial infarction. This condition, known as stroke-induced heart injury (SIHI), has been well characterized for the four chambers of the heart and myocardial tissue, but the timeline and extent of injury in the coronary arteries remain unclear. To study the timeline of ischemic SIHI in the left coronary artery (LCA), we documented how the thickness, cross-sectional area, and immune cell recruitment in the LCA changed following an insular ischemic stroke in a rat model. Rats were divided into a stroke (endothelin-1 injection) and control (phosphate-buffered saline injection) group and sacrificed at various time-points post-treatment (6h, 24h, 7d, 14d, & 28d). Following a standard H&E staining protocol, we observed a general thickening and constriction in the vessel until 7 days post-stroke, corresponding to eutrophic remodelling. After immunohistochemistry staining for pan-leukocytes (CD45), neutrophils (myeloperoxidase), and B lymphocytes (CD45R), we observed a maximal recruitment of innate and adaptive immune cells occur concurrently at 24 hours post-stroke, indicating possible immunological memory in inflammation from stroke. These results extend our understanding of stroke-induced heart injury to vascular changes in the heart and lay the groundwork in addressing the relevant clinical problem of cardiac complications after stroke.

# 1. Introduction

## 1.1. Background

*Stroke and the heart-brain relationship*

Stroke affects individuals worldwide and is the most common cause of morbidity and a significant source of mortality in industrialized countries[1]. Strokes have been traditionally categorized into either ischemic strokes, which are more common and involve occlusion of a cranial blood vessel, or hemorrhagic strokes, which are more severe than ischemic strokes and involve bleeding and increased intracranial pressure causing reduced blood flow[2]. Both of these categories of strokes ultimately result in a lack of blood flow to the brain causing cell death.

Following an ischemic stroke, there is a complex pathophysiological response that takes place in the body. This begins in the brain with the obstruction of nutrient transport, like glucose, which limits the energy available for the cell to maintain ion gradients and initiates an ischemic cascade consisting of several cellular pathways[3]. Impaired maintenance of ion gradients across the cell membrane causes an accumulation of intracellular $Na^+$ and $Ca^{2+}$ ions, drawing water into the cell by osmosis, and causing cytotoxic edema[4-5]. Minutes after occlusion, irreversible cell death begins to occur causing tissue necrosis, and subsequent loss of brain function[6].

Stroke, among other neurological disorders, has previously been reported to be associated with structural and functional impairment to the heart, such as neurocardiac arrhythmias and lesions[7-10]. Damage to the heart from stroke-induced heart injury (SIHI) was first identified when hemorrhagic stroke patients were found to have electrocardiogram abnormalities[11]. Subsequent work has then found stroke-induced arrhythmias and myocardial lesions in mouse models of hemorrhagic stroke[12]. In addition to early research focused on hemorrhagic SIHI, ischemic stroke patients were observed to have similar cardiac consequences, such as atrial fibrillation manifesting

in approximately 25% of cases [13-15]. Previous work in our lab has also identified a similar immune cell recruitment profile following an insular ischemic stroke in rats and the results of Sheridan *et al*. (1996) for canines following a myocardial infarction. Clinically, patients who have had an ischemic stroke are at an increased risk of a heart attack in the following months[16]. Both hemorrhagic and ischemic stroke have a similar prevalence of cardiovascular changes following stroke, however, the focus of our study is on ischemic stroke because of its greater incidence in the population[17].

*Mechanism of Stroke Induced Heart Injury (SIHI)*

Currently, the two major proposed mechanisms of SIHI involve the release of pro-inflammatory chemokines into blood circulation[18], and/or autonomic dysregulation triggering a catecholamine surge[19] (Figure 1).

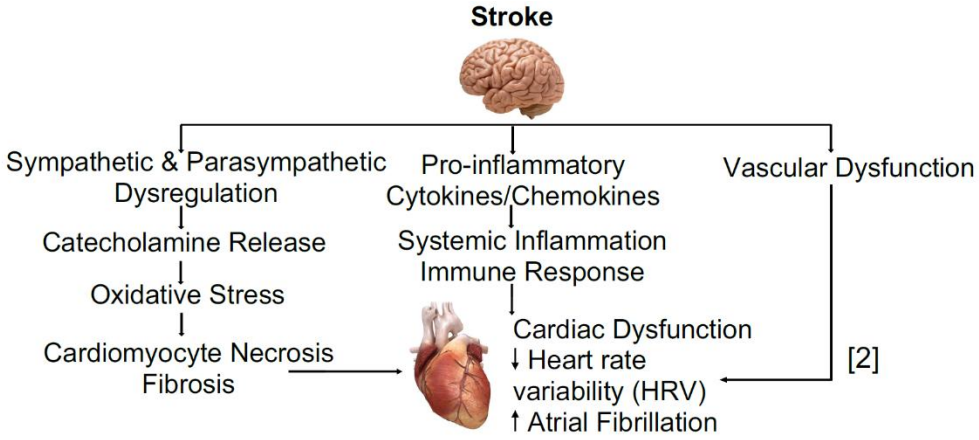


**Figure 1. Potential mechanisms for stroke-induced heart injury.**

The release of chemokines as part of the inflammatory response initially recruits neutrophils to infiltrate into the damaged brain tissue and macrophages to phagocytose debris and pathogens. Following the activation of neutrophils and macrophages of the innate immune system, this further triggers an adaptive immune response from T and B lymphocytes[20]. However, inflammation is not exclusively localized to the brain, as macrophages, lymphocytes, and neutrophils are found in

greater abundance in the heart following a hemorrhagic stroke in patients[21]. When inflammatory cells are recruited to the heart, they can release cytokines that induce cardiac fibroblasts to become active and proliferate, which may increase levels of extracellular collagen, leading to fibrosis[22].

The other mechanism can be explained by the catecholamine surge hypothesis. Following stroke, the autonomic nervous system, including regions of the brain like the insular cortex, may become dysregulated leading to a surge of catecholamines released from myocardial nerve endings from overactivation of the sympathetic nervous system (SNS)[23]. When catecholamines bind to the heart's β receptors[24], they increase mitochondrial $Ca^{2+}$ concentration, causing oxidative stress and myocardial cell necrosis[25].

*The rat insular cortex*

My project focuses on heart injury induced by a stroke occurring in the right insular cortex (IC). The rat insular cortex lies on the dorsal bank of the rhinal fissure and receives different types of inputs from the sensory thalamic nuclei (Figure 2). The insular cortex is known to have dense reciprocal innervations with the limbic structures, such as the amygdala and hypothalamus, and play a significant role in the control of the autonomic nervous system[26]. In humans, it is involved in a variety of functions, ranging from pain perception and emotion processing to speech production. Previous studies have reported mixed results on lateralization differences, but seem to suggest that the right IC may control SNS activity in particular[27-29]. Even though previous work in our lab has established that ischemic stroke in the left IC is linked to heart injury as well, this study so far has focused only on the right IC.

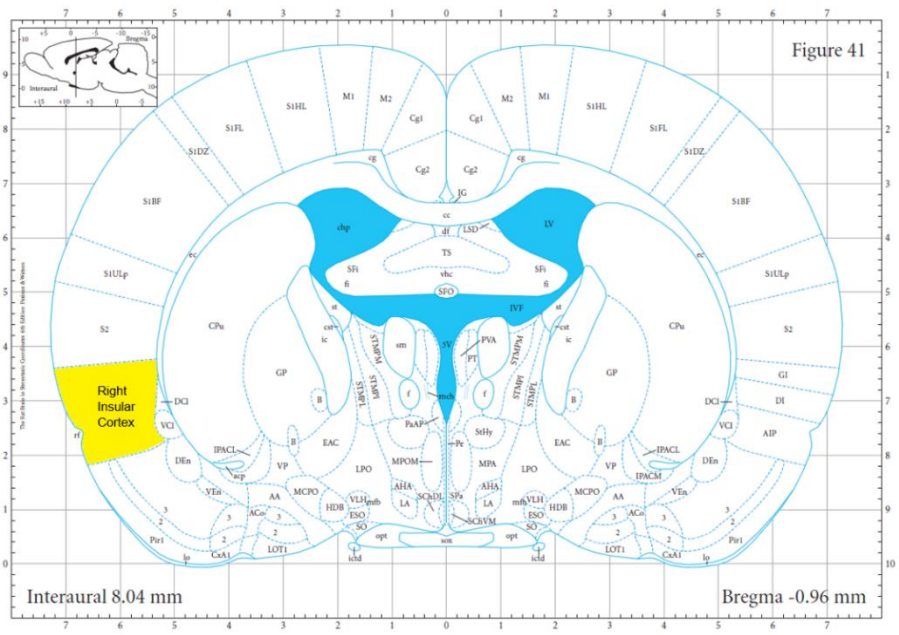


**Figure 2. Anatomical position of right insular cortex in the rat brain.**

*The left coronary artery and heart disease*

The left coronary artery branches from the aorta, within the sinus of Valsava, and passes through the pulmonary trunk and the left atrial appendange, until it divides into the left anterior descending artery and the left circumflex artery (Figure 3). The function of coronary arteries is to supply blood to the heart tissue, which delivers oxygen and nutrients for its proper functioning. The left coronary artery and its branches supply most of the blood to the ventricular myocardium and left atrium of the heart. An occlusion of the left coronary artery, known as the Widow Maker heart attack, reduces or prevents the flow of blood from tissue typically perfused by this artery and has one of the greatest risks of long-term injury and death among myocardial infarctions[30]. Furthermore, the left coronary artery in the atrioventricular groove is densely innervated by autonomic nervous system[31] and presents a good target area to examine the contributions of the ANS to SIHI. Hence, we chose this area to examine any injury to the heart vasculature following a selective insular ischemic stroke.

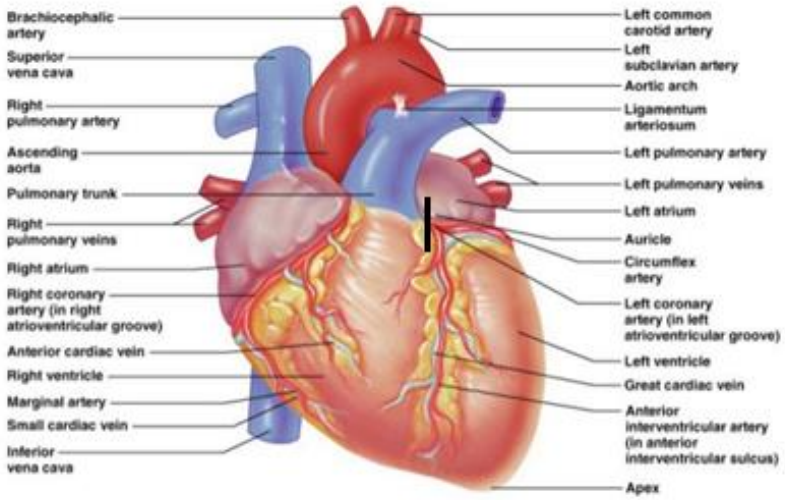


**Figure 3. Anatomical region of left coronary artery in the left atrioventricular groove.** Histological sections obtained from the region indicated by a black vertical bar.

*Characterization of SIHI in the heart's chambers and pulmonary vein-left atrium (PVLA) border*

To provide a broader understanding of the heart-brain relationship, our research group has been actively investigating the implications of stroke-induced heart injury in the chambers and myocardial tissue of the heart. Thorburn (2018) in our lab has previously established a novel rodent model of focal insular ischemic stroke to evaluate behaviour and pathological outcomes of SIHI. She found that at 28 days post-stroke, rats with left IC damage demonstrated impaired sensorimotor gating, compared to rates with right IC damage and control groups. All stroke injury groups were found to have an increase in microglia activation in the IC and white and grey matter regions, in addition to cardiac fibrosis expressed in the left atrium tissue. Jaramek (2018) in our research group further extended these findings by discovering neutrophil infiltration and endothelial dysfunction at 6 hours following selective IC stroke. This is followed by T cell infiltration at 24 hours and fibrosis at 14 and 28 days at the pulmonary vein-left atrial (PV-LA) border.

Hence, although previous studies have indicated the connection between stroke in the right insular cortex and heart injury, the degree and timeline of injury, specifically with regards to inflammation, fibrosis and stenosis of coronary artery vasculature changes, have been uncharacterized so far. Evaluating several histological characteristics can help us further understand the temporal relationship between stroke and heart injury in the left coronary artery.

Firstly, immune cell recruitment can be used to observe contribution of the inflammatory pathway to SIHI, occurring specifically in the left coronary artery. Secondly, artery cross-sectional area can be measured as an indication of how overactivation of the SNS may cause dilation in this blood vessel. Additional responses and remodelling of the artery may occur due to fibrosis, myocardial necrosis or edema.

### 1.2. Hypothesis

Following stroke, the heart vasculature is expected to experience an increased amount of injury, as indicated by a dilation and thickening of the coronary arteries, as well as increased inflammatory cell recruitment in surrounding tissue.

### 1.3. Objective

The objective for my project is to document the timeline of stroke-induced heart injury (SIHI) in the left coronary artery in the endothelin-1 insular ischemic stroke induced rat model using a histology-based approach.

## 2. Methods

Sixty Wistar rats aged 6 months and weighing 500-600g were randomly assigned to either the endothelin-1 (ET-1) right insular cortical (IC) stroke group or the phosphate-buffered saline (PBS) control group and at each time-point for observation (6h, 24h, 7d, 14d, and 28d). Each treatment group at one time-point was n=6 initially.

A standard procedure for the unilateral injection of ET-1 or PBS, heart removal, fixation, sectioning and immunohistochemistry (IHC) staining was conducted by Victoria Jaremek, as per the details outlined in her undergraduate thesis (Figure 4). The specific IHC antigens we stained for are CD45R to detect B lymphocytes, CD45 to detect pan-leukocytes, and myelin peroxidase (MPO) to detect neutrophils. A standard H&E staining procedure was performed to distinguish acidophilic and basophilic cellular structures to quantify blood vessel thickness and cross-sectional area.

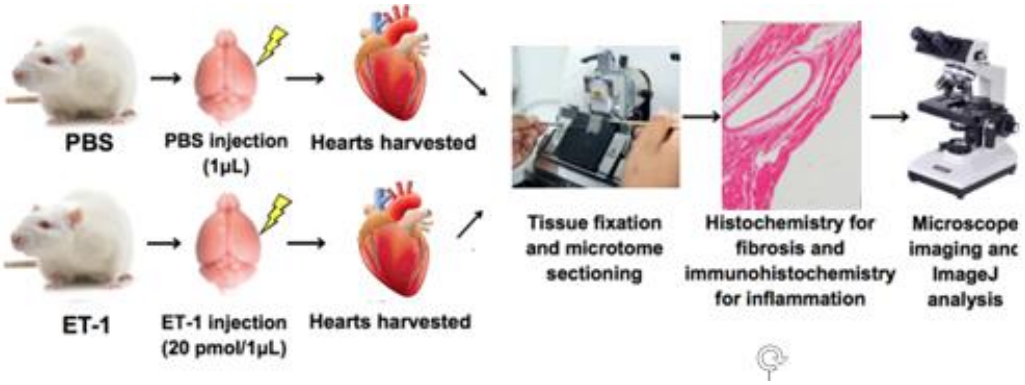


**Figure 4. Schematic outline of the injection, heart removal, fixation and sectioning, and IHC staining protocol for the study.**

Cell counter on ImageJ was used to identify inflammatory cells by their intense brown staining. Several IHC stained samples were excluded from statistical analysis as their staining was uneven (such that inflammatory cells could not be identified).

Area of the tissue was measured with Freehand selection on ImageJ. For artery thickness analysis, the Segmented line tool was used to measure six different thicknesses along the circumference of the artery. A mean was obtained from values recorded in Excel to provide a representative estimate of the overall vessel thickness.

All statistical analyses were performed using GraphPad Prism 6.0. Means between PBS and ET-1 groups, and between each of the time-points, were compared using two-way analysis of variance (ANOVA) with Bonferroni correction. Means between PBS and ET-1 groups for each time-point were compared using multiple t-tests with a Holm-Sidak correction. A p-value of less than 0.05 was considered significant for all statistical tests.

## 3. Results

*Responsiveness and remodelling of the left coronary artery*

To examine the contribution of stroke-induced heart injury to the immediate response and long-term remodelling that may be occurring at the left coronary artery, I first studied how the vessel thickness changes in the time following an endothelin-1 induced stroke. When a two-way analysis of variance (ANOVA) was performed, no significant differences were found in arterial thickness when comparing across the time or injection group variable, or an interaction between these two factors. Multiple t-tests to compare differences between stroke and control groups at each time-point also found no significant differences.

Preliminary observations of trends in the data, without statistical evidence to support them, can be noted. There is a considerable increase in the mean artery thickness between the 24H and 7D time-points (Figure 5A). Additionally, a decrease in mean artery thickness was observed between the 14D and 28D time-points. However, there is also large variation in the control thicknesses at various time-points.

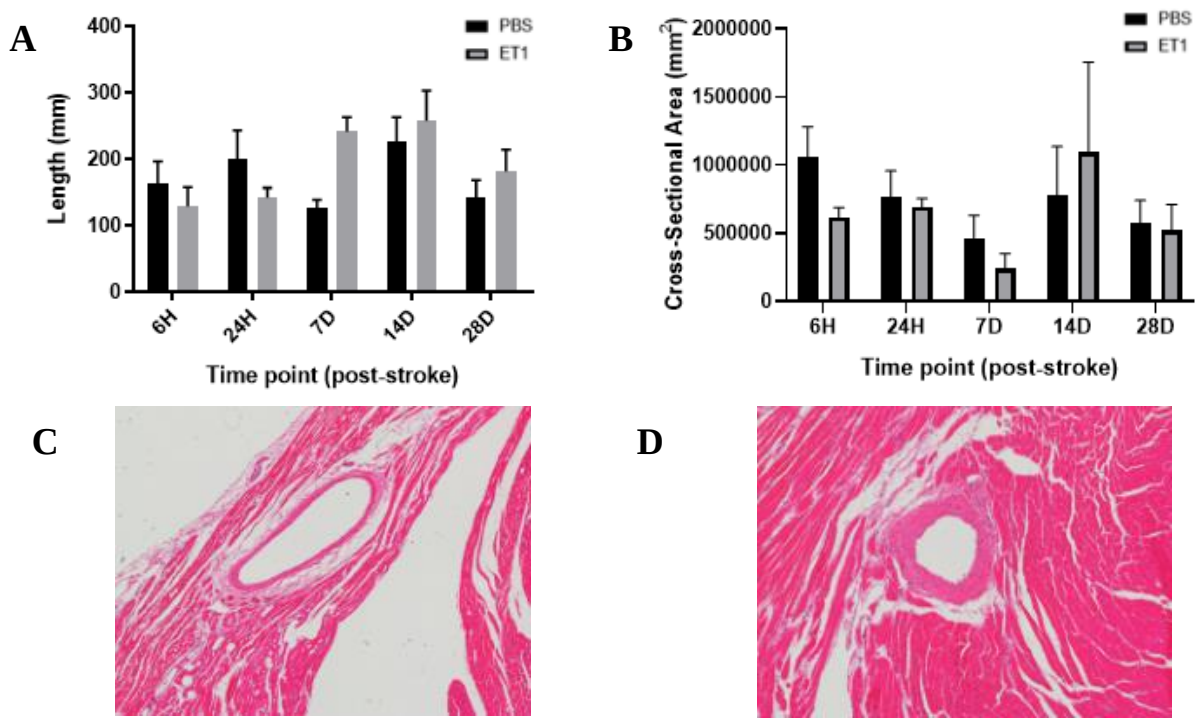


**Figure 5.** Comparison of left coronary artery thickness (A) and cross-sectional area (B) beside the atrioventricular junction between PBS (control) and ET-1 (stroke) groups over varying time-points (6h, 24h, 7d, 14d, & 28d) following injection. Means +/- SEM were plotted for each stroke injection group and time-point. Sample size in each time/injection condition was five (n=5). Representative images taken from one rat at 7D in control (C) and endothelin-1 (D) group.

Next, to further examine how a dysregulated autonomic response and long-term artery remodelling manifest in SIHI, I studied how cross-sectional area of the left coronary artery changes in the time following stroke. No significant differences were found in cross-sectional area between both factors studied when a two-way ANOVA was performed, and between stroke and control rats when multiple t-tests were subsequently conducted. Cross-sectional area of the vessel appears to decrease from 6H to 7D, with the mean area lower for ET-1 than PBS groups (Figure 5B). The trend beyond 7D is unclear, with large variation and an increase in cross-sectional area at 14D.

Hence, taken together, the overall response and remodelling that occurs in the left coronary artery following stroke involves a thickening and narrowing of the blood vessel (Figure 5C, 5D).

*Inflammation at myocardial tissue adjacent to the left coronary artery*

Following an analysis of the responsiveness and remodelling of the left coronary artery, we next sought to quantify inflammatory cell recruitment following a right selective IC stroke. Similar to thickness and cross-sectional area, no significant differences were found in all immune cell densities when a two-way ANOVA was performed, and between stroke and control rats when multiple t-tests were subsequently conducted.

To observe the response from all leukocytes of the immune system, I first compared IHC images stained for CD45, an antigen found in pan-leukocytes. There is greater pan-leukocyte cell density at all time-points in the stroke animals, compared to the control group (Figure 6A). From 6H to 7D, there is a consistent activation of pan-leukocytes, with a decrease beginning at 14D.

Following quantification of pan-leukocytes, the next goal was to focus on documenting the innate and adaptive immune response following insular stroke separately. To study the innate immune response in the left coronary artery following a right IC stroke, neutrophil density was analysed in the tissue adjacent to the vessel (Figure 6C). There is an increase in neutrophil recruitment following stroke, with an apparent maximal recruitment occurring at 24H. However, there is also a large variation between different time-points that obscures the trend seen for stroke animals.

Finally, to study the effects of a right IC stroke on the left coronary artery through the adaptive immune response, B-lymphocyte density was analysed in the tissue adjacent to the vessel. Similar to the neutrophils of the innate immune response, the B-lymphocytes had an increase in recruitment following stroke, with the same apparent maximal recruitment occurring at 24H (Figure 6B).

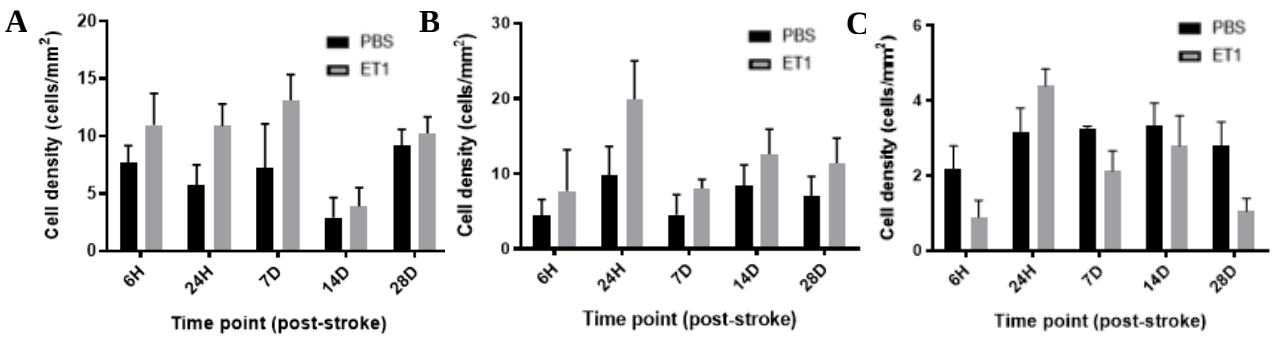


**Figure 6.** Comparison of pan-leukocyte (A), B lymphocyte (B), and neutrophil (C) cell density between PBS (control) and ET-1 (stroke) groups over varying time-points (6h, 24h, 7d, 14d, & 28d) following injection. Sample size in each time/injection condition varied from three to five (n=3-5), due to exclusion of certain samples from improper staining. Means +/- SEM were plotted for each stroke injection group and time-point.

## 4. Discussion

In my Scholar's Electives project, I used a histology-based approach to study the physical remodelling and immune response occurring in the left coronary artery following a right insular cortical ischemic stroke in a rat model. The timeline of changes was documented and compared with previous work in our lab that has extensively studied damage to the chambers of the heart and myocardial tissue following a stroke. Ultimately, the goal of this study was to document changes to the left coronary artery and extend our understanding of stroke-induced heart injury to vascular changes of the heart following an insular ischemic stroke.

For the majority of comparisons, there was no statistical significance found in the two-way ANOVA. This is likely because of the small sample size and large variation that exists when using different biological replicates. Different biological replicates must be used for my study as rats must be sacrificed for heart histology measurements to be taken. The sample size is constrained by the number of rats that we began with in the study, and the exclusion of several IHC stained samples because of improper staining. Increasing the sample size should increase

the power of the statistical tests used, which would allow smaller effect sizes to be detected when conducting a hypothesis test, such as a t-test or ANOVA. Hence, this should reduce the probability of a type II error should indeed there be a real difference that the statistical test has not detected in this study.

Instead of studying how stroke causes pathophysiological changes in the left coronary artery over time, I shifted the focus of my project to studying differences between stroke and control groups at each of the time points following stroke as statistical power was insufficient to draw conclusions for the time course. After performing multiple t-tests to compare each of the PBS and ET-1 groups at different time-points, no statistical significance was found for this test also. Although there is no statistical evidence to support the conclusions drawn, some preliminary observations of trends in the data can be discussed in the context of the overall project goals.

First, I set out to quantify the responsiveness and remodelling that occurs in the left coronary artery following a right selective IC stroke. For thickness of the left coronary artery, there appears to be a considerable increase from 24H to 7D. This is consistent with the hypothesis for my study, which anticipates fibrosis and cellular edema occurring in the coronary artery wall following stroke[22]. There is a large variation in the control thicknesses at various time-points, which suggests that this trend may be due to chance or perhaps a factor in the injection procedure common to both experimental groups.

For cross-sectional area of the left coronary artery, there appears to be a decrease from 6H to 7D, with the mean area lower for ET-1 than PBS groups, which is inconsistent with the coronary artery dilation that I expected in my hypothesis, due to overactivation of the sympathetic nervous system[23]. Activation of the SNS usually results in dilation of the coronary arteries to supply the

heart with more oxygen and nutrients during physiological stress. However, this discrepancy may be attributed to vascular remodelling.

Sonoyama *et al*. (2007) describes how myogenic tone, a functional property of the blood vessel wall to regulate its fluid pressure, can contribute to eutrophic and hypertrophic remodelling. Eutrophic remodelling is the usual physiological response to hypertension and involves a reduction in lumen diameter and an increase in wall thickness. This description of eutrophic remodelling is what was seen in our study from the first few hours following stroke to the end of the first week. However, extended periods of hypertension that damage myogenic autoregulation can cause hypertrophic remodelling that causes an increase in lumen diameter and decrease in thickness, which is what was observed beyond the 7D time-point following stroke. Hence, in the context of current knowledge of vessel myogenic autoregulation, these results are consistent and may be a greater factor to the observed response and remodelling than autonomic dysregulation.

The inflammatory cell recruitment profile is generally consistent with what I hypothesized for my study. Activation of the innate and adaptive immune response are central to the proposed mechanism for how stroke can cause injury to the heart, and this is reflected by a greater density and recruitment of pan-leukocytes in the tissue adjacent to the left coronary artery for stroke animals, compared to the control group. Furthermore, the innate and adaptive immune response were noted to occur in close temporal proximity to each other as maximal immune cell recruitment was seen at 24H for both neutrophils and B-lymphocytes.

During a normal infection with an external pathogen, the adaptive immune response is usually delayed after the innate response[33]. However, upon subsequent infections with the same pathogen, the innate and adaptive immune responses occur nearly at the same time. This is due to immunological memory from a large precursor pool of memory T and B cells that can quickly

differentiate upon contact with the same antigen. In the case of a stroke, there is a large release of damage-associated molecular pattern molecules (DAMPs)[34] causing an overactivation of the innate response by binding innate cell receptors found on macrophages and dendritic cells, which subsequently activate adaptive T and B lymphocytes. Hence, what may explain the concurrent response between the innate and adaptive immune system is that there may be the prior existence of memory T and B cells that recognize DAMPs, which reduce the delay between the activation of the innate and adaptive immune responses.

Taken together, following a selective insular cortical stroke, it appears that artery eutrophic remodelling occurs over a longer duration of time until 7D, and switches to hypertrophic remodelling after there is damage to the myogenic autoregulation of the blood vessel (Figure 7). On the other hand, the innate and adaptive immune response appear to occur closely together, with a maximal recruitment of immune cells at 24H. The time of maximum inflammation seems to precede the remodelling effects taking place in the left coronary artery following stroke.

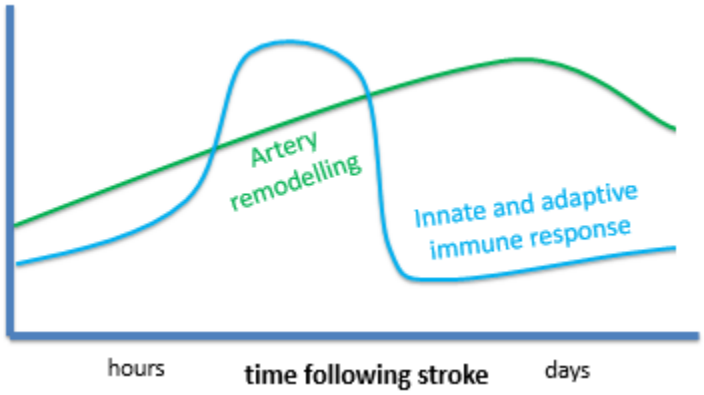


**Figure 7.** Summary of proposed remodelling and immune cell activation changes in the left coronary artery following right IC stroke. Artery remodelling (green) occurs for a longer duration of time until 7 days post-stroke, whereas the innate and adaptive immune response (blue) occurs concurrently and in a shorter window of time at approximately 24 hours post-stroke.

There are a variety of future extensions to this project and research area that logically stem from my findings. Firstly, this study was considerably under-powered due to a lack of a sufficient sample size and biological variability due to the use of a rat model and their differential responses to cranial vessel occlusion. Increasing the sample size of each of the treatment groups would likely result in being able to support conclusions with statistical evidence. If using a greater number of rats is not possible due to financial or time constraints, the goal of the study can be revised to instead study how stroke causes pathophysiological changes in the heart at specific times following stroke, as opposed to observing how time influences these changes.

Furthermore, it may be of interest to study cardiac outcomes in models of left insular cortical occlusion, which can be compared with those of right IC. There is currently uncertainty in the research community about the lateralization effects of autonomic control for the insular cortex, so noting any differences in cardiac pathology, or lack thereof, may yield further insight into lateralization effects of the IC and the autonomic dysregulation mechanism of SIHI.

Finally, with regards to the inflammatory response, further immunological and biochemical research studying possible interaction of DAMPs with memory cells of the immune system may strengthen the theory of concurrent activation of the innate and adaptive system we found, and aid in developing possible therapeutic targets to reduce cardiac pathologies following an ischemic stroke.

In conclusion, my project determined a preliminary timeline of the pathological changes occurring in the left coronary artery following an insular ischemic stroke. I found that both remodelling and immune response effects were present, which is consistent with the mechanisms for stroke-induced heart injury. Eutrophic remodelling, due to a combination of autonomic dysregulation and myogenic autoregulation from hypertensive conditions, likely contributed to a

sustained change until 7D. Following 7D, hypertrophic remodelling became dominant after damage to the myogenic response. Immune recruitment was seen for all leukocytes, but in particular, immune cells of the innate and adaptive immune response experienced their maximal recruitment almost concurrently. This finding suggests that immunological memory may play a role in the inflammatory mechanism for SIHI. Ultimately, these documented changes to the left coronary artery following an insular cortical stroke extend our understanding of stroke-induced heart injury to vascular changes in the heart, seeking to address the major clinical problem of cardiac complications following stroke.

**Word count:** 4885 words

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