Safety Assessments of Therapeutic Hypothermia for Intracerebral Hemorrhage

by

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ABSTRACT

Therapeutic hypothermia (TH) is a common intervention used in a variety of injury profiles. However, when applied to intracerebral hemorrhage (ICH), TH can worsen outcome. Previous research from our lab has shown that TH can aggravate intracranial bleeding both during cooling induction and rewarming phases.[1] Here, I evaluate aspects of the safety of using TH in ICH.

First, I tested if intra-arterial chilled infusate (ICI), one method of local cooling, is safe to use when active bleeding is present. The experimental group, anesthetized male adult rats, received an infusion of heparinized room temperature saline over 20 minutes into the internal carotid artery at the external carotid bifurcation. Brain temperature change from this infusion was determined using implanted temperature probes in naïve animals. There was an average drop of ~0.1 °C, with a maximum drop of 0.5 °C in the ipsilateral hemisphere, when correcting for contralateral temperature change. Then, the infusion was given following the initiation of collagenase-induced ICH, during a time of active bleeding. Hematoma volume was assessed 24 hours after collagenase injection using a hemoglobin-detecting spectrophotometric assay. There was no difference between ICI and control groups for hematoma volume ($p = 0.64$). Brain water content (edema) was measured in the ipsilateral and contralateral cortices and striata, as well as the cerebellum, using wet/dry weights. Elemental concentrations were measured using inductively coupled plasma mass spectrometry, and activity and core temperature were monitored using telemetry probes. There was no difference between the ICI and control groups in edema ($p = 0.81$) or elemental concentrations ($p > 0.1$) in any region of interest. There was also no difference between the experimental and control group for temperature ($p = 0.56$), or

activity ($p = 0.61$). These results suggest ICI is safe when implemented in collagenase-induced ICH.

Clotting factors are inhibited by TH, and this effect may underlie TH-induced bleeding. Also, coagulopathy in late stages of ICH injury could aggravate ongoing bleeding. Therefore, I hypothesized next that late-onset coagulopathy would increase hematoma size. Warfarin (0.4 mg/kg loading dose, 0.25 mg/kg/day maintenance for the following 5 days) was orally administered to naïve rats. Tail bleed time was used to assess anticoagulation 6 hours after the last warfarin dose (day 6). Tail bleed time showed warfarin successfully produced a state of anticoagulation between drug treated and placebo group animals ($p = 0.02$). A spectrophotometric assay determined that there was no difference ($p = 0.28$) in the concentration of hemoglobin within the brains of each of the warfarin treated and placebo naïve animal groups. This meant that the warfarin doses successfully induced anticoagulation, without causing spontaneous bleeding. Following this validation, the warfarin dosing regimen was administered to rats, with the loading dose starting 24-hours after collagenase-induced ICH. Tail bleed time between warfarin and placebo treated groups, measured 6 hours following the last dose (day 6), showed warfarin successfully induced longer bleed times ($p = 0.008$). Additionally, the diameters of blood blots collected during the tail bleed test were measured every 5 minutes. These showed that the warfarin treated group also bled at a faster rate than the placebo group ($p = 0.03$). Hematoma volume, as determined using a hemoglobin spectrophotometric assay, was not different between the warfarin and placebo treated groups ($p = 0.39$). These findings suggest that anticoagulation, an isolated factor of TH, does not cause late or re-bleeding in collagenase-induced ICH.

Prolonged cooling, even if it does not worsen bleeding through coagulopathy, may still negatively affect outcome through other means. Depending on the depth and duration, TH has the potential to broadly affect brain plasticity, especially given the spatial, temporal, and mechanistic overlap with the injury processes that cooling is used to treat. I review experimental and clinical evidence to evaluate whether application of prolonged TH has any adverse or positive effects on post-stroke plasticity. The available data suggest that mild TH does not appear to have any deleterious effect on neuroplasticity; however, there is a need for additional high-quality preclinical and clinical work in this area.

PREFACE

This thesis is an original work and received research ethics approval from the University of Alberta Animal Care and Use Committee for Biosciences, Project AUP960.

Chapter 1 and 3 are my original writing and experimental work, completed with guidance from lead researcher of our lab group, Dr. Fred Colbourne. Some of the research conducted for this thesis forms part of a research collaboration, also led by Dr. Fred Colbourne. F. Colbourne was involved in concept formation and study planning for all chapters.

Chapter 2 is my original writing, although L.J Liddle was lead author for a publication that incorporates data from Chapter 2.[2] L.J. Liddle helped in planning the study for Chapter 2 with F. Colbourne, as well as completing all surgical procedures, making guide cannula, and aiding in data collection. L.J. Liddle continued the project in Chapter 2 by completing a long-term behavioural study with another co-author of the publication, C. Dirks. Doctors Andrew Demchuk and Mohammed Almekhlafi were also collaborators for Chapter 2.

I was responsible for the data collection, analysis, and write-up for Chapter 3. The pilot work using phenylephrine referenced in Chapter 3 was led by K. Dietrich.

Chapter 4 is compiled from excerpts of a published literature review that A. Kalisvaart was lead author for.[3] F. Colbourne was the supervisory author and was involved with concept formation, study planning, and manuscript composition. I was second author for this review, and was responsible for original drafting of Sections 4.2, 4.3, 4.7, and 4.8 included here. A. Kalisvaart and F. Colbourne both contributed greatly to the editing and re-working of my originally drafted sections.

DEDICATION

For the friends and family who supported me; especially my fiancé, Gregory Funston, and my parents, Stacey and Tammy Prokop.

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Thank you to my lab mates for helping me grow not only as a researcher, but as a colleague. Thank you to my family for their support and motivation to continue and complete my education. Thank you to my friends for the insurmountable support and encouragement that came always at the crucial moments. They always found ways to help me handle my stress and move on.

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List of Acronyms

AWB	Autologous whole blood
BBB	Blood brain barrier
BP	Blood pressure
CNS	Central nervous system
CT	Computed tomography
EuroHYP-1	European multicentre, randomised, phase III clinical trial of hypothermia plus best medical treatment versus best medical treatment alone for acute ischemic stroke
ICA	Internal carotid artery
ICH	Intracerebral hemorrhage
ICI	Intra-arterial chilled infusate
ICP	Intracranial Pressure
ICPMS	inductively coupled plasma mass spectrometry
LTP	Long-term potentiation
MMPs	Matrix metalloproteinases
MRI	Magnetic resonance imaging
rFVIIa	recombinant factor VII activated
STAIR	Stroke Treatment Academic Industry Roundtable
TH	Therapeutic hypothermia
TOBY	Total Body Hypothermia for Neonatal Encephalopathy
tPA	Tissue plasminogen activator

CHAPTER 1

1.1 Introduction

This thesis describes a variety of safety considerations regarding the use of therapeutic hypothermia (TH) for treatment of intracerebral hemorrhagic stroke (ICH). The safety considerations include the use of intra-arterial chilled infusate (ICI), as well as anticoagulation effects in a rodent model of ICH. Also included is a review of potential changes to plasticity in both clinical and pre-clinical models.

ICH is characterized by bleeding within brain tissue from damaged blood vessels, and accounts for approximately 10-15% of strokes worldwide.[4] TH is the cooling of tissue to prevent or impede damage from injury, and is used in a variety of injury profiles—notably, ischemic stroke.[5] Given that ischemic and hemorrhagic stroke overlap in pathophysiology, previous research has investigated the use of TH for ICH.[1,6–8] Although some of these investigations in TH show promise for ICH, some have also found associated risks that limit the efficacy of this treatment. Thus, I evaluated a subset of factors thought to limit the efficacy of this therapy for ICH treatment. I hypothesized that ICI administered during a time of active bleeding would negatively affect outcome in ICH by worsening the bleed size. I also hypothesized that anticoagulation, one of the many effects of TH, would worsen bleed size in isolation. Further, I reviewed the literature to evaluate the risk TH poses to plasticity following both ischemic and ICH stroke. The rationale behind the experiments is reviewed in the following sections. Background information on relevant topics such as stroke epidemiology, experimental models of ICH, and TH, are provided.

1.2 Stroke

1.2.1 Epidemiology

Stroke occurs when there is a disruption of blood supply to the brain. This disruption leads to brain tissue damage, causing disability or death. Stroke is the second leading cause of death worldwide, after ischemic heart disease, accounting for 10.2% of total deaths.[9] Although death rates have decreased over time as diagnoses and treatments improve, more people are living with the consequences and disabilities caused by the injury.[10–12] Survivors require post-stroke care, which has been estimated to cost ~18,849 CAD per patient year in Canada.[13] One study found that only 21% of people who had their first-ever stroke survived to 15 years; of the survivors, 1 in 10 had lived with moderate-severe disability since their stroke.[10] At 15 years, the prevalence of cognitive impairment, depressions, and anxiety was ~30-39% among survivors.[10]

Ischemic stroke is the most common type of stroke, accounting for approximately 80% of incidents. Ischemic stroke occurs when there is a blockage of blood flow to the brain from a blood clot. Although ICH is less prevalent, causing ~10-15% of all stroke incidents, it is more lethal, with a mortality rate of 36-50%.[4,14] ICH is characterized by a rupturing blood vessel within the brain parenchyma, and can be caused by trauma, arteriovenous malformations, tumors, or aneurysms.[15] Another type of hemorrhagic stroke is a subarachnoid hemorrhage, which occurs in the sub-arachnoid space, and is half as common as ICH, but just as lethal.[15]

Effective stroke treatments improve outcome through injury prevention, minimizing damage when it does occur, providing neuroprotection, and/or rehabilitation. Despite research efforts on effective pharmacological treatments and interventions, only tissue plasminogen activator (tPA) has been used in the clinical setting to minimize damage effectively. However, tPA is only used in ischemic stroke, often with mechanical thrombectomy, to break up clots. It is

limited by its narrow therapeutic window of effectiveness, which is ~4.5 hours from the start of the ischemic event.[16] There is no comparable pharmaceutical intervention for acute ICH. Instead, efforts are made to reduce the amount of bleeding within the brain, with the aim of minimizing overall hematoma size. These attempts can include hemostatic treatments like recombinant factor VII activated (rFVIIa), methods to decrease blood pressure (BP), or in extreme cases, surgery to remove the hematoma.[17–21] However, adverse interactions can complicate these strategies and require consideration. For example, hemostatic treatments require close monitoring of coagulation balance to minimize risk of ischemic events due to clotting.[18,19,22] Likewise, lowering BP has the potential to affect cerebral blood flow and cell death; although lowering BP has been shown to be safe and ultimately neuroprotective through minimizing damage and preventing hematoma expansion in ICH.[23–28] Commonly, ICH survivors participate in extensive rehabilitation, often learning to compensate for their functional deficits rather than achieving true neurological recovery. New treatments and therapies are needed to minimize damage in stroke, and to allow for the best possible recovery, not only to improve mortality rates, but to also decrease the long-term burden on survivors, their families, and the healthcare system.

1.2.2 Intracerebral Hemorrhage

Although ICH is less common than ischemic stroke, it has a higher mortality rate, especially for those with comorbidities like diabetes.[4] Ischemic patients can also experience hemorrhagic transformation, which can be worsened if the patient is taking anticoagulant medications.[29] Anticoagulants, like warfarin, can make asymptomatic microhemorrhages turn into symptomatic ICH.[30] Typically, intervention in the acute phase of ICH is for the purpose of mitigating life-threatening events. Patients can succumb to autonomic failures due to brain

swelling, suffer epileptic seizures, or have fever that contributes to poor outcome.[17] Many factors affect long-term outcome after an ICH, with the most predictive being the size of the hematoma.[31] Other factors include age, comorbidities, state of coagulopathy, leakage of blood into the ventricular system, and BP.[32–34] Hematoma expansion can also worsen prognosis, and occurs in ~33% of patients.[35] Strategies to mitigate hematoma expansion include lowering BP and promoting clotting to stop active bleeding.[17,21,36] Clinical studies into clotting methods, such as rFVIIa, show that hematoma expansion can be reduced, but with no change to functional outcome or mortality.[37–39] Likewise, clinical studies of BP reduction found this helped mitigate hematoma expansion[40], but did not reduce death or severe disability; however, lowering BP did improve functional outcome.[23] These clinical studies have been challenged with accurately predicting which patients would benefit most from these interventions, considering that only a subset of patients with ICH experience hematoma expansion.[35,39,41,42]

1.2.2.1 Risk Factors

Underlying disease precedes and contributes to many non-traumatic ICH events.[17] Generally, acute stroke is the result of multiple interacting risk factors.[43,44] Many factors that contribute to the risk of experiencing ICH are modifiable life-style habits that are also associated with comorbidities of ICH.[43,44] These include poor diet and sedentary lifestyle, smoking, alcohol consumption, cholesterol levels, anticoagulation, and use of drugs such as cocaine, heroin, and amphetamines.[44,45] Common and potentially modifiable risk factors are associated with an estimated 90% of population attributable risk for stroke worldwide.[43] Other factors are non-modifiable: aging, having hereditary cerebral amyloid angiopathy, and being male or of Asian ethnicity.[44,45]

Up to 66% of ICH cases are due to hypertension, or high BP.[46] Subcortical regions where a large vessel feeds small vessels, like in the striatum or thalamus, are most susceptible to hypertensive hemorrhages.[47] Chronic hypertension also causes microaneurysms and small vessel damage.[48,49] The lobar areas are the second most common region for ICH, which is often the result of cerebral amyloid angiopathy.[50] Deposits of the amyloid protein weakens blood vessels in a similar way to hypertension, accounting for ~33% of ICH in elderly patients.[51] Further, β-amyloid deposits are indicative of Alzheimer's disease, and these patients are at higher risk for ICH.[52] Hemorrhages caused by other factors, such as tumors, aneurysms, arteriovenous malformations, or coagulopathy are classified as 'secondary' ICH, whereas those caused by hypertension and cerebral amyloid angiopathy are deemed 'primary' ICH.[53]

1.2.2.2 Clinical Manifestation

Both ischemic and hemorrhagic stroke present similar symptoms at onset and therefore require imaging to determine the best course of intervention for that patient. Both types of stroke present focal neurological deficits in the acute phase, which depend on the location and size of the area in the brain where the stroke is occurring. These symptoms develop over the minutes to hours that the stroke first occurs. The basal ganglia is often where ICH occurs, but it can also occur in the cerebral lobes, thalamus, cerebellum, pons, and rarely in the medulla.[51,54] Symptoms can consist of decreased consciousness, vomiting, headache, visual deficits, fever, increased BP, and seizures, many of which can also present during ischemic stroke.[17,36,55] Seizures themselves can increase BP, increasing the risk of re-bleeding or increased bleeding, further complicating the injury and increasing mortality.[55,56] Indeed, hemorrhage size and location are key predictors of outcome in ICH.[31,47] Hematoma volumes are associated with the site of the hemorrhage, with

hemorrhages being larger in lobar areas and smallest in the brainstem, partly owing to the available tissue volume in each area.[57] Hematoma volumes larger than 30 mL are associated with worse prognoses for mortality and morbidity[58], and can be considered large.[59] Hematoma volumes are considered medium sized when they fall between 15-30 mL, and small below 15 mL.[59] Hemorrhages in the brainstem, although smaller, can be more fatal because they affect an area of autonomic control. Typically, however, when the hematoma is larger, the patient will present with lower consciousness.[51] Hematomas larger than 4 cm in diameter tend to be fatal, owing to the increased intracranial pressure (ICP) that often leads to brain stem compression.[36,51] The variability of ICP better predicts outcome than just the mean pressure, and is thus an important factor to control in the acute clinical setting.[60] Moreover, hemorrhage in deeper areas of the brain, including the cerebellum and medulla, often causes coma and is associated with poor outcomes.[51] Likewise, patients with lobar hemorrhages tend to have better functional outcomes than those with hemorrhages in deeper brain areas.[61]

Patients presenting with larger ICH are more likely to have more extreme interventions than patients with smaller hematomas.[62] This is because patients with large hematomas will have the greatest potential for improvement, outweighing the risks involved with invasive procedures. For example, surgical interventions such as a craniotomy or hematoma evacuation will likely only be attempted in patients whose hemorrhages are severe enough that these procedures would be life-saving.[62,63] Further, coagulopathy is corrected prior to attempts at surgical interventions, owing to bleeding risks.[64–66] Use of anticoagulant medication becomes more common as the population ages and atrial fibrillation detection increases.[67] This is because anticoagulation medicine is a protective measure against the five- to sevenfold increased risk of ischemic stroke associated with atrial fibrillation.[68] However, anticoagulant use threatens to exacerbate bleeding

in ICH, is a predictor of poor outcome following ICH, and can even cause an ICH itself.[21,22,31,69–71] The annual rate of ICH in patients taking oral anticoagulants, including warfarin, is 0.3% to 0.6%.[22,72,73] However, this number is lower (0.1% to 0.2%) when examining only the more recently developed direct oral anticoagulants.[72–74] As distribution of these medications increases, the percentage of ICH associated with these anticoagulants is also increasing.[21,71,75,76] Therefore, the investigation of the management of risks associated with these medications is of continued importance.

1.2.2.3 Pathophysiology

In ICH, damage is categorized as either primary or secondary. Primary damage is the initial injury caused by the rupturing of cerebral blood vessels. Blood pushes through brain tissue, mechanically dissecting the brain parenchyma.[77] This injury impedes the normal functioning of vessels and neuronal cells both in the immediate area and in connecting networks. This dysfunction leads to ongoing metabolic imbalances and cell death. Secondary damage comprises the cascading effects of this initial injury, occurring in the perihematoma zone. Secondary damage involves cell death, oxidative damage, and inflammation, among many other deleterious processes (iron toxicity, excitotoxicity, etc.). Although a complete profile of secondary damage has not yet been fully described, it is hypothesized that it is mediated by multiple pathways that are activated by blood that infiltrates the brain tissue from primary damage.[77,78] A mass effect of the growing hematoma, blood brain barrier (BBB) damage, toxicity from blood products, and cerebral edema all contribute to secondary damage. Furthermore, the ruptured vessels in the core of the hematoma area no longer supply blood to that region, and compounding ischemic injury can occur here.[79] However, the compounding effects of reduced

blood flow and hematoma expansion do not appear to induce ischemic injury in areas outside the hematoma.[25,80]

Expansion of the hematoma over time adds to the primary damage, and worsens secondary injury processes. Bleeding usually stops within 3 hours of the initial injury, but a potential exists for extended bleeding or re-bleeds weeks later.[57,58,70,81,82] Initial hematoma development of >10.2 mL / hour has been shown to be a strong indicator of later hematoma growth.[81] This suggests that more blood extrusion in the early phase can determine the amount of secondary bleeding later, leading to hematoma expansion and/or slowing hematoma resolution. This is one reason why therapies to limit bleeding in the early phase of injury, like BP control and rFVIIa, have been pursued. However, use of rFVIIa for anticoagulation reversal is not recommended, as it does not affect bleeding duration and volumes compared to a placebo when tested for warfarin reversal.[21,83] There is debate over how hematoma expansion should be defined, with different research groups using varying quantitative cut-offs to define hematoma expansion either as an absolute amount or relative to the initial volume of injury.[35] Regardless, ~73% of ICH patients show some degree of hematoma expansion between brain scans within 3 hours and a 24-hour follow up scan from symptom onset.[70] Also, ~38% of patients show more than a 33% increase in hematoma volume over 24 hours.[84] Evidence suggests that hematoma expansion is a result of multiple ruptured vessels that stretch and break from the force of the initial growing hematoma, in addition to ongoing bleeding from the initial site of injury.[48,77] The risk for vessels to break appears to be highest closer to the primary bleeding site, as these vessels are not only more likely to be brittle or diseased, but also to have opposing pressures placed on them from the growing hematoma and the counter pressure of surrounding tissue.[85–88] Considering this, hematoma expansion does not occur in all ICH cases, and secondary breaking

of vessels appears to be dependent on a larger volume of blood intruding over a shorter period of time.[89] Small volumes of blood over longer intervals put less pressure on the surrounding tissue, and could allow ongoing resolution processes to counteract a slow leak.

1.2.2.4 Rodent Models

A number of different species are used in research, but the most commonly used are rodents, especially rats and mice.[90] A variety of methods can be used in preclinical ICH research to induce the ICH itself, but no method perfectly mimics the full range of characteristics in human ICH.[91] The two most common methods are autologous whole blood injection (AWB) and collagenase injection.[92,93] Other, less commonly used models of ICH include balloon inflation[94], cerebral blood vessel avulsion[95,96], focused ultrasound[97], injection of specific blood components, and spontaneous ICH.[98] Models of spontaneous ICH can use etiologically relevant methods, such as anticoagulant therapy, hypertension, amyloid angiopathy, and high sugar and fat diets to induce ICH. Each model can be used to study different characteristics of human clinical ICH, but each model also has its own limitations when compared to human ICH.[99] Here, I focus on the collagenase injection model because it is used in the experiments described later in this thesis. I also briefly review the AWB model, as this is the other of the two most commonly used ICH models, next to collagenase injection.

Both AWB and collagenase injection models use stereotaxic surgery to access the infusion sites. The striatum is often chosen as the infusion site, as this is the most common area of the brain where ICH occurs and it can easily contain small to large hemorrhages without extension into the ventricular system.[54] The AWB model uses an infusion of blood from the animal, often taken from a superficial vessel (e.g., tail). This blood is injected into the brain

parenchyma at a clinically relevant volume, which can vary, to form a hematoma immediately after the injection. For example, the striatum of a rat is capable of containing hematomas of approximately 50-100 μL, which is relatively equal to a large hematoma in humans, scaled to brain size.[100] This type of accumulation of blood within the brain tissue can reduce cerebral blood flow and causes edema.[101] This mass effect means that the AWB model has the potential to lead to secondary hematoma expansion if the blood infusion caused surrounding vessels to rupture. However, there have been mixed results regarding the ability of the AWB model to reliably produce hematoma expansion via secondary bleeding.[91,99,102–104] Although vessels break, young healthy animals tend to clot quickly, ceasing bleeding. Research suggests this model is poorly suited for studying the effects of rebleeding.[99]

The collagenase infusion model uses the bacterial enzyme collagenase to degrade the basal lamina of blood vessels, which contain collagen.[92] This leads to spontaneous bleeding within about 10 minutes from the injection, and a full hematoma develops within 24 hours.[92] The collagenase model typically causes more edema than the AWB model, and is currently the ideal model for studying hematoma expansion and small vessel rupture.[99,105] Differences between the AWB and collagenase models extend beyond the bleeding patterns and edema formation to how these models react to rehabilitation and TH, as discussed further in following sections.[106,107]

Success in clinical translation from pre-clinical research is limited for numerous reasons, among which is the lack of a model of ICH that encompasses the complexity of the human condition.[99] This failure to translate is abundant and concerning in ICH research. Issues such as flawed experimental designs and poor statistical use play major roles.[100] Studies on safety and dose responses should be incorporated regularly, along with pre-planning of power analyses, randomization, and blinding. Additionally, most ICH research uses young, male rats, limiting the

generalization of findings to both sexes. Furthermore, because ICH is more common in older populations, using young animals further reduces the accuracy of the model in reflecting the clinical condition. However, the logistics of experimental implementation and minimizing mortality can be barriers to using older animals with comorbidities in stroke research. Treatment window is another consideration when examining how treatments alter outcome, because treatments can be delayed in the clinical setting.[108] Preclinical studies often examine treatment effects when given immediately or soon after injury. Multiple factors can interplay in various ways to cause ICH and other forms of comorbid injury. Therefore, researchers should be testing therapies in multiple models and settings to get a holistic characterization of the effects. Further concern for preclinical research stems from a limited 12% of experimental ICH studies reporting no significant treatment effect.[100] This suggests a bias in publication towards positive results, and increases the risk for different researchers to invest time and resources into studying unfruitful treatments that have already been studied but remain unreported.

1.3 Therapeutic Hypothermia

Therapeutic hypothermia is an intentional decrease in body temperature for the purposes of slowing or preventing injury. Historical reports show that TH has been used for resuscitation, and to effectively treat trauma, cardiac arrest, neonatal hypoxia/ischemia, schizophrenia, cancer, and typhoid fever.[109–113] Consensus on the optimal parameters of TH for various injuries is difficult to establish, as each parameter is unique, with its own complications. TH is divided into different phases: induction, maintenance, and rewarming. However, there is no standardized classification for the depth of TH into mild, moderate, or severe cooling. Approximate ranges consist of <33°C for severe, 33–34°C for moderate, and >34°C for mild cooling.[114] Further

investigations are needed to determine optimal cooling depths, rates, timing of onset, rate of rewarming, and management of side effects for the various applications of TH. The efficacy of TH is dependent on these parameters.[115] TH to a cooling depth of 32-35°C has been shown to be neuroprotective after ischemia and trauma in animal models, leading to meaningful reductions in damage and better recovery.[116–118] These findings have influenced clinical investigations using different methods, including surface and endovascular cooling, to evaluate safety and feasibility in ischemia.[119–122] TH has also been applied in ICH clinical studies.[123,124] The different injury profiles of ICH and ischemia could be differentially affected by decreased temperature, altering the overall efficacy of TH.[125] Investigations are ongoing to understand the full range of mechanisms by which TH affects these similar but differing injuries.[126] Hypothermia has numerous mechanisms of action, both protective and injurious (complications of this therapy), depending on how it is used. The broad effects of TH allow it to be more effective than an intervention that targets a more limited set of processes, but also relate to a broad set of potential complications.

1.3.1 Mechanisms of Hypothermia

The overarching benefit of TH is neuroprotection.[127] The mechanisms of stroke injury and cell death are extensive, with much overlap between ICH and ischemia.[128] Cooling is an effective neuroprotectant in a variety of brain injury profiles, particularly ischemic injury, because it targets numerous mechanisms of injury. Among many effects, TH has been shown to mitigate excitotoxicity, oxidative stress, BBB breakdown, inflammation, and apoptosis in ischemia.[129–133] These processes contribute to edema formation, and the management of these with TH can be life-saving in ICH, where edema and raised ICP is often fatal. Many of the

damaging processes TH impedes are those triggered by anoxia, or a lack of blood and oxygen. Because ICH has a different injury profile than ischemia, it is possible that the effectiveness of TH in treating ICH could differ. For example, a previous study from our lab has shown TH does not directly influence iron-induced injury in rats. This contributes to the concerns for effectiveness of TH for ICH because iron toxicity is a factor in ICH, but not ischemia.[134] However, TH has been shown to have some benefit for ICH[6,7,135–139], amongst other brain injury types, which may result from the various interplaying factors affected by this therapy.

1.3.1.1 Metabolism

Cerebral metabolism decreases by about 5% for every 1°C drop in temperature.[115,140] This reduction in metabolism is beneficial when the oxygen supply is impaired, as it leads to lower demands for oxygen and glucose.[109,115] This change in glucose demand affects cell death when intracellular metabolism switches to anaerobic glycolysis as a function of interrupted blood supply.[141] Anaerobic glycolysis leads to intra- and extracellular acidosis and calcium influx, for which the cell is unable to compensate.[115,142,143] TH has been shown to improve the capacity of cells to manage this calcium influx, and reduce neurotoxicity.[142,144] Some animal studies have demonstrated a decrease in cerebral blood flow parallel to the cerebral oxygen consumption decline when body temperature decreases to ~28-30°C.[140,145,146] This decrease in cerebral blood flow can affect ICP, which is important when brain compliance is reduced.[147]

1.3.1.2 Inflammation and Free Radicals

Tissue injury activates an inflammatory cascade that involves a variety of pro-inflammatory cells infiltrating the injured area. The balance of this inflammation response is

important for patient outcome. Less inflammation could mean less intrinsic repair is triggered, but too much inflammation could lead to swelling, brain stem compression, and possibly death. Studies in rats have shown that TH attenuates the inflammatory response following stroke, although most of this work has been done in ischemia. The anti-inflammatory effect of TH works through a number of potential mechanisms. It reduces neutrophil infiltration[148,149], microglial activation[148,149], and inflammatory mediators like nitric oxide, and cytokines[150–152]. These effects could be influenced by the inhibition of inflammatory transcription factors (NF-κB), either by lowering translocation and activation, or by prolonging the accumulation in the nucleus of cells.[115,153–156] The inflammatory response can also activate the production of free oxygen radicals.[157] The number of free radicals can be lowered by TH, while also mitigating endogenous antioxidant mechanisms.[158] Free radical production is positively related with temperature; decreasing temperatures correlate linearly with less free radical production.[142]

1.3.1.3 Edema

Brain injury can lead to BBB breakdown and edema, contributing to the mass effect in ICH. Edema contributes to increases in ICP and the potential for brain herniation. TH has been shown to decrease vascular permeability and mitigate edema in both clinical and pre-clinical ICH research.[8,159–161] One proposed mechanism for edema reduction by TH is through the preservation of the basal lamina by inhibiting matrix metalloproteinases (MMPs), and increasing the expression of endogenous MMP inhibitors.[162–164] This is supported by preclinical research in rodents, which found lower levels of MMPs in TH treated groups compared to normothermic controls.[165,166] Thus, the structural integrity of the BBB is better preserved when using TH, lessening the impact of edema.

1.3.2 Methods of Cooling

Brain injuries differ in injury cascades, suggesting the need for various TH cooling methods and regimens to reach maximum efficacy and protection for each. However, each cooling method is associated with its own set of benefits and risks, which must also be considered. Hypothermia initiated prior to ischemic injury has been shown to provide optimal protection[117]; however, this is unrealistic for spontaneous injuries and could pose issues for bleeding in ICH. Initiating TH early following injury and maintaining for longer is suggested as the best option[131], but starting too early after hemorrhagic stroke could pose the same bleeding complications mentioned.[167] Interestingly, longer cooling durations can recover some of the benefits typically lost due to delays in cooling in rats.[168] In terms of temperature depth required, some meta-analyses suggest that brain temperatures of 30-34°C can provide protection similar to when temperatures reach below 25°C for ischemic stroke.[117,131]

Cooling methods can be generalized into two main categories: surface cooling and core cooling. Conventional surface cooling refers to cooling with ice packs or air- or water-circulating cooling blankets. Cold water immersion can also be used for surface cooling. Core cooling uses devices like intravascular catheters, saline filled balloons, and cold fluid infusions (e.g., ICI). Some pre-clinical research also examines alternative methods of cooling, like cooling circulating blood[169], and using drug-induced hypothermia as an alternative to physical hypothermia.[170–172]

1.3.2.1 Focal versus Systemic Cooling

Systemic cooling refers to cooling the whole body, whereas focal cooling is only applied to a localized area. Although systemic surface cooling is common for inducing TH, it is risky

because it has numerous side effects and implementation challenges.[173] Coagulation factors and platelet functions can be impaired when body temperatures are below 35°C.[174] Additionally, lowering core body temperature can induce vasoconstriction and shivering, thereby increasing BP, cardiac output, and possibly ICP.[175] Systemic TH also has a slower rate of cooling because it requires cooling a larger volume, and the body has thermal resistance mechanisms.[119,173] The increased time to target temperature limits treatment efficacy. Localized cooling can avoid many of the complications associated with systemic TH, and is therefore considered advantageous to systemic cooling.[176] Many research groups have developed a variety of local cooling devices for either animal research or clinical use. One example is a cooling coil previously developed in our lab for inducing hemispheric-specific hypothermia in rats.[177] Studies using this coil show that local cooling can been effective in reducing edema and improving functional recovery in rats with ICH, although it does not provide neuroprotection.[135,136] Other local cooling devices have also been shown to avoid some of the complications of systemic TH, including rebound hyperthermia and ICP, infections, and coagulopathy.[176–180]

1.3.2.2 Hypothermia in Patients

Surface and endovascular cooling are two methods for clinical TH.[175] Although surface cooling is relatively non-invasive, temperature maintenance can be challenging when the body activates its autonomic resistance to temperature change. Increases in sympathetic tone, vasoconstriction, discomfort, and shivering all affect the ease of temperature induction and maintenance.[175,181] Automated surface cooling systems have relatively high cooling rates and more precise levels of temperature control compared to other methods of cooling patients, such as ice packs, fans, convective air blankets, water mattresses, and alcohol bathing.[182]

Endovascular cooling by infusion of cold saline (4°C) also induces hypothermia quickly, although the invasive nature requires expertise for its implementation, potentially delaying onset time.[175,183] Regardless, interest in this method for the application of selective trans-arterial brain cooling remains.[184–186]

As mentioned above, selective cooling is ideal for avoiding systemic complications of TH. Although there are many devices for selective brain cooling, some, like helmets, head caps, and neckbands, only cool the surface. This means that deep brain areas do not reach target temperatures below 36°C, and to monitor temperature in those deep areas requires sensor implantation, countering the non-invasiveness of the cooling technique.[187]

1.3.2.3 Hypothermia in Rodents

Use of anesthetic is required for brain-selective methods of TH in rodents, and is therefore implemented for a short duration. These include the use of a cooling blanket[188], a coil attached to the head[177], or intra-arterial cold saline[189,190]. However, some devices have been developed to locally cool brain tissue without long-term anesthetic.[177] Long term TH is not ideal under anesthetic, as it increases the risk of overdose and mortality.[177] Most systemic methods of cooling rodents involve cooling blankets or spraying the animals with water or alcohol, which can be combined with the use of fans in either awake or anesthetized rodents.[131,191] Other methods include the use of a cold room or pharmacological induction.[192–194]

As previously mentioned, the AWB and collagenase models of rodent ICH differ in many characteristics, one of which is their reaction to therapeutic hypothermia. For example, TH in the AWB model, starting at 1 or 4 hours for a duration of two days post-ICH, reduces inflammation,

edema, and BBB disruption, without change to functional deficits or bleeding.[138] Conversely, TH starting within 12 hours following collagenase infusion increases hematoma volume.[1,195]

1.3.3 Rewarming and Other Limitations and Complications

Rewarming rate is critical for preserving the neuroprotection gained from TH.[196] Rewarming following systemic cooling can lead to more severe effects compared to rewarming from local cooling, given the whole-body application of these methods. Rapid rewarming after TH risks increases in brain edema and ICP that could lead to brain stem compression or other ischemic injury, and possibly death. Slow rewarming is beneficial for avoiding sudden vasodilation, increased cardiac output volume and rate, and ICP changes.[197] Indeed, a slow rewarming rate is correlated with slower increases in ICP and decreases in CPP (i.e., it mitigates sudden changes in ICP and CPP).[198] Slow rewarming may also ease the restart of metabolic pathways that were altered by TH.[199]

Side effects of TH need to be considered and managed as well, because their effects can worsen outcomes for patients. As mentioned previously, cooling before ischemic injury provides optimal neuroprotection. However, if cooling were to be applied before ICH, this could cause more bleeding and a larger hematoma, as TH contributes to coagulopathy. As mentioned above, the timing of cooling onset after injury is also important if there is active bleeding to prevent worsening hematoma size from hypothermia induced anticoagulation.[167] Depth of cooling also appears to have an impact on platelet function. Research in both human patients and baboons found that temperatures below 35°C can induce mild platelet dysfunction, and cooling below 33°C also affects the synthesis and kinetics of clotting enzymes and plasminogen activator inhibitors.[174,200–204] Furthermore, coagulopathy worsens when acidosis co-occurs.[205,206]

Hypothermia is a stressful procedure for patients. Animal studies have shown that cooling without sedation counters the neuroprotective effects that would otherwise be generated.[207,208] Shivering can occur when anesthetics are not used, leading to increased metabolism and oxygen consumption, countering the metabolic gains of TH. Cooling also leads to immune suppression and pneumonia[121], increased risk of infection, cold diuresis and hypovolemia, electrolyte disorders, insulin resistance, and impaired drug clearance.[196]

Although evidence of TH efficacy varies, especially in ICH, there is still much interest in the potential of TH.[209–211] However, several problems remain. First, the mechanisms of injury vary between diseases, which necessitates different approaches and treatment parameters, such as depth, duration, and delay in order to achieve optimal neuroprotection. Second, side effects complicate the use of TH, both by reducing the potential benefits of TH and increasing the risk of poor outcome. These complications require investigations into effective management strategies to optimize patient outcomes. In summary, each injury profile, including ICH, likely requires its own set of TH parameters for optimization, and has its own set of complications to consider.

1.4 Purpose

This thesis has three aims, which I investigate with two experiments that use the collagenase model of ICH in rats, and a literature review. First, I will assess the safety of applying ICI at a time of active bleeding using spectrophotometry and wet/dry tissue weights. I hypothesize that ICI will worsen hematoma volume measured at 24-hours post-ICH onset. This will be the first time that ICI is experimentally applied during a time of known active bleeding in ICH. Second, I will investigate the risk of late or re-bleeding from the administration of the

anticoagulant medication warfarin, starting 24-hours post-ICH onset, and dosing every 24-hours. I hypothesize that warfarin will worsen the spectrophotometry assessment of hematoma volume 7 days post-ICH. Lastly, I review potential effects of TH on plasticity in both clinical and pre-clinical models.

CHAPTER 2: Assessing Safety of Intra-Arterial Chilled Infusate in Intracerebral Hemorrhage

Data from this chapter is included as part of a larger study, plublished as: Liddle LJ, Prokop BJ, Dirks CA, Denchuk A, Almekhlafi M, Colbourne F (2020). Infusion of Cold Saline into the Carotid Artery Does Not Affect Outcome after Intrastriatal Hemorrhage. Therapeutic Hypothermia and Temperature Management.[2]

2.1 Introduction

Intra-arterial chilled infusate (ICI) is used for selective cooling in clinical and pre-clinical ischemic stroke.[184] Most research has investigated this technique for ischemic stroke mitigation.[184] However, ICI may also be beneficially applied to ICH to help remove or flush out harmful inflammatory cells and metabolites (see below), and/or locally cool to reduce injury through TH. That being said, there is a possibility that ICI could be harmful when applied to ICH, as the infusate often requires heparin to prevent the creation of emboli, and could induce coagulopathy and more bleeding. Adding to this effect, lower temperatures alone can also induce coagulopathy in a dose-dependent manner. However, ICI is brief compared to other methods of cooling (see Section 1.3.2), and may not create biologically significant temperature-induced changes to clotting. Furthermore, the infusion of saline into the vascular system could increase arterial volume, raising BP, and potentially cause clot failure and more bleeding. For these reasons, the use of ICI in ICH is important to investigate for potential benefits or harm.

Using ICI as a method of selective cooling mitigates the complications associated with whole-body systemic cooling while implementing the protective benefits of hypothermia (see Section 1.3.2.1), which is one of the reasons for interest in investigating these methods. Focal cooling of the brain helps avoid pneumonia and uncontrollable shivering that is often seen in systemic cooling protocols.[121,212] Examples of success in implementing these cold saline infusions in humans come from pilot studies that aimed to avoid body cooling, while attaining brain cooling. One clinical pilot study used 50 mL of 4°C saline (10 mL/min) prior to clot extraction, and an additional 300 mL over 10 minutes afterwards, to reach an estimated brain temperature of 35.2-37.2°C (not directly measured from brain).[186] Another pilot study in humans infused ~660 mL of 4-17°C saline over 20 minutes.[213] Systemic cooling does not appear to be

the only benefit of ICI—it also flushes out metabolites such as lactate, prostaglandins, and carbon dioxide, as well MMPs[214], that can worsen ongoing injury or impede recovery.[189,215,216] Inflammatory markers like leukocytes and cytokines can also be flushed away with ICI.[217] This can be helpful if the infiltration of inflammatory cells is causing further injury from overexpression (see Section 1.3.1.2). Compared to the clinical studies, some pre-clinical studies of these infusion methods have used much larger volumes of infusate than would be used clinically, and at temperatures much lower than what is feasible in patients.[212,218,219] For example, some animal studies in rats have used 10-36 mL/hour infusion rates of 0-20°C saline over 10-30 minutes.[185,190,218–221] The use of such large volumes being infused at fast rates introduces higher risk for volume overload, increased BP, and could therefore lead to the rupturing of vulnerable blood vessels. Furthermore, the colder the infusate used, the more likely there will be induced vasospasms from abrupt and drastic temperature changes.

The aforementioned clinical and pre-clinical work has focused on ischemic injuries. To my knowledge, the only study that has evaluated the safety of arterial infusions in hemorrhage did so in the context of ischemia with hemorrhagic transformation.[222] This study mainly assessed the relation of other drug or contrast agent deliveries and safety assessments, and used saline as a control.[222] They found that hemorrhagic transformation was associated with the size of the ischemic infarct volume, and there was no difference in prevalence of this complication between the different infusion-type groups.[222] However, a no-infusion group was absent in this study, and hemorrhagic transformation could not be assessed based on presence or absence of infusion in this instance. Thorough investigation of these infusion protocols is needed to ensure safety, especially given the modest-to-poor translational quality of existing work, and the lack of investigations into hemorrhagic complications.

The goal of the research presented here is to assess if ICI is safe when used with ICH, at a time of active bleeding. We begin by determining the change in brain temperature of rats resulting from an infusion of 3 mL of room temperature (~20°C) heparinized saline. Heparinized saline is necessary to prevent clotting within the catheter, thus ensuring stable flow and minimizing the risk of creating emboli. We chose to study room temperature saline, rather than chilled saline, because, clinically, cold (5°C) saline is infused into an artery in the groin area, and the body has warmed it to around 20°C by the time it reaches the catheter tip in the neck area.[213] Furthermore, 3 mL was calculated as the rat-equivalent volume that would be on the high-end of feasibility in patients, considering whole-body blood volume to avoid volume overload (see Section 2.2.2.1).[186] We evaluate effectiveness of the infusion from the brain temperature change that occurs during these infusions. Following this, we assess if bleeding is worse when ICI is applied during a time of active bleeding in the collagenase model of intracerebral hemorrhage. We use this as a safety test to gauge risk in ICH. During a time of active bleeding, changes in temperature[195] or infusion of anti-clotting agents like heparin[223] could be dangerous.[29] We hypothesized that heparinized ICI saline would worsen bleeding, because heparin (an anti-clotting agent) and cooling both increase bleed time (see Section 1.3.3), and the infusion of saline could dilute the blood and slow clot time. Therefore, we review whether the ICI dose increased risk of hematoma expansion and, if so, by how much. We subsequently assess brain water content, an indication of edema. This model of pure ICH (in contrast to ischemia with hemorrhagic transformation) provides an indication of the safety of ICI in the case of hemorrhage. Future studies can take these findings into consideration for safety and other analyses of hemorrhagic transformation after ischemia.

2.2 Methods

2.2.1 Subjects

All protocols followed the Canadian Council of Animal Care Guidelines and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta, Animal Use Protocol #960. Group sizes were calculated *a priori* to estimate 80% power to detect a 20% increase in hematoma volume and a 1% decrease in edema at an alpha level of 0.05. We used randomization and blinding throughout our experimentation. A total of 61 (Exp. 1 = 9; Exp. 2 = 20, Exp. 3 = 32) male, Sprague Dawley rats (225-300 g; ~3 months old) were obtained from Charles River Laboratories (Saint-Constant). Animals spent a minimum of 7 days acclimating from transport before undergoing any procedures. All animals were individually housed after surgery with *ad libitum* access to food and water. Individual housing was necessary for animal safety with surgical attachments to the skull (Experiment 1), intensive monitoring purposes (Experiment 1, 2, and 3), and limitations of the telemetry device receivers for Experiment 3. Housing rooms were temperature, humidity, and light (12-hour light–12-hour dark cycle) controlled.

2.2.2 Procedures

In all surgical procedures, including core temperature telemetry probe implantation, cannula implantation, ICH induction, and ICI induction, rats were anesthetized with isoflurane (4% induction, 1.5-2.5% maintenance in 60% N_2O and remainder O_2). For cannula implantation and ICH induction, animals were placed in a stereotaxic frame. A rectal temperature probe connected to a heated water pad, along with a removable electric heating pad, maintained core temperature at ~37°C throughout all surgical procedures. All procedures were done in a sterile

manner. For ICH induction in Experiments 2 and 3, an incision was made at the midline on the scalp (~2.5 cm), and a burr hole was drilled (3.5 mm right and 0.5 mm anterior to Bregma).[92,224,225] A 26 G Hamilton syringe (10 µL) was lowered 6.5 mm below the surface of the skull into the striatal area. The collagenase (1 µL of 0.6 U/µL, Sigma-Aldrich, type 4-S Lot# SLBL855 or 1 µL of 0.2 U/µL, Sigma-Aldrich, type 4-S Lot# 108M4100V) was infused over 5 minutes, with a 5-minute 'wait-time' before the needle was slowly removed to prevent backflow. A solid metal screw (stainless steel, plain finish, binding head, slotted drive, 1/8" length, #0-80 threads) was inserted into the burr hole. The incision site was closed with surgical staples, and a few drops of Bupivacaine hydrochloride (5 mg/mL, SteriMax Inc.) were applied to the site for local pain management.

2.2.2.1 Experiment 1: Determining ICI brain temperature effect

In Experiment 1 (n = 9; weight at surgery: 550 g ± 46 g), temperature was measured from the cooled striatum (Ipsi), the contralateral cortex (Contra), and rectum (Body) of anesthetized naïve rats to test the efficacy of cooling the brain from ICI via the internal carotid artery (ICA). Figure 2-1(A) shows the timeline of procedures for Experiment 1. Rats were anesthetized with isoflurane (4% induction, 1.5–2.5% maintenance in 60% N_2O, balance O_2) and two guide cannulas (23-gauge, 4 mm long) were implanted (AP=0.5, ML=+3.5 and ML=-3.5 from Bregma) and anchored with dental cement. Two other cannulas were placed posterior to the guide cannulas and secured with dental cement. These acted as anchors for attachment of the temperature probes to prevent movement when the animal was turned onto its back. Rats were given three days to recover from this procedure. Following this, rats were once more anesthetized, and two thermocouple probes (HYP1- 30-1/2-T-G-60-SMP-M, Omega, Stamford,

CT, USA) were lowered below the skull surface, into the striatal areas of the brain, and anchored with tape to the posterior cylinders to measure temperature from both the ipsilateral (side of cooling) and contralateral cortices during ICI induction. Once the thermocouple probes were securely anchored, the animal was turned onto its back to undergo the ICI procedure.

Aseptically, a small (<3 cm) incision was made in the neck, slightly off to the left side of the midline of the animal. Both common carotid arteries were isolated, and auxiliary vessels were electro-cauterized (ME 102; KLS Martin). Following isolation, the common carotid and ICA were temporarily occluded using S&T vascular clamps (Fine Science Tools). Catheter tubing (PE-10) was inserted into the carotid system near the carotid bifurcation and secured with silk suture. The vascular clips were removed prior to the infusion. Heparinized (10,000 USP/L) saline (3 mL of room temperature, ~20°C) was infused with a micro infusion pump over 20 minutes. This infusion protocol was based on clinical studies[186], and assumes that a maximum 1L of saline can safely be infused into a patient's middle cerebral artery at a rate of 30 mL/minute, within a reasonable period of time. Here, the dose is scaled to rats based on brain weight (1 L =~70% of human brain weight), total blood volume (~20% blood volume), and body mass (~1.25% body mass).[2] A 3mL dose was ultimately selected, falling between a ~1.4 mL (scaled to brain weight) and a 4.4 mL infusion (scaled to body mass). Following the infusion, the internal and common carotid arteries were briefly occluded to remove the catheter. The external carotid artery was sealed using sterile suture and electrocoagulation. The incision was then sutured closed and treated with Bupivacaine hydrochloride (5 mg/mL, SteriMax Inc.), and the temperature probes were removed. During the procedure, temperature was recorded continuously with video for later entry and analysis.

Animals were euthanized 24 hours after ICI induction, and brain samples were collected

for histological analysis. Briefly, animals were injected with Euthanyl (pentobarbital sodium; 80 mg / kg; I.P; Bimeda-MTC) and brains were formalin-perfused fixated (0.9% saline and formalin). Brains were left in formalin for 1 week, after which they were transferred to a formalin-sucrose (30%) cryoprotective solution for a minimum of 3 days. Once equilibrated, brain samples were flash-frozen in isopentane chilled with surrounding dry ice. Brain samples were sectioned with a cryostat to 40 µm thick and stained with Cresyl violet. Sections were then examined for signs of damage due to the procedure (possible ischemia, hemorrhage, etc.) and to confirm correct placement of brain temperature probes.

2.2.2.2 Experiment 2: Hematoma Volume Assessment in ICI

A timeline of Experiment 2 is shown in Figure 2-1(B). An ICH was induced using an infusion of collagenase (1 µL of 0.6 U/µL, Sigma-Aldrich, type 4-S Lot# SLBL855 or 1 µL of 0.2 U/µL, Sigma-Aldrich, type 4-S Lot# 108M4100V) into the striatum, ipsilateral to the ICI infusion side, as described above (see Figure 2-4). Upon completing the ICH induction, the animal was turned onto its back and transferred from the stereotaxic frame to a nose cone for the ICI procedure, as described above. Animals (weight at surgery: 381.75 g ± 56 g; ~4 months old) were randomized by pulling group allocations out of an opaque box into ICI infusion (n=10) or control groups (n=10) once the infusion catheter was in place. Control animals had a 20-minute wait time instead of the heparinized saline infusion. Average time between collagenase infusion and start of ICI was 59 minutes. Animals were euthanized 24 hours after collagenase injection, and brains collected for blood volume spectrophotometric analysis.[105,226,227] For this, animals were anesthetized with 4% isoflurane (60% N_2O, balance O2) and immediately decapitated (<30 seconds after removal from anesthetic). Briefly, brain hemispheres were separated into

ipsilateral, contralateral, and cerebellar. These were each weighed and proportional amounts of dH_2O were added to create a homogenate with a ratio of brain tissue to water of 1:4. After being left on ice for 7 minutes, this mixture was aliquoted into centrifuge tubes and spun (15800 g for 35 minutes). The supernatant (100 µL) was mixed with Drabkin's solution (600 µL, Sigma-Aldrich) for spectrophotometric analysis of hemoglobin content (540 nm wavelength).

2.2.2.3 Experiment 3: Brain Water Content Assessment in ICI

A timeline of the procedure followed in Experiment 3 is shown in Figure 2-1(C). Core temperature telemetry probes were implanted as previously described.[135,167] Briefly, a sterilized calibrated probe (model TA10TA-F40; Data Sciences Int., MN) was inserted through an abdominal incision (<3 cm). Analgesia was achieved using Bupivacaine (0.5 mg S.C.; SteriMax Inc.). Muscle and skin were sutured closed following core probe implantation, and Meloxicam (0.2 mg S.C., Boehringer Ingelheim Ltd.) was applied. Animals (weight at surgery: 300 g ± 26 g) were given three days to recover from this first procedure prior to further manipulations. During these three days, baseline temperature and activity readings were recorded using Advanced Research Technologies software (ART; version 2.30). Hourly moving averages were taken from 30-second sample intervals for analysis, with each hourly value (corresponding to circadian cycle) averaged from data collected over the course of multiple days. Differences were taken for comparing post-procedure values to baseline values for temperature and activity ((post procedure value) – (baseline value)).

Following this, an ICH was induced using an infusion of collagenase into the striatum, ipsilateral to the ICI infusion side (see Figure 2-4) as described above. Upon completing the ICH induction, the animal was placed onto its back and transferred from the stereotaxic frame to a

nose cone for the ICI procedure, as described above. Animals were randomized into ICI infusion or control groups once the infusion catheter was in place. Again, control animals had a 20-minute wait time instead of the heparinized saline infusion. Average time between the collagenase infusion and the beginning of the ICI infusion was 43 minutes.

Approximately 24 hours after collagenase injection, animals were anesthetized with isoflurane (described above), and injected with Magnevist® (gadopentetate dimeglumine; 2.5 mL/kg; Bayer, Mississauga, ON) into the femoral vein, which was allowed to circulate for 10 minutes. Magnevist® is a Gadolinium-based contrast imaging agent that is impermeable to a healthy BBB.[228] This was for the purpose of sample preparation for inductively coupled plasma mass spectrometry (ICPMS) analyses. Following this 10-minute wait period, animals were euthanized (as described above in Experiment 2) 24 hours after collagenase injection, and brains were dissected for water content (indicative of edema; Figure 2-7), and ICPMS analyses (Thermo Scientific ICAP-Q quadrupole ICP-MS, University of Alberta) as previously described.[224]

For brain water content measurements, extracted brains were cut coronally in a matrix 4 mm posterior and 2 mm anterior to the needle mark (from the collagenase injection). The ventral portion, inferior to the lateral ends of the corpus callosum and about 7 mm ventral to the dorsal cortex surface, was removed. Hemispheres were then divided. Fine tools were used to gently separate the cortex from the striatum along the corpus callosum tract. The 4 regions of interest (ipsilateral and contralateral cortices and striata were weighed to assess ‘wet weight’. These were then placed in an oven at 100°C for 24 hours, removed, and weighed again to assess the ‘dry weight’. Water content was calculated as a percent of the region of interest using the equation below.

$$Percent\ Water\ Content = \frac{(Wet\ Weight) - (Dry\ Weight)}{Wet\ Weight} \times 100$$

The striatal brain regions that were used for brain water content measurements were also used for ICPMS analysis after the dry weights were recorded. For this, each dried striatal sample was digested separately in 2 mL of high purity nitric acid (trace metal grade, Fisher Scientific) for at least 1 week. These samples were submitted for analysis through the Canadian Centre for Isotopic Microanalysis at the University of Alberta. Handling of samples was such that cross contamination was minimized.

2.2.3 Statistical Analysis

All statistical analyses were completed using GraphPad Prism (version 6.0c) software, except the 2-way repeated measure ANOVAs that analyzed the brain water content, temperature, and activity measurements (Experiment 3), which were done using Stata (IC 13.1). All significance levels are set at $p < 0.05$. Data from Experiment 2 was analyzed with a two-tailed unpaired t-test with Welch's correction to assess if there was a difference in hematoma volume between the control and ICI groups, as well as if there was a difference in weight loss post-ICI procedure between the groups.

2.3 Results

2.3.1 Experiment 1: Determining ICI brain temperature effect

There were two animal exclusions in this phase, where the temperature probes did not reach low enough in the brain for accurate recording. Therefore, data from seven total animals were collected and used here. Temperature probe location was confirmed using histology to ensure it was recording from the correct brain regions (Figure 2-2). When comparing the

temperature change of the ipsilateral hemisphere to the temperature change of the contralateral hemisphere ((ipsilateral temperature change) – (contralateral temperature change)), the average change was a decrease of 0.1°C during the 20-minute infusion time. The maximum cooling ((ipsilateral temperature change) – (contralateral temperature change)) in any one animal was a drop of 0.5°C for a duration of 2 seconds. The maximum cooling in any one animal in the hemisphere ipsilateral to the infusion (not controlled for temperature change in other body areas) was a drop of 0.8°C for a duration of 22 seconds.

2.3.2 Experiment 2: Hematoma Volume Assessment in ICI

There was no significant difference in hematoma volume between the infusion and control groups ($p = 0.6424$, Figure 2-5). There was also no significant difference in weight loss between the groups ($p = 0.0918$, Figure 2-6). There was no mortality in the assessment of hematoma volumes.

2.3.3 Experiment 3: Brain Water Content Assessment in ICI

There were three mortalities in Experiment 3. The first mortality was attributed to coprophagic asphyxiation (as determined by a post-mortem assessment guided by the suspicion that this may have been the cause of the observed blocked airway) after core probe implantation, but before the ICI procedure. The second mortality was attributed to surgical error during the ICI procedure. The last mortality was attributed to a fatal-sized hemorrhage after ICH procedure as determined by a pathology exam (this animal was in the control group). This mortality, although concerning, can be explained by the variable nature of the collagenase-ICH model being used.[105] Furthermore, temperature and activity data from one animal was not able to be collected due to

battery failure of the telemetry probe after implantation, although this animal's edema data was still included for assessment. Lastly, one ICI animal was excluded from ICPMS analysis due to suspected sample contamination during tissue processing (elemental concentrations were 5-15 x higher than average, and > 3 SD away from the mean). Overall, there were 29 animals included for brain water content assessment (n= 14 ICI, n= 15 control), 28 for temperature and activity (n= 14 in each of ICI and control groups), and 27 for ICPMS analysis (n= 13 ICI, n= 15 control).

There was no significant difference between ICI and control groups in brain water content at any of the regions of interest ($p = 0.8081$; Figure 2-8). There was an expected effect of stroke, exemplified by the significance seen between regions of interest ($p < 0.0001$). Also, temperature ($p = 0.5636$; Figure 2-9) and activity ($p = 0.6107$; Figure 2-10) were not significantly different between the groups during the 24-hour post-procedure time. Differences from baseline measurements observed are likely an anesthetic effect and an impact of the stroke, since the changes are approximately equal between the groups.

Elemental concentrations were determined by ICPMS, and calculated as parts per million per dry weight of sample (grams) (Figure 2-11). Data were analyzed by ANOVA, which showed no difference between groups for any region of interest ($p > 0.1$). However, there was a main effect of region (ipsilateral vs. contralateral striatum) for gadolinium (Figure 2-11 (A); $p < 0.0001$) and sodium (Figure 2-11 (B); $p < 0.0001$), with the ipsilateral striatum containing more of each element than the contralateral striatum. There was also a main effect of region for potassium (Figure 2-11 (C); $p <0.0001$), which was present in lower concentrations in the ipsilateral striatum than the contralateral striatum.

2.4 Discussion

This data suggests that chilled heparinized saline infused during the acute period of collagenase-ICH in rats is safe, although it does not reduce injury. Both hematoma volume and edema levels were similar between ICI-treated and control groups. Further, ICPMS measurements suggest no change to BBB integrity between the groups. Additional experimentation by Liddle *et al*.[2] found there were neither histological nor behavioral benefits associated with this cooling protocol (see publication for further details).

Descriptive observational data from Experiment 1 demonstrates that, although there is an overall average change in brain temperature during ICI infusion, the change in brain temperature is small and transient. Brain temperature returns to baseline levels immediately upon cessation of the infusion. We used 3 mL of room temperature saline over 20 minutes, as this is a clinically comparable dose.[186,213] Other researchers who used greater doses (colder, more volume, or faster infusion rates) have reported a larger drop in temperature of 2.4-7.5°C.[185,218–221] Although using colder saline could achieve these lower brain temperatures, it risks adverse effects like vasospasm. Additionally, 3 mL was calculated to be a reasonable and comparable infusion volume in a rat, in relation to what is safe and feasible as a human-equivalent volume to infuse through a narrow catheter and avoid volume overload (see Section 2.2.2.1).[186] Colder, higher volume, and higher infusion rates should be considered with caution for these reasons.

Hematoma assessment from Experiment 2 shows that ICI does not increase hematoma size in this model during a time of active bleeding. This is important because hematoma size is the main predictor of outcome following ICH.[31] Therefore, ICI appears to be safe when applied in a setting of active bleeding. This is a positive outcome, but it is contradictory to our expectation, because the saline was heparinized to prevent the catheter from being blocked. One

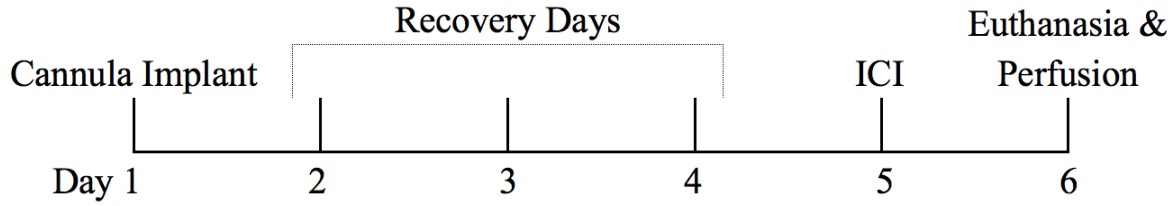
possible explanation for the lack of effect on hematoma volume is that the severity of the stroke was inappropriate. However, the hematoma volumes in this study are similar to those in other recent studies from our lab, suggesting that the lack of effect seen here is not due to the insult being either too large or too small.[224,227,229] To investigate this unexpected outcome further, Experiment 3 was implemented. In Experiment 2, hematoma volume was assessed with an assay that measures hemoglobin concentration within brain tissue. We wanted to check if the blood of the ICI group was diluted by the saline, and therefore underestimating the blood volume, as determined by hemoglobin amounts. Experiment 3 showed no difference between the ICI and control groups in brain water content or post-procedure temperature or activity change. These findings suggest that the similarities between groups in Experiment 2 were not confounded by diluted blood in the ICI group. Additionally, analysis of elemental concentrations within both ipsilateral and contralateral striata found no difference in levels between the ICI and control groups. This provides further evidence that ICI is safe to use during a time of active bleeding in the brain, and does not change elemental concentrations within tissue. Notably, there were higher concentrations of gadolinium and sodium in the ipsilateral striata (seen in both groups), which is indicative of BBB damage, edema, and ion dyshomeostasis caused by the hematoma.[224] Although other methods of measuring BBB integrity exist (e.g., Evans Blue assay) and may yield different results, gadolinium has also been used as an acceptable screening test.[224] Conversely, there were lower concentrations of potassium in the ipsilateral striatum of both groups. Lower levels of potassium in the ipsilateral striatum are indicative of edema in that area.[230] These results replicate findings from previous experiments measuring these elements in ICH.[224] Because the differences found were consistent between the groups, we can conclude that ICI does not affect these elemental concentrations on a regional level.

Here, we use a scaled dose from what is believed to be clinically achievable (in terms of temperature and infusion rate) to test the safety of ICI when active bleeding is present. Previous research has shown the benefit of ICI in ischemic injury models, but their dosing regimens should be critically considered for clinical feasibility, as mentioned previously.[185,190,218–221] Additionally, ischemic injury can undergo hemorrhagic transformation, and studying ICI therapy in a setting of active bleeding is necessary to inform hemorrhagic scenarios. To our knowledge, this is the first study examining the safety of ICI that includes a no-infusion control group in an active bleeding setting. Considered together, the data from these experiments suggest that ICI is safe to use during a state of active bleeding within the brain. Further investigation into the potential benefits of ICI for ICH, as well as effects using an ischemic model with hemorrhagic transformation, is needed. Selective cooling and extent of regional wash-out are benefits of ICI in ischemia, and the details of these effects in ICH should be determined.

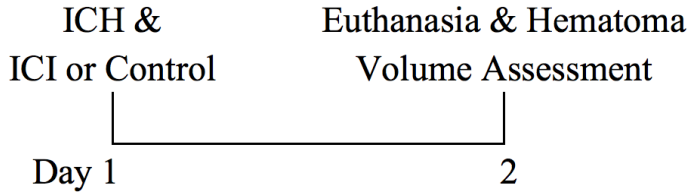
2.5 Conclusions

We show that using a clinically feasible regimen of ICI during a time of active bleeding is safe. This finding was not expected, as the saline used as infusate was heparinized to prevent blockage of the catheter, and was expected to create coagulopathy and potentially more bleeding. Although the temperature effect in the brain was modest, ICI did not worsen bleeding, brain water content, behavioral activity levels, or change elemental concentrations compared to a no-infusion control group. Future investigations are needed to determine if and how ICI may be beneficial for ICH.

A-Exp. 1



B-Exp. 2



C-Exp. 3

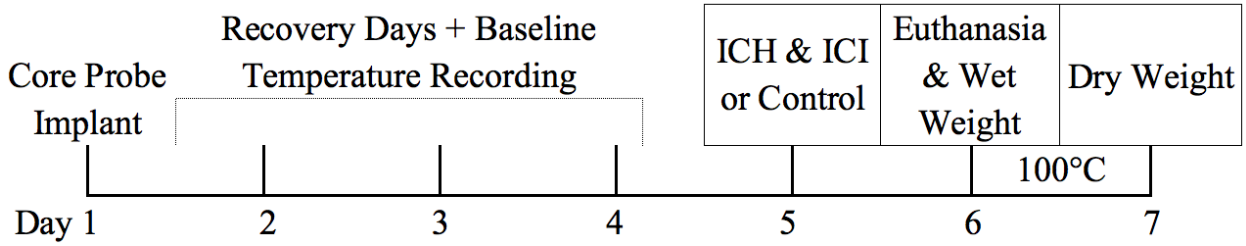


Figure. 2-1. (A-Exp. 1) Timeline of Experiment 1. **(B-Exp. 2)** Timeline of Experiment 2. **(C-Exp. 3)** Timeline of Experiment 3.

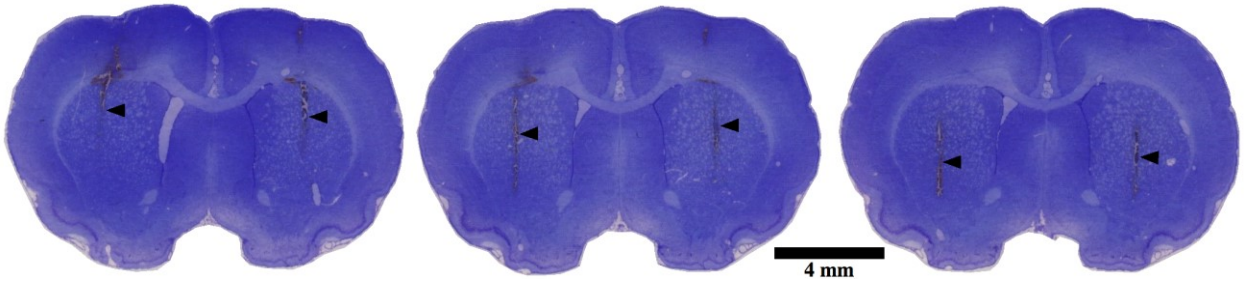


Figure. 2-2. (Exp. 1) Two thermocouple probes were lowered into the striatal areas of the brain to measure temperature from both the ipsilateral (side of cooling) and contralateral cortices during ICI induction in naïve animals. These three slices (40um thick; 120um between each slice shown in figure; Cresyl violet stained) show where the thermocouple probe needles were recording from during the procedure. Black arrow heads indicate where the needle tracts are.

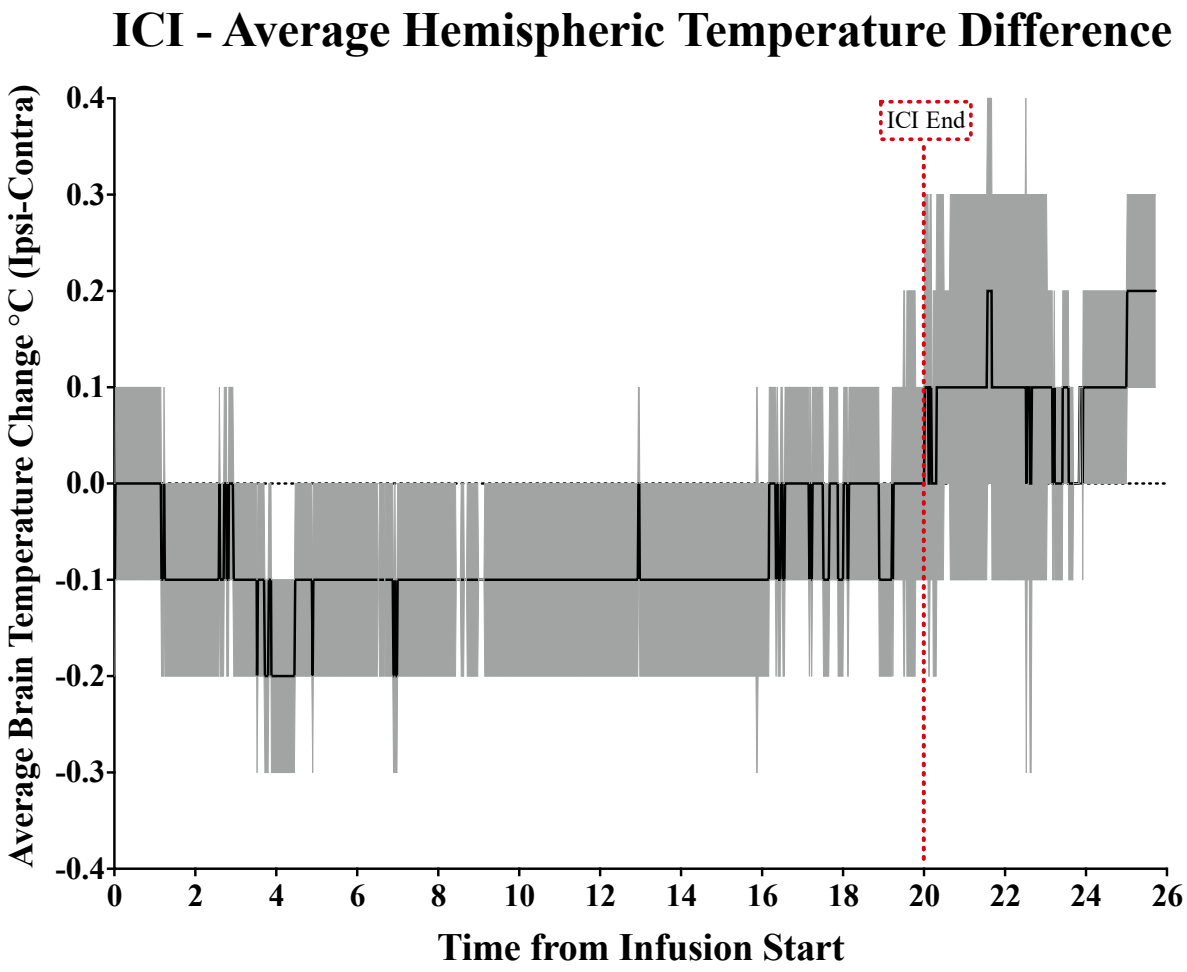


Figure. 2-3. (Exp. 1) Change in brain temperature (°C) averaged over seven animals during ICI procedure. Corrected for baseline temperature. The black line represents the average temperature change difference from baseline values, using the contralateral hemisphere to the infusion side as an internal control across animals [(ipsilateral hemisphere) – (contralateral hemisphere)] over time with continuous sampling. The gray area represents the 95% confidence interval for all points.

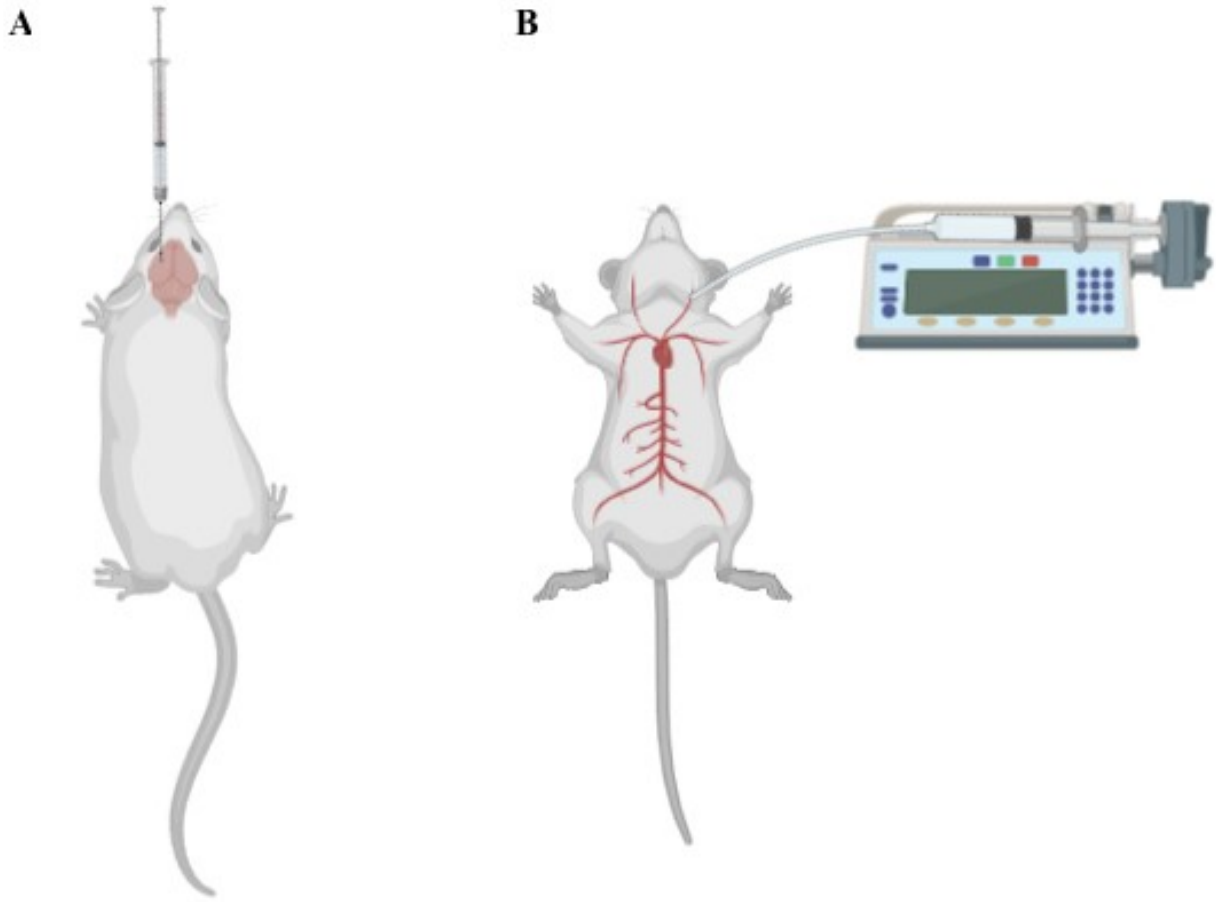


Figure. 2-4. (Exp. 2) Representative animal orientation when receiving ICH induction (A) and when undergoing ICI procedure (B). Collagenase is infused while the animal is in a stereotaxic frame. ICI is given on the same side the animal was given an ICH. (Images made through biorender.com)

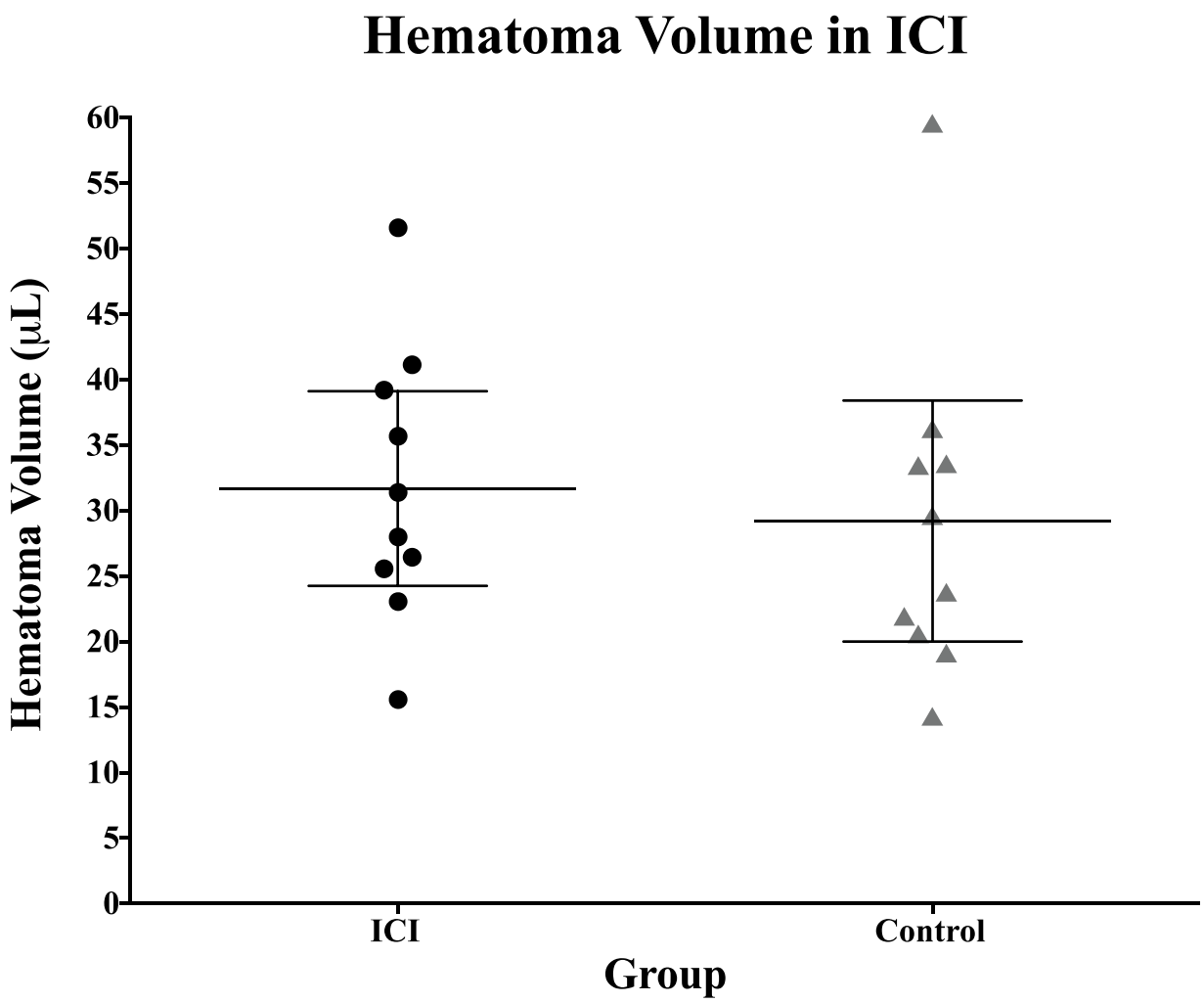


Figure. 2-5. (Exp. 2) Hematoma volume assessments measured with a spectrophotometric Drabkin's-reaction assay. Bars are mean with 95% confidence interval. There was no significant difference between the infusion and control groups hematoma volumes (p = 0.6424).

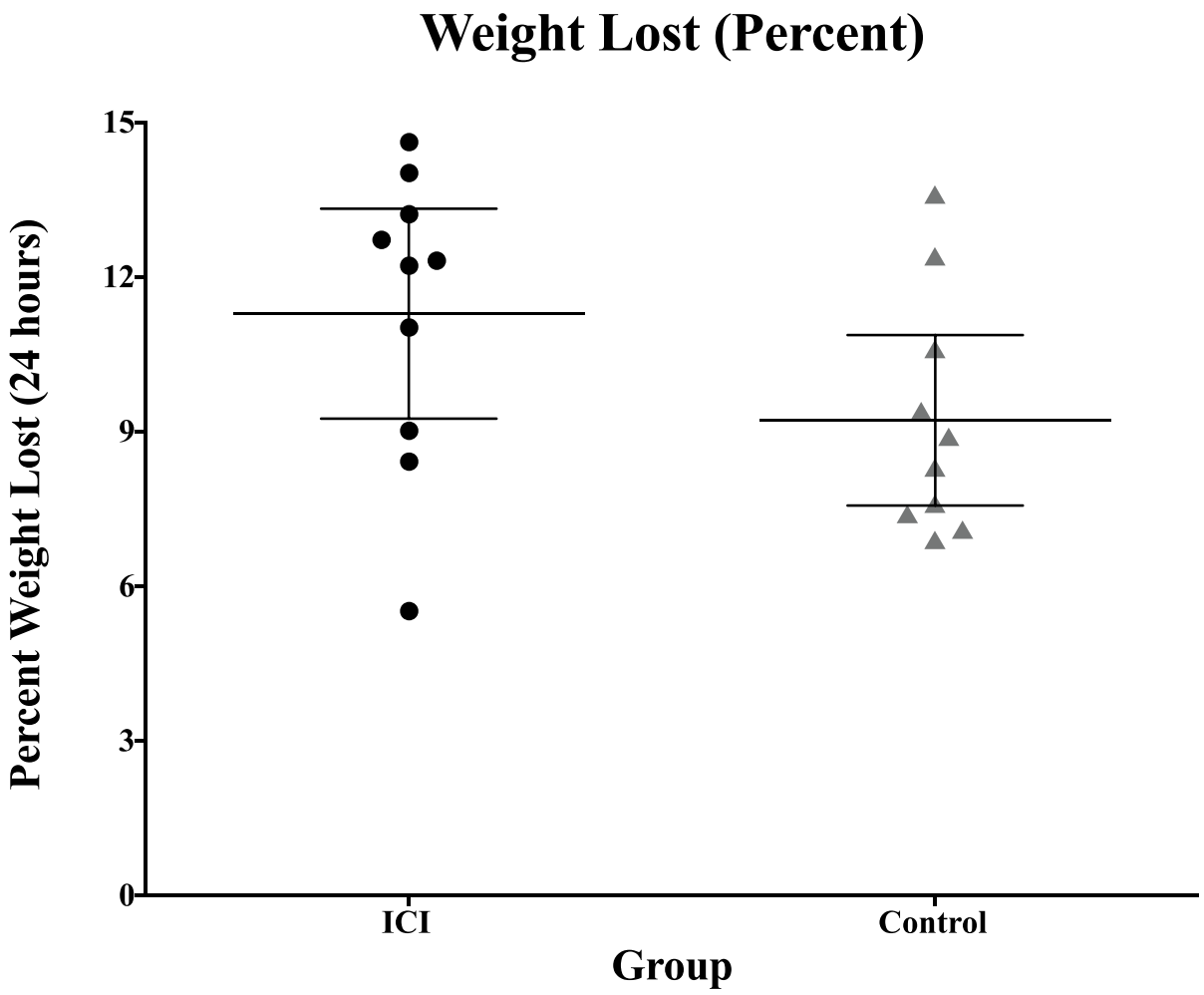


Figure. 2-6. (Exp. 2) Weight loss in ICI, presented as percent loss from time of ICI procedure to time of euthanasia. [1- (weight at euthanasia/surgery weight)]. Bars represent the mean with 95% confidence interval. There was no significant difference in weight loss between the groups (p = 0.0918).

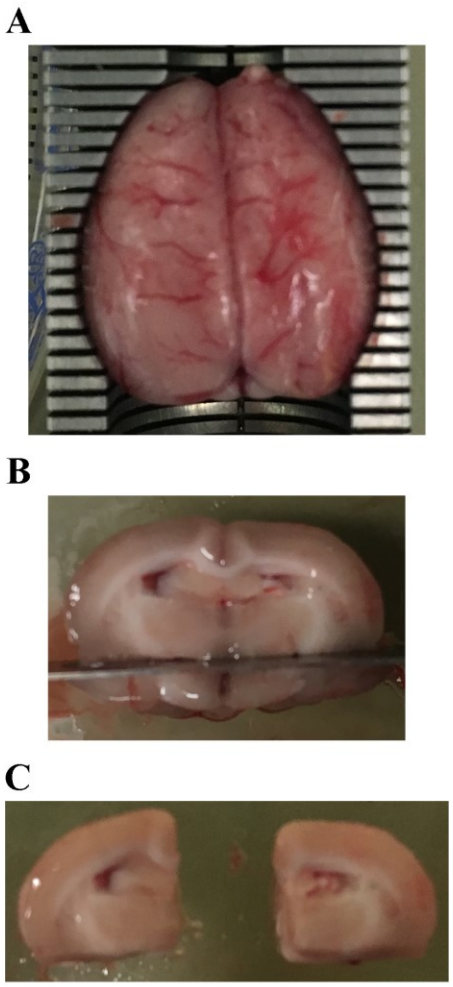


Figure. 2-7. (Exp. 3) Striatal dissection stages.

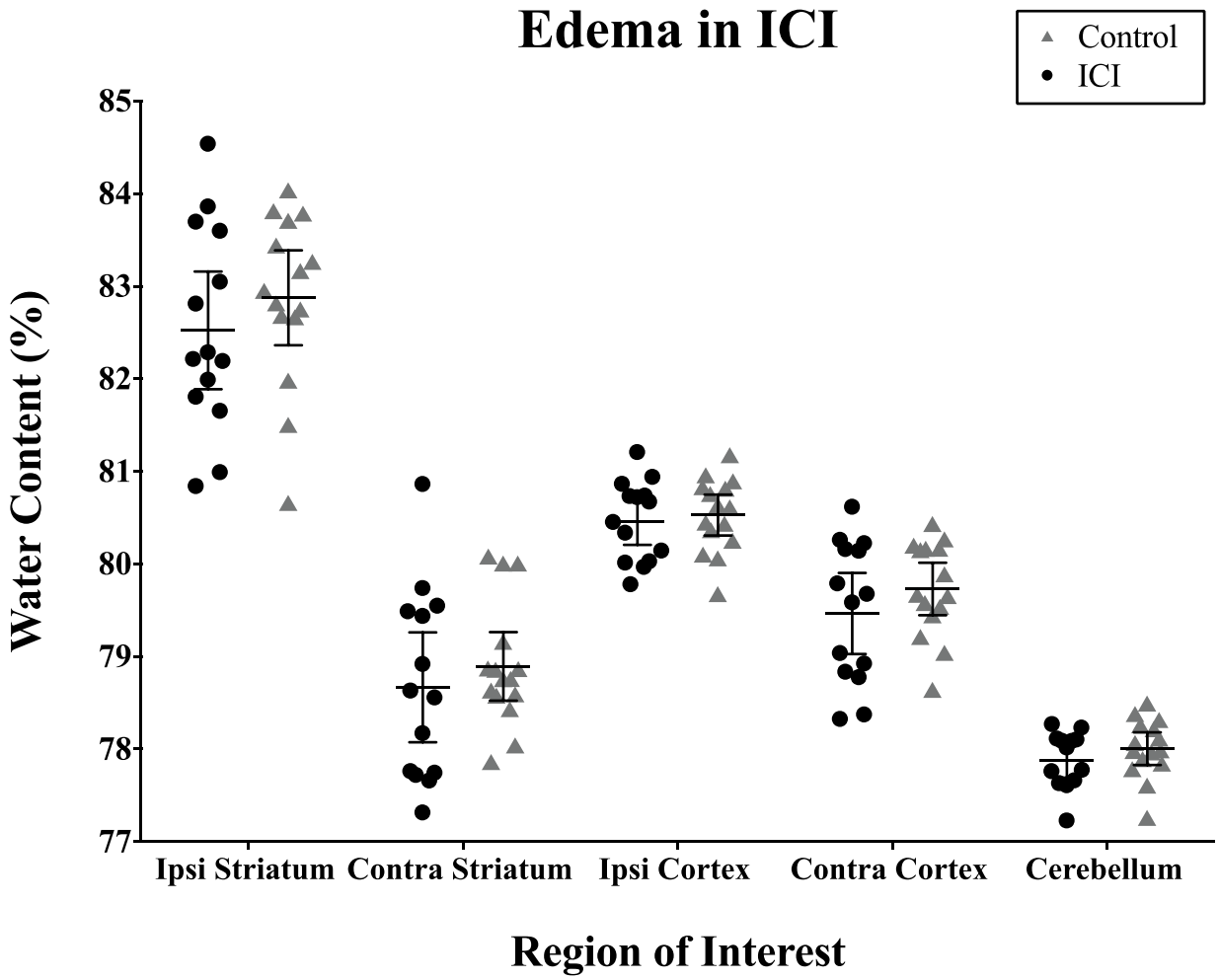


Figure. 2-8. (Exp. 3) Percent water content (indicative of edema) assessment in ICI compared to control groups (no infusion). Bars are mean with 95% confidence interval. There is no significant difference between groups at any region of interest. There was an effect of stroke, exemplified by the significance seen between regions of interest ($p < 0.0001$).

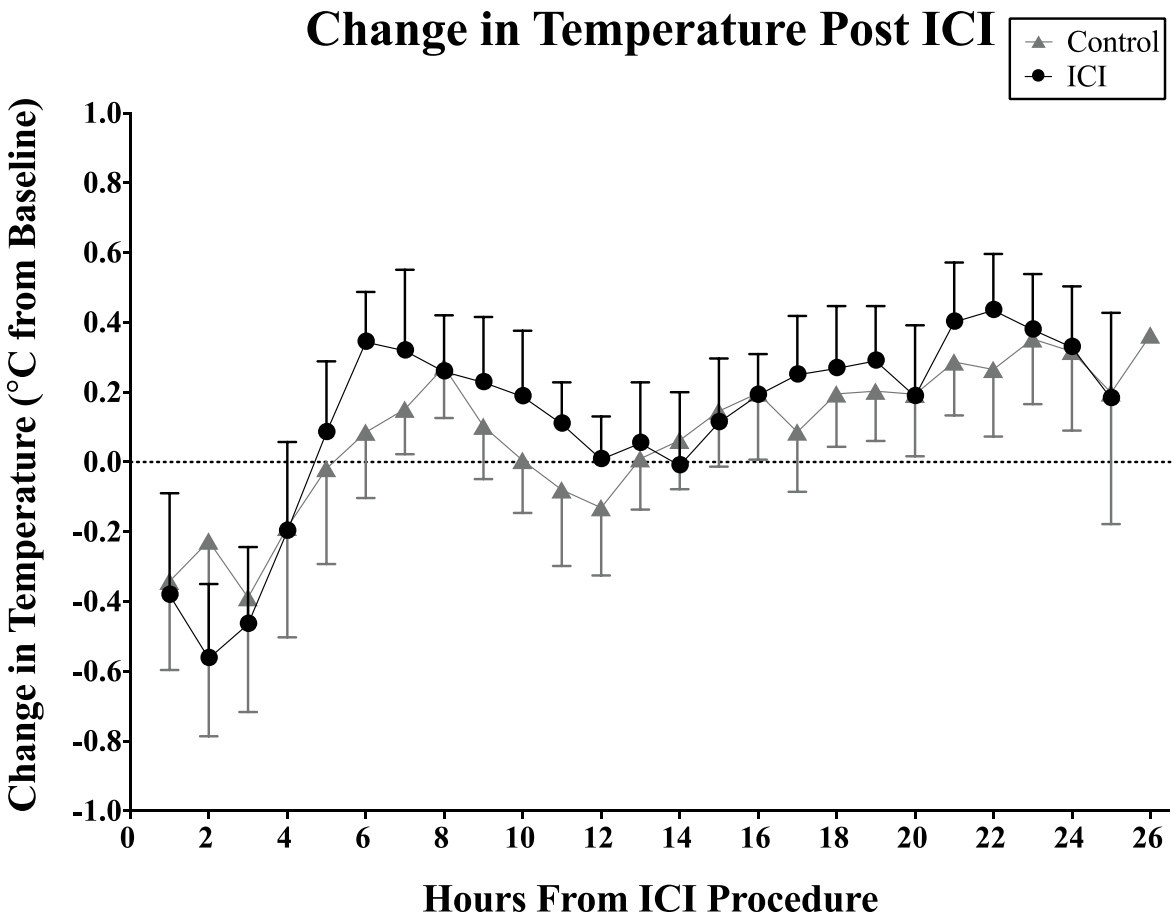


Figure. 2-9. (Exp. 3) Symbols represent the mean, and the bars represent the 95 % confidence interval. Change in animals' core temperature (°C) compared to baseline measurements between ICI and control (no infusion) groups. A positive value means that the groups were, on average, warmer than their pre-procedure temperatures, when corrected for circadian rhythms. Changes seen are likely an anesthetic effect and an impact of the stroke, since the changes are approximately equal between the groups ($p = 0.5636$).

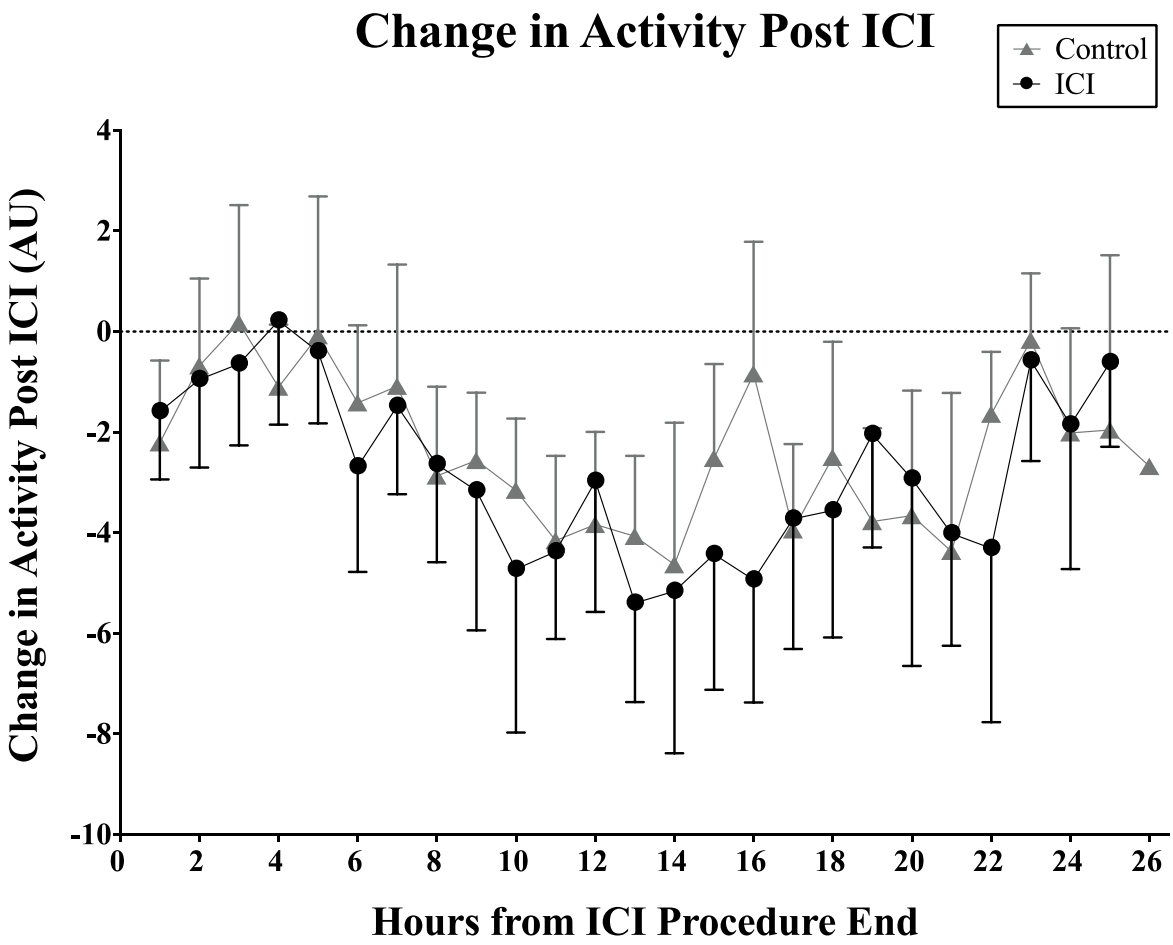
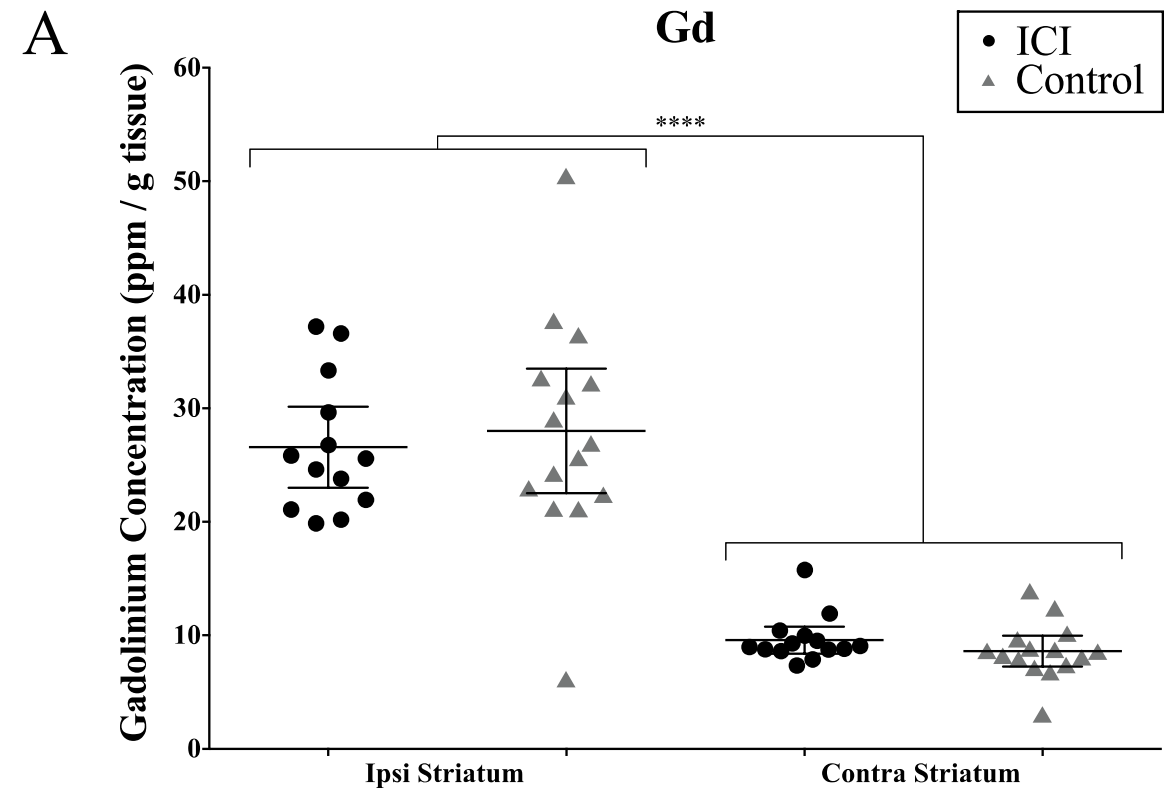
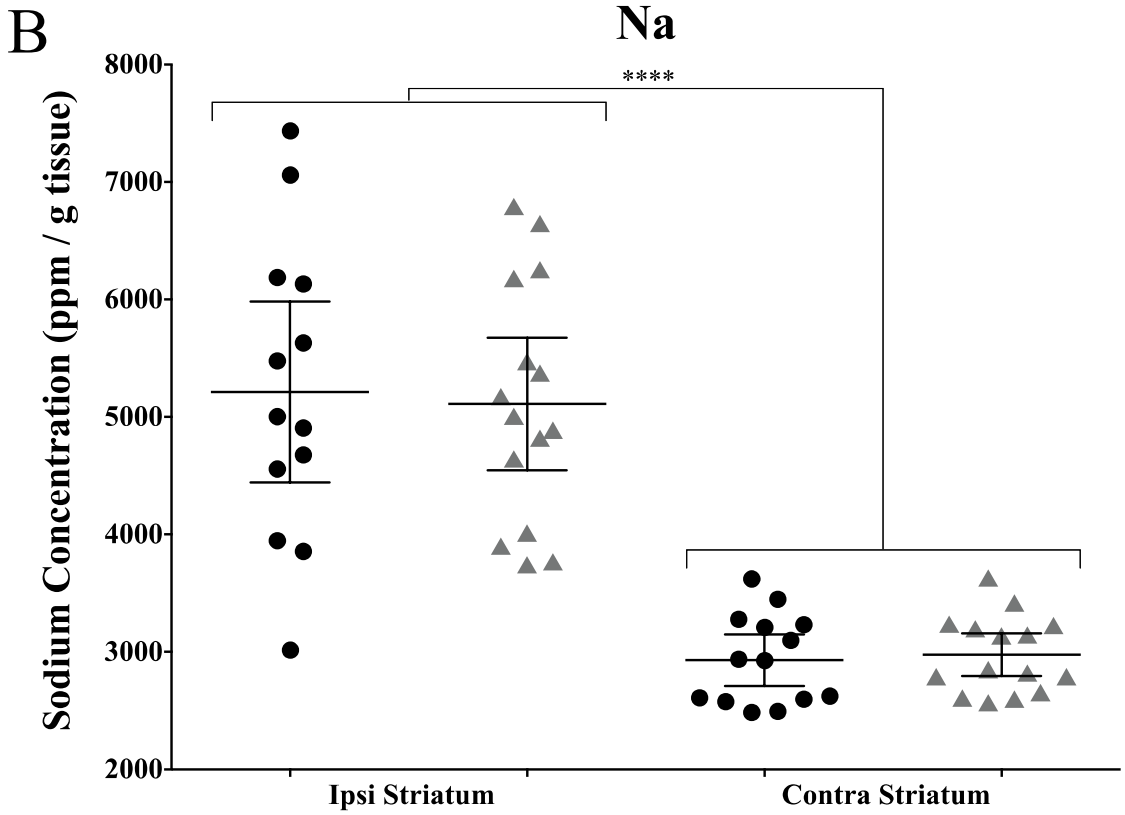


Figure. 2-10. (Exp. 3) Change in animals' activity post-ICI compared to baseline measurements in arbitrary units (AU; determined from detecting transmitter movements over the receiver). Symbols represent the average, and the bars represent the 95% confidence interval. This is calculated by taking the difference between the post-ICI activity and the pre-ICI activity, matched for circadian rhythm. A negative value indicated that activity of the group decreased after the ICI procedure, compared to the pre-procedure measurements. Like temperature above, changes here are likely an anesthetic effect and an impact of the stroke, since the changes are approximately equal between the groups ($p = 0.6107$).


A
Gd
ICI
Control
****
Gadolinium Concentration (ppm / g tissue)
60
50
40
30
20
10
0
Ipsi Striatum
Contra Striatum


B
Na
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Sodium Concentration (ppm / g tissue)
8000
7000
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4000
3000
2000
Ipsi Striatum
Contra Striatum

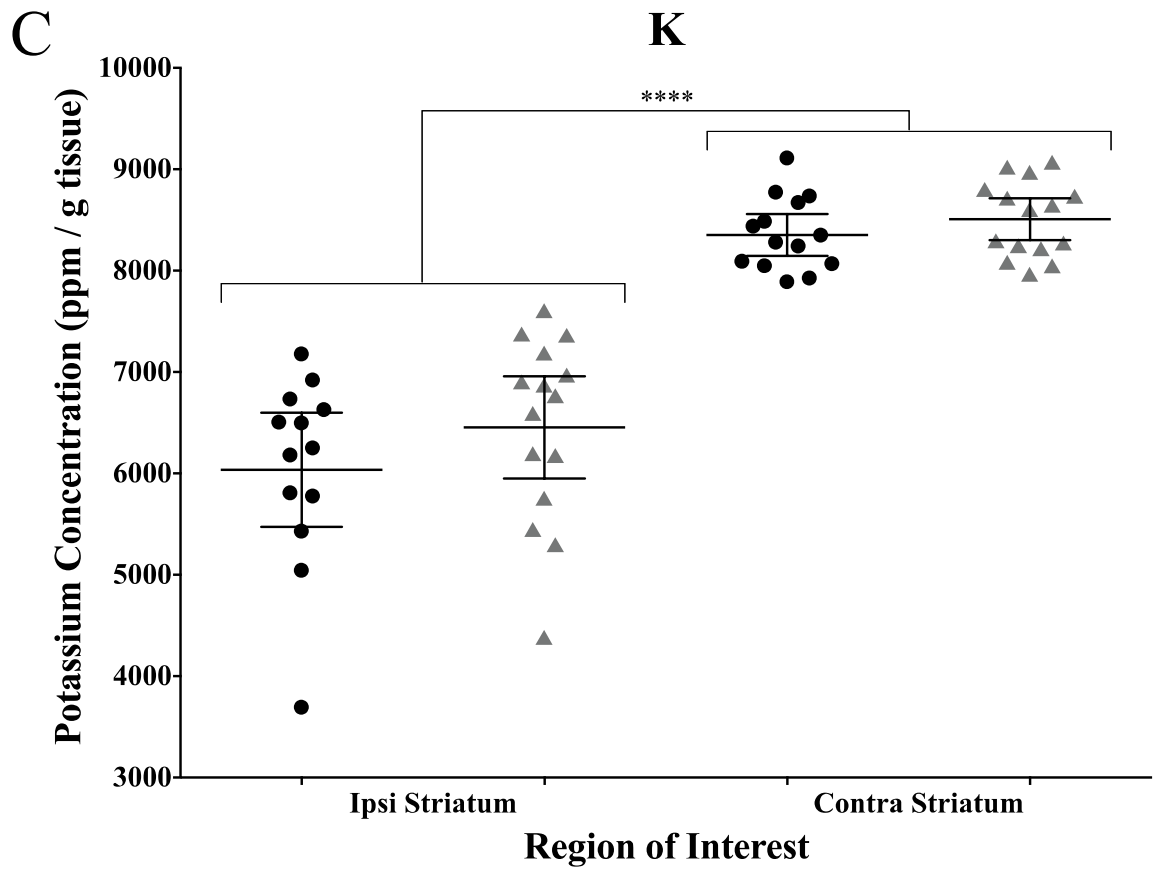


Figure. 2-11. (Exp. 3) ICPMS results of elemental concentration between ICI and control groups, between ipsilateral and contralateral striatum's. Values are calculated in units of parts per million (ppm) per dry weight of tissue sample in grams (g). Bars represent the average, with 95% confidence intervals. There was no difference between ICI and control groups at any region of interest for any element, considering the data both with and without outliers. There was a significant difference between ipsilateral and contralateral regions of interest within each type of element analyzed here (****$p < 0.0001$), exemplifying a main effect of stroke on these elemental changes.

CHAPTER 3: Investigating Late Re-Bleeding in Intracerebral Hemorrhage

3.1 Introduction

The rupturing of blood vessels that is characteristic of ICH leads to BBB dysfunction, which is suspected to increase susceptibility for continued leaking of blood into brain tissue.[46] Breakdown of this barrier provides areas for blood to enter brain tissue, furthering injury and prolonging recovery.[31] This can be due to leaking or a re-bleed from the main damaged vessel, or smaller vessels surrounding the primary injury site that have lost structural integrity. This potential for ongoing or late re-bleeding is a concern, because the main predictor of patient outcome is the size of the bleed (hematoma).[31,58,231] Early hematoma expansion occurs in approximately 30% of patients. Hematoma expansion is the addition of any amount of blood into brain parenchyma past the initial bleed. Hematoma expansion is seen most often within the first 3 hours, but can also appear at times 6 hours or later.[58,77] Detecting late bleeding can be made more difficult if clinicians and researchers are not looking for it, or if blood is entering already damaged areas, or if there is ongoing hematoma resolution that may offset the appearance of new blood.

One factor that could contribute to this secondary hematoma expansion is coagulopathy. Coagulopathy can be induced when temperatures drop, as in TH (see Section 1.3.3), or from anticoagulant medication use.[1,196] Warfarin is one of the top five most commonly prescribed anticoagulant medications, and is used to prevent and secondarily control risk for thromboembolisms.[232] Patients who experience an ICH when on these medications have larger hematomas, and are more likely to have hematoma expansion and worse outcomes.[21,67,69,233,234] These medications are stopped when a hemorrhagic event occurs, and no consensus has been

determined about when it is safe to restart these patients on these medications to prevent an ischemic insult, since this decision relies heavily on individual circumstances of the patient. Some guidelines say that some form of treatment can be instituted within the first 72 hours after ICH, whereas others suggest waiting longer than 4 months; all stress the dependence on the patient's individual circumstances.[235–238]

It is difficult to establish if this late bleeding regularly occurs after ICH in animal models commonly used, such as rodents. Although clinical assessments of re-bleeding use computed tomography (CT) or magnetic resonance imaging (MRI)[239–241], these methods pose logistical difficulties in use with animals, as they would need to be anesthetized, introducing potential confounds to bleeding, like changes to BP.[242] Considering this, animal researchers can turn to easily implemented assays to assess bleeding post-mortem.[226] However, if late bleeding occurs, it may be from bleeds that are too small to easily detect with routine pre-clinical experimental methods. For example, microbleeds in rodents are difficult to detect. Microbleeds are clinically defined through neuroimaging as lesions with a size of 2-10 mm.[243–246] Considering the brain volume of male rats are ~0.047% of male human brain volumes (~599 mm^3 for rats[247], and ~1273.6 cm^3 for humans[248]), a microbleed in a rat would be substantially smaller and therefore harder to detect accurately, especially with the methods available to pre-clinical researchers. A physiological state of decreased coagulation could be used to increase the amount of blood leakage (without causing spontaneous bleeds), and therefore increase the likelihood of detecting smaller bleeds using these assays. One way of doing this is to use the anticoagulation drug warfarin, which could prolong any ongoing bleeding, making any small bleeds easier to detect.[249] Warfarin is a common and affordable vitamin K inhibitor. This medication is used for decreasing coagulation in patients that have a predisposition for clotting complications that can lead to an

ischemic episode. By using an anticoagulant medication at a time when bleeding is assumed to have stopped, detection of late bleeding will be enhanced.

As mentioned above, one of the many effects of TH is coagulopathy. A previous study investigating TH in ICH found that about 40% of rats cooled for a week, rather than 1-3 days, had a four-fold increase in hematoma volume.[1] The current study investigates the effect of coagulopathy alone on hematoma expansion at later times, when coagulation is assumed to have stopped active bleeding in a rat model. This approach will help to explain the role coagulopathy may have had in the results from the previous pre-clinical study.[1]

First, the dose of warfarin required needs to be validated to ensure that it is producing a sufficient level of anticoagulation, without causing spontaneous bleeding itself, which has been previously seen.[30,250] Once a dose is found to be safe and valid for the needs of this study, the dosing regimen can be applied in a post-ICH setting to study late bleeding. This study compromises on quantifying how much bleeding would be naturally occurring, to allow us to simply detect it. An infusion of collagenase is used here to surgically induce an ICH. This model of ICH involves injury to multiple blood vessels. Compared to the AWB model, the collagenase model produces a larger primary injury, more distal injury, and has slower degradation.[105] This injury model is the best option for studying the possibility of late bleeding, because without the severity and nature of the injury it provides, it is unlikely that late bleeding would be severe enough to detect.

Taking previous research findings of late bleeding in TH into consideration[1], we hypothesized that late-onset coagulopathy, an isolated factor of TH, would increase hematoma size assessed 1 week after ICH. The finding of a larger hematoma size in a late-onset anticoagulated group would indicate that late bleeding is occurring regularly in a rat animal

model of ICH, but at normally undetectable levels. An alternative outcome is that hematoma size could be unchanged between anti- and normal coagulation groups. This alternative outcome would indicate that factors other than, or in addition to, coagulopathy likely play a larger role in the late bleeding seen with TH.

3.2 Methods

3.2.1 Subjects

All protocols followed the Canadian Council of Animal Care Guidelines and were approved by the Biosciences Animal Care and Use Committee at the University of Alberta. A total of 44 male, Sprague Dawley rats were used for Experiments 1 and 2. All of Experiment 1 animals, and 12 animals for Experiment 2, were obtained from Charles River Laboratories (250-275 g; age ~7 weeks). The other 20 animals used in Experiment 2 were obtained from the University of Alberta's Science Animal Support Services (SASS) colony (250-300 g; age ~7 weeks; Charles River origin). This change in supplier was due to a cessation of SASS's breeding colony program. Animals spent a minimum of 7 days acclimating from transport from Charles River, and a minimum of 5 days acclimating from the University of Alberta's Science Animal Support Services colony before undergoing any procedures or being transferred to single housing. All animals were individually housed for the duration of the experiments with ad libitum access to food and water. Housing rooms were temperature, humidity, and light (12-hour light–12-hour dark cycle) controlled. Randomization into warfarin and saline treated groups was determined using a set of red and black playing cards, shuffled, and pulled randomly at the time of the first dosing (24 hours post-surgery).

3.2.2 Warfarin Administration

A 0.4 mg/kg loading dose was used on day 1, starting 24 hours following collagenase induced ICH. Timing was chosen based on previous TH work in our lab that showed cooling before 24 hours, using either local or systemic cooling, increased bleeding.[1,137,195] On each of days 2-6, a dose of 0.25 mg/kg was given every 24 hours to maintain an anticoagulated state. This maintenance dose was chosen by consulting previous literature, an verified with a pilot dosing control study (see appendix).[251–255] Warfarin sodium (Sigma-Aldrich, product code PHR1435, Lot# LRAA2743) was mixed into dH_2O at a concentration of 3.5 mg/mL. Saline at proportional volumes was used for the control group to try mimic the salty taste of the sodium in the warfarin. The solutions were separately mixed into cookie dough (Pillsbury 'Ready to Bake' sugar cookies) for oral consumption. This cookie dough was given to the rats in the days prior to the animal receiving an ICH to avoid neophobic reactions post-ICH. All oral doses were given on a tray placed on the floor of the animal's cage. The animals consumed these voluntarily within ~1-10 minutes under experimenter observation to ensure complete consumption. This route of administration was chosen to minimize stress (e.g., raised BP associated with gavage procedures) and remove the need for anesthetic use, both of which could introduce confounds to the experiments.[256,257]

3.2.2.1 Experiment 1: Dose Validation

A total of 12 animals were used for this phase of the study, with 6 in each of the warfarin treated and control (saline) groups. Figure 3-1(A) shows the experimental timeline. Every 24 hours, the animals were weighed to calculate and prepare accurate doses (described above). Approximately 6 hours after the last dose was consumed, animals were anesthetized and a tail

bleed assessment completed. The core temperature of the animal was maintained at ~37°C with a heated water pad and a removable electric heating pad. The tip of the tail was transected 2 mm from the tip, and a timer started. Blotting (filter, Whatman #1) paper was used to blot the incision every 10 seconds to absorb new blood. Bleeding was considered to have stopped when two consecutive blots showed no additional blood, and the timer was stopped at this point. The maximum duration permitted was 30 minutes.

Animals were euthanized on day 6 of dosing, after the tail bleed assessment (~6 hours after the last dose), and brains were collected for blood volume spectrophotometric analysis.[2,105,226,227] Animals were anesthetized with 4% isoflurane and quickly decapitated (<30 seconds after removal from anesthetic). Brain hemispheres were immediately separated into ipsilesional, contralesional, and the cerebellum. These were each weighed, and proportional amounts of dH_2O were added to create a homogenate with a ratio of brain tissue to water of 1:4. After being left on ice for 7 minutes, this mixture was aliquoted into centrifuge tubes and spun (15800 g for 35 minutes). The supernatant (100 µL) was mixed with Drabkin's solution (600 µL, Sigma-Aldrich) for spectrophotometric analysis of hemoglobin content (540 nm wavelength).

3.2.2.2 Experiment 2: Late Bleeding Assessment

A total of 32 animals were used for this experiment. On day 0, an ICH was induced using an infusion of collagenase (1 µL of 0.6 U/µL, Sigma-Aldrich, type 4-S Lot# SLBL855) into the striatum (see Figure 3-1(B) for timeline).[2,92,224,225] Rats were anesthetized with isoflurane (4 % induction, 1.5-2.5 % maintenance in 60 % N_2O and remainder O_2). A rectal temperature probe connected to a heated water pad, along with a removable electric heating pad, maintained core temperature at ~37°C throughout the surgical procedure. An incision was made at the midline on

the scalp (~2.5 cm), and a burr hole was drilled (3.5 mm right and 0.05 mm anterior to Bregma). A 26 G Hamilton syringe (10 μL) was lowered 6.5 mm below the surface of the skull into the striatal area. The collagenase was infused over 5 minutes, with a 5-minute 'wait-time' following before the needle was slowly removed to prevent backflow. A solid metal screw (stainless steel, plain finish, binding head, slotted drive, 1/8" length, #0-80 threads) was inserted into the burr hole. Surgical staples were used to close the incision site, and a few drops of Bupivacaine hydrochloride (5 mg/mL, Pfizer Canada) were applied to the site for local pain management.

Every 24 hours, the animals were weighed to calculate and prepare accurate doses (as described above). Approximately 6 hours after the last dose was consumed, animals were anesthetized and a tail bleed assessment was completed as described above. For this, the animal's body temperature was monitored by a rectal probe and maintained (including tail) at ~37°C with a heated water pad and a removable electric heating pad. Diameter of the blood blot taken was measured at each 5-minute time mark as a quantitative measurement of the amount of bleeding occurring over time. Animals were euthanized on day 6 after collagenase injection, after the tail bleed assessment, and brains were collected for blood volume spectrophotometric analysis, as described above.

3.2.3 Statistical Analysis

T-test analyses were completed using GraphPad Prism (version 6.0c) software. Data are presented as mean with 95% confidence intervals. All significance levels are set at $p< 0.05$. Whole brain blood volume, hematoma volume, and tail bleed times were each assessed with a one-way, unpaired t-test with Welch's correction. Blot size over time was analyzed with a 2-way repeated measures ANOVA using Stata (IC 13.1) software. This ANOVA was followed up with

an analysis of the simple effects of the significant factors identified in the ANOVA.

3.3 Results

There were no mortalities nor violations of protocol in either experiment.

3.3.1 Experiment 1: Dose Validation

There was a statistically significant difference in tail bleed times between the warfarin treated and control group in otherwise naïve animals ($p = 0.0243$; Figure 3-2). Warfarin animals bled for 1.5x longer than the control group, reaching the maximum ethical time limit allowed for this procedure of 30 minutes (i.e. a ceiling effect). There was no significant difference in whole brain blood volume between the warfarin treated and control groups in naïve animals ($p = 0.2817$; Figure 3-3).

3.3.2 Experiment 2: Late Bleeding Assessment

There was a statistically significant difference in tail bleed times of animals with ICH between the warfarin treated and control group ($p = 0.0084$; Figure 3-4). One animal from each of the warfarin and saline treated groups had tail bleed times less than 5 minutes, so the 2-way repeated-measure ANOVA on blot diameter was completed with 15 animals per group (instead of the 16-total included in tail bleed time analyses, given diameter was measured once every 5 minutes starting at the 5-minute mark). There was a main effect of treatment ($p = 0.0325$), with no other significant outcomes for size of tail bleed blot (Figure 3-5). The simple effects analysis of treatment (at each time interval) revealed treatment to have the most prominent effect at the 15-minute measurement time ($p = 0.0130$). All other times, except at 20-minutes ($p = 0.1386$),

were trending towards significance ($0.06 < p < 0.09$). There was no significant difference in hematoma volume between the warfarin treated and the control group ($p = 0.3905$; Figure 3-6); however, the power to detect small effects is limited here.

3.4 Discussion

Warfarin increases tail bleed time by at least 1.5x—an indication of an anticoagulated state. We could not detect bleeding times longer than 30 minutes in this experiment, as 30 minutes is the maximum time ethically allowed in this procedure. However, no difference was seen in whole brain blood volume between the warfarin and control groups. This indicates that there was no spontaneous bleeding caused by warfarin in these naïve (no ICH) animals. Therefore, the dosing regimen used (0.4 mg/kg loading dose on day 1, and 0.25 mg/kg given every 24 hours on days 2-6) induced a state of anticoagulation without causing spontaneous hemorrhaging, and was determined to be safe. Following this dosing validation, the regimen was applied in Experiment 2 of this study to increase any late bleeding that was already occurring. If bleeding was occurring, the warfarin should theoretically improve the detectability of these (potentially micro-sized) bleeds. In short, this dosing procedure was used to determine if late bleeding occurs in the rat animal model of ICH used, specifically due to an anticoagulated state alone.

Analysis of the tail bleed data in Experiment 2 shows that warfarin significantly increased the duration of bleeding, as well as revealed significance and trends over time for the amount of bleeding occurring (blot diameter over time) compared to saline-treated control animals. Together, these are indications that warfarin is successfully inducing an anticoagulated state, matching findings from previous research at comparable doses.[251,253–255] For example, one

group also measuring tail bleed time found a warfarin-dose-to-bleeding-time curve estimating a 0.25 mg/kg/day dose to correlate with a tail bleed time approximately double that of the control group.[251] This group also followed a maximum of 30-minutes allowed for the tail bleed test. However, analysis of the hematoma volume results here shows that, despite the increased bleed time and amount, warfarin-treated animals did not have differently-sized hematomas. Therefore, it would seem that anticoagulation, as an isolated factor of TH, is not affecting late re-bleeding in this model of ICH. In other words, clotting dysfunction 24-hours after induced ICH does not increase brain bleeding in this model.

Considering that it is well known that anti-coagulant medications used during a time of known active bleeding produce larger bleeds, we would expect any late bleeding to be enhanced.[21,67,69,233] Therefore, because the hematoma sizes were not different between the treatment and control groups, this suggests that there is no late bleeding occurring in the collagenase model used here.

Although our results indicate that anticoagulation alone does not cause late re-bleeding, we cannot rule out the possibility that late bleeding would occur under different conditions, or with multiple factors acting together. For example, higher doses of medication, a larger hematoma, a different model of ICH induction, high BP, or co-morbidities like diabetes could all produce different late bleeding results. Dysfunction of the BBB caused by ICH leads to susceptible tissue, which when physiologically strained, could lead to further damage.[224] Increased BP is common in ICH patients, and hypertension is the leading cause of ICH. Furthermore, post-ICH interventions such as rehabilitation have been shown to be harmful if started too early.[258] This may be due to increases in BP from physical activity during rehabilitation leading to clot failure and bleeding into the brain. However, when our lab

investigated this in the same animal model of ICH described here, we found modest changes in BP due to routine lab assessments and no effect on hematoma volume (L. Liddle, R. Reinders, B. Prokop, C. Nadeau, & F. Colbourne, unpublished data). These results should be considered with caution, as the animals used were young and healthy. A model more realistic to clinical presentations, such as hypertensive or other comorbid animals, should be used in the future.

As mentioned earlier, hypothermia causes numerous physiological changes that have the possibility to cause re-bleeding in a hemorrhagic setting.[1] Some of these changes include increases in BP[175], vasodynamic changes[196], and decreased coagulation[174,200–203]. Increases in BP and/or vasotonic changes (i.e., a narrowing or widening of vessel diameter that is closing off the damaged area) could induce clot failure and secondary bleeding in damaged and vulnerable blood vessels. Compounding this, a state of anticoagulation would increase the amount of any bleeding. An investigation of raised BP was attempted as a follow-up to the experiment presented here. A previous pilot study by K. Dietrich of our lab had no success using injected phenylephrine under anesthetic to produce sustained BP increases in rats. Phenylephrine produced a large abrupt change in BP, with quick regression to baseline (within ~2 minutes), despite continued treatment and progressively raised doses. In another pilot study (n=2) by myself and F. Colbourne, midodrine hydrochloride (a vasopressor) was orally administered twice daily (~3.5-4.5 hours apart) at various doses (0.167, 1, 2, 3, 4, 5, and 10 mg/kg). The oral dosing procedure was the same as the warfarin administration procedure described above. Animals were monitored, and BP was recorded. None of the midodrine doses showed temporally sustained increases in BP (see appendix). The increases in BP produced by midodrine were no longer than about 30-60 minutes. Furthermore, the 10 mg/kg dose quickly produced signs of toxicity (e.g., piloerection, shivering, porphyrin) in the rats, and was immediately discontinued (no

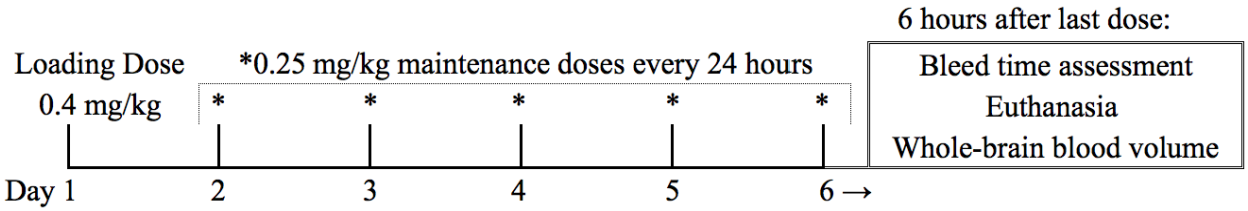
mortalities). Therefore, the effect of BP on late bleeding could not be studied using this method. The logistical difficulties and the poor responses to these pharmacological methods of manipulating BP makes it difficult to study these factors. Future investigations of these factors should consider using a spontaneously hypertensive rat animal model to study the effect of BP, and its combination with anticoagulation, on late re-bleeding. Furthermore, the results of clinical studies that have found that interventions to lower BP had little to no impact on outcome should be taken into account when planning future studies of mitigating this damage.[23,42]

Following this, investigations into the treatment of these complications should be pursued. Possibilities include substances such as milrinone (a vasodilator). Milrinone has been shown to be protective against rewarming shock (acidosis), afterdrop (when warm blood shunts to the periphery and cold blood to the core), rebound vasodilation, and vascular dysfunction associated with the rewarming phase of TH.[259,260] Although milrinone has the potential to be beneficial in protecting the body from these adverse rewarming effects, it may have harmful effects if its vasodilating properties induce clot failure. After a clot forms and closes off a leaking vessel, changes to the size of that vessel could potentially dislodge that clot, causing another bleed. Full investigation of the help or harm of this type of substance is important for our understanding and improvement of patient care. These studies could also inform researchers on the validity of experimental animal models used reflect clinical outcomes. This knowledge is important for gauging risk of side effects, in addition to telling us how well a treatment may work. It is important to persevere with these studies, as they could inform decisions on timing and methods of patient care, and to avoid critical times where there is a risk of aggravating damage.

3.5 Conclusions

Anticoagulation alone does not cause larger hematoma sizes 24-hours post-ICH. By extension, this suggest that late bleeding is not regularly occurring in a rat animal model of ICH. Of the factors involved in TH, coagulopathy alone does not appear to be the explanation for previous studies that found TH to increase bleed size.[1] Future studies are needed to determine which factors, or combinations thereof, can cause the re-bleeding complications seen in previous work.[1] This knowledge will help in patient care decisions and avoiding situations that worsen patient outcome.

A



B

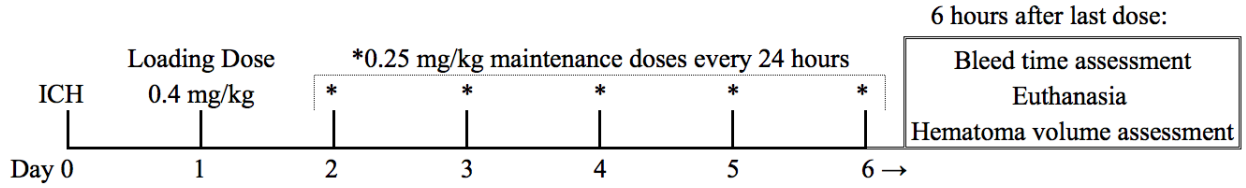


Figure 3-1. (A-Exp. 1) Experimental timeline for dose validation. **(B-Exp. 2)** Experimental timeline for late bleeding assessment.

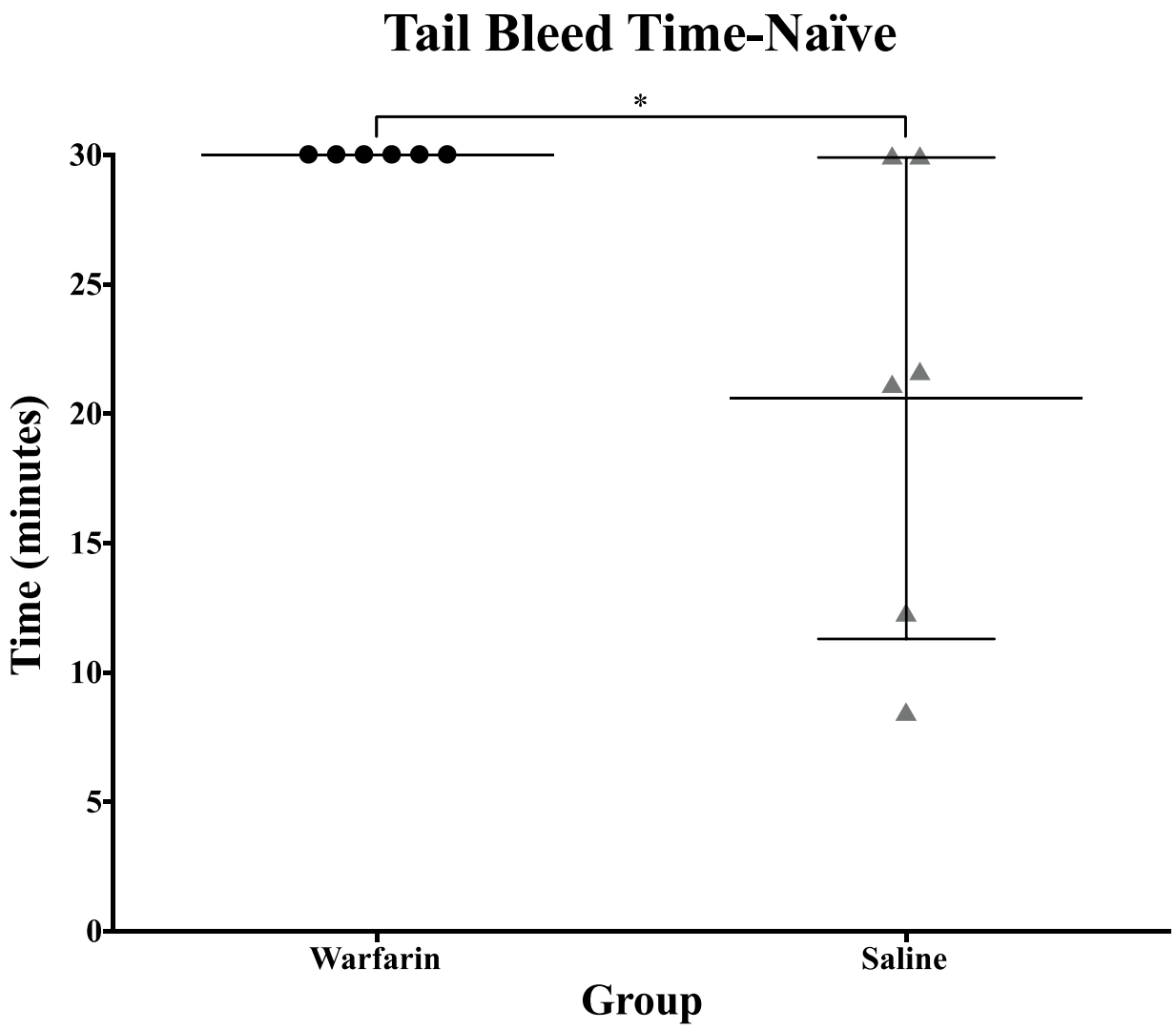


Figure 3-2. (Exp. 1) Tail bleed time assessment of coagulopathy between warfarin treated (0.4 mg/kg loading dose, 0.25 mg/kg maintenance dose every 24 hours) and control (saline) groups in naïve animals (* $p = 0.0243$). Bars are average with 95% confidence interval. These data demonstrate that warfarin effectively increased time to clotting.

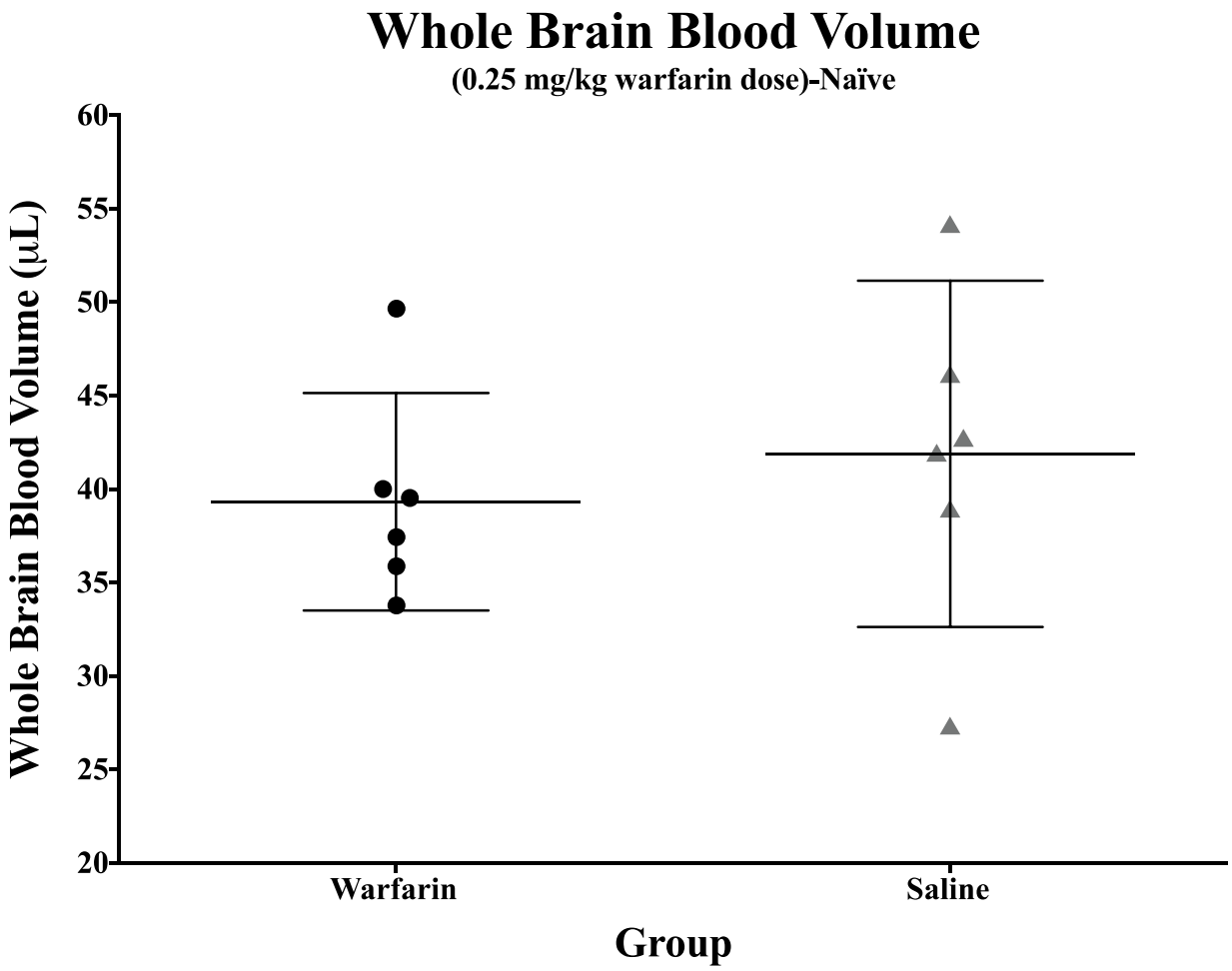


Figure 3-3. (Exp. 1) Whole brain blood volume (µL) of naïve animals. Comparison between warfarin treated (0.4 mg/kg loading dose, 0.25 mg/kg maintenance dose every 24 hours) and control (saline) groups (p = 0.2817). Bars are average with 95% confidence interval. These data demonstrate that warfarin did not cause increased bleeding in the brain.

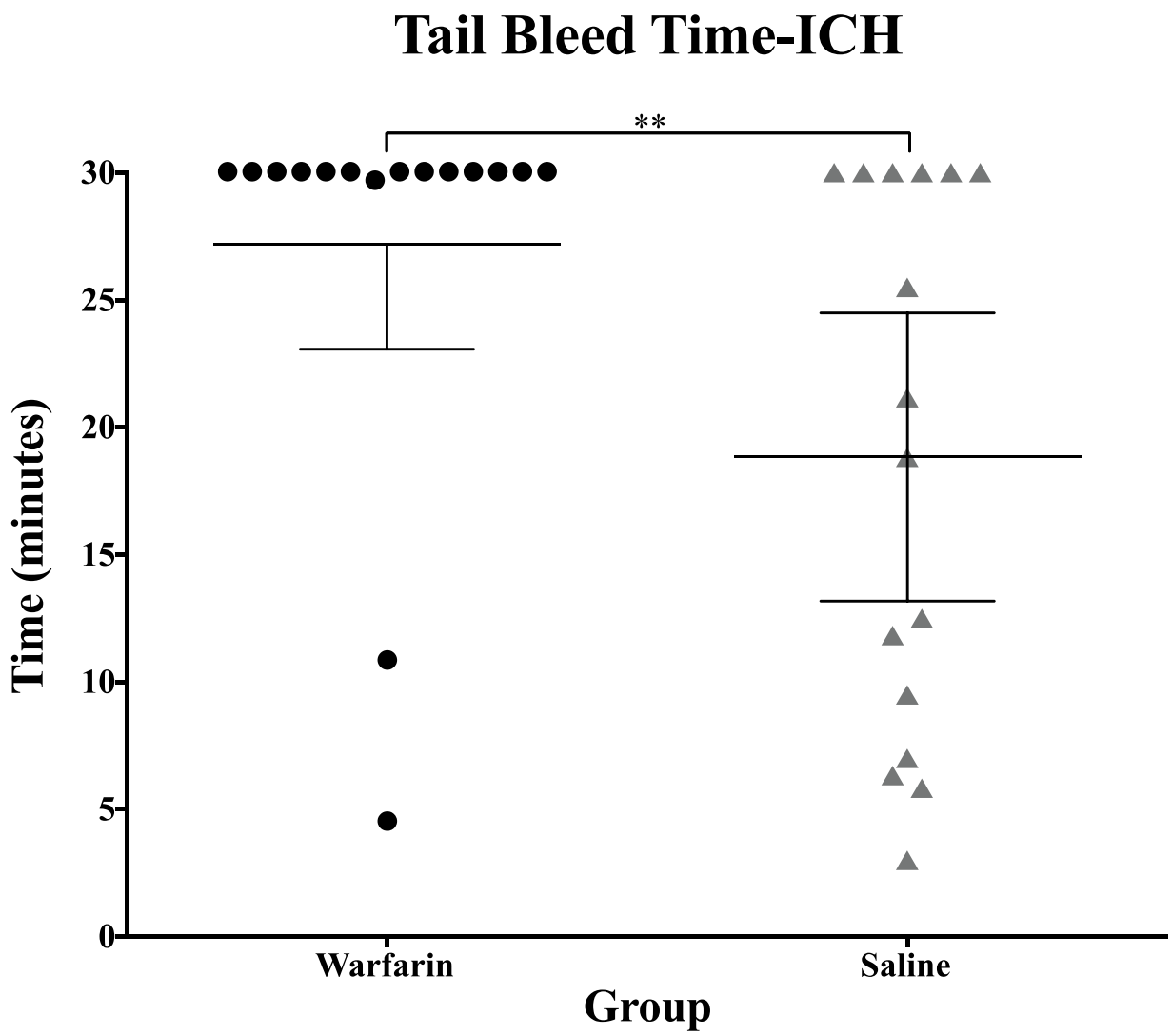


Figure 3-4. (Exp. 2) Tail bleed time assessment of coagulopathy between warfarin treated (0.4 mg/kg loading dose, 0.25 mg/kg maintenance dose every 24 hours) and control (saline) groups. Maximum time allowed for this test is 30 minutes. Bars are average with 95% confidence interval (**p = 0.0084). There was a significant difference between the groups, demonstrating the effectiveness of warfarin to increase time to clotting.

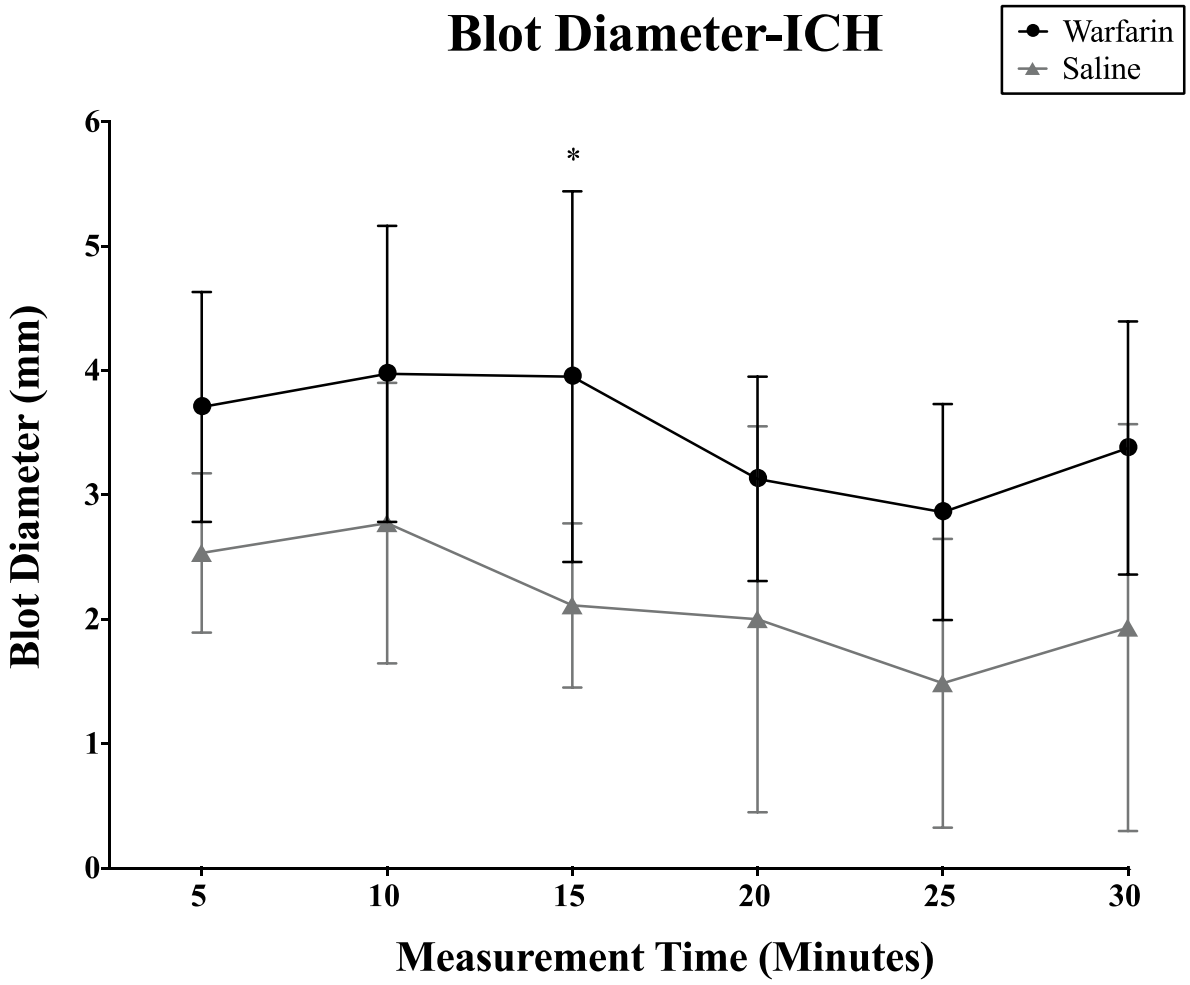


Figure 3-5. (Exp. 2) Size of tail bleed blot, over time, between warfarin treated (0.4 mg/kg loading dose, 0.25 mg/kg maintenance dose every 24 hours) and control (saline) groups. Symbols represents the average, and bars show the 95% confidence interval. There was a main effect of treatment ($p = 0.0325$). Simple effects analysis of treatment (at each time interval) showed treatment to have the most prominent effect at the 15-minute measurement time (*$p = 0.0130$). All other times, except at 20-minutes ($p = 0.1386$), were trending towards significance ($p < 0.09$). These data demonstrate that warfarin increased the amount of bleeding (not only the time of bleeding).

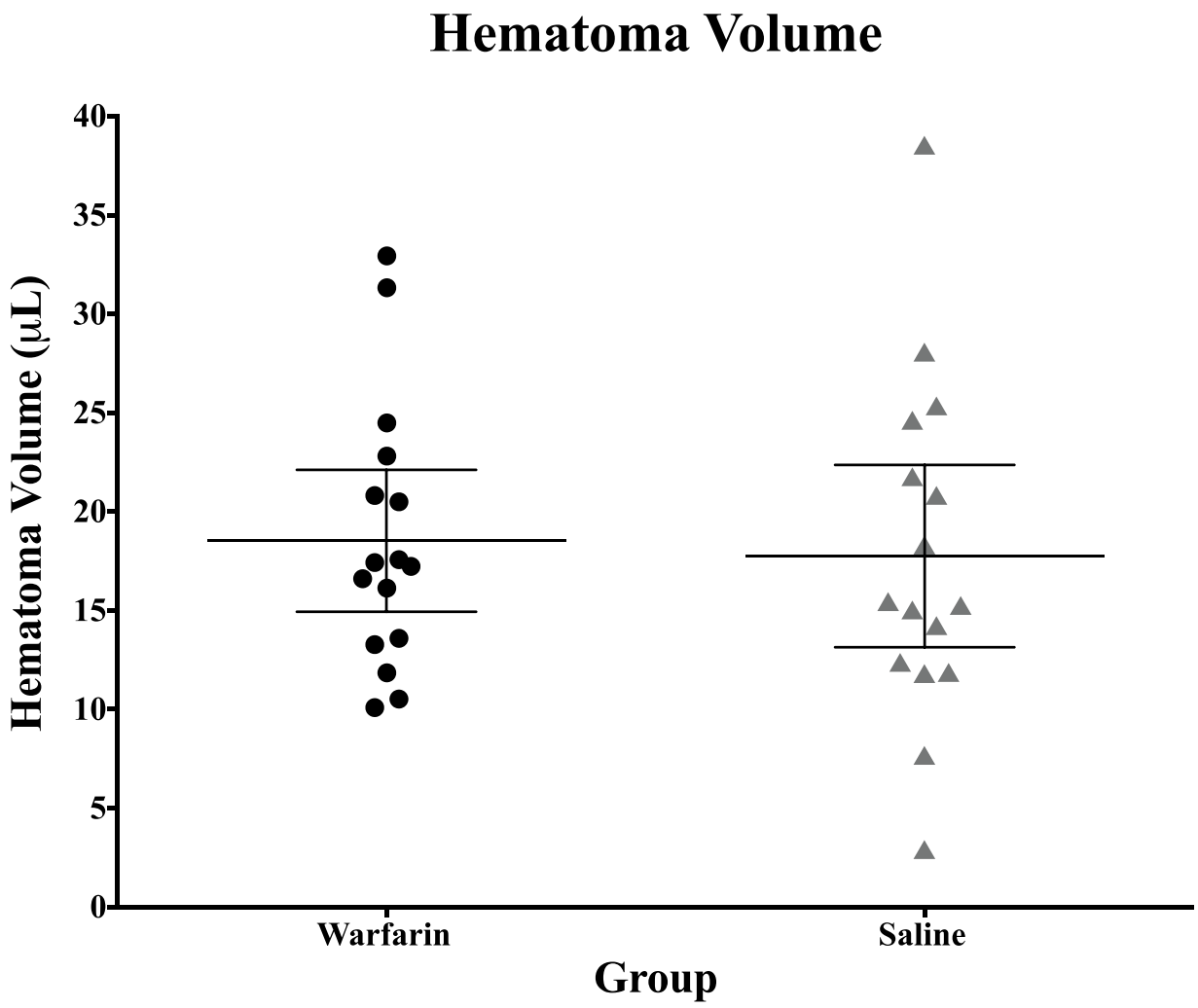


Figure 3-6. (Exp. 2) Hematoma volume (µL) between warfarin treated (0.4 mg/kg loading dose, 0.25 mg/kg maintenance dose every 24 hours) and control (saline) groups. Bars are average with 95% confidence interval. There was no significant difference between the groups (p= 0.3905). These data demonstrate that warfarin did not increase the amount of bleeding within the brain, suggesting that bleeding after the initial hematoma formation is unlikely to be occurring.

CHAPTER 4: Hypothermia: Impact on Plasticity Following Stroke

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This chapter is a review paper that has been published, with irrelevant sections removed.[3] Sections not included here are those which focus on injury profiles not consistent with the theme for this thesis (i.e. not stroke). Kalisvaart ACJ, Prokop BJ, Colbourne F (2019). Hypothermia: Impact on plasticity following brain injury. Brain Circulation 5(4)169-178

4.1 Introduction

Therapeutic hypothermia (TH) counteracts many deleterious mechanisms of ischemic and hemorrhagic brain injuries, and there is considerable preclinical evidence that this leads to meaningful reductions in brain damage with concomitantly better recovery.[117] Although clinical evidence of efficacy is less impressive and sometimes contradictory, there is still much interest in the potential of TH.[209–211] However, several problems must be resolved before that goal is fully realized. First, mechanisms of injury vary between different types of stroke (see Chapter 1), which may necessitate different approaches and treatment parameters, such as depth, duration, and delay in order to get optimal neuroprotection. Second, side effects have long complicated the use of TH, both by reducing the potential benefits of TH and increasing the risk of poor outcomes. For example, cooling increases the risk of infection and can cause coagulopathy. These systemic complications have been extensively studied in an attempt to avoid them or find effective management strategies, thereby optimizing patient outcomes. For instance, using brain-selective cooling can avoid many systemic complications.

As discussed in Chapter 1, TH has many adverse effects. Here, we review another potential side effect of TH - the possibility that TH will adversely affect neuroplasticity, thereby delaying or weakening neurological and functional recovery. Such deleterious effects are possible with TH, especially given its broad-spectrum effects, which could directly impact neural repair processes that spatially and temporally overlap with mechanisms of injury.[261–263] For instance, neuronal metabolism is dampened by TH, which is advantageous for mitigating brain injury; however, this same mechanism may weaken neuroplastic changes that require heightened metabolic activity.[264,265] One can assume that the duration of TH affects this risk, with days of TH potentially having the greatest chance of impeding repair compared to brief cooling. The

same concerns apply to the depth and the method of cooling. Nonetheless, even local application of mild TH may affect brain regions vital to neural repair (e.g., peri-infarct cortex), with likely greater impact if deeper levels of cooling are achieved.

Despite these concerns, only a few of the TH efficacy studies done in animals have considered such risks. This chapter explores the literature on brain repair when TH has been applied following ischemia and intracerebral hemorrhage (ICH). No clinical studies have directly assessed whether TH impacts neuroplasticity, only whether cooling improves the final outcome or a specific mechanism of injury (e.g., raised ICP). Therefore, these studies do not exclude the possibility that TH may have had harmful effects on brain repair.

4.2 Method of Article Collection

Primary collection for articles reviewed here were found using the University of Alberta's Library journal article search engine and Google Scholar. Main terms were used in a variety of combinations. These included "hypothermia", "neuroplasticity", "hibernation", "neurogenesis", "brain-derived neurotrophic factor," "plasticity", "cooling", "therapeutic cooling," "therapeutic hypothermia", "angiogenesis", "synaptogenesis", "neuronal sprouting", "dendritic branching", "BrdU", "doublecortin", "golgi stain", and "targeted temperature management" in conjunction with the pathologies of interest, with English, full text and peer reviewed being requirements. Following this, we explored references within these and other readings that were deemed applicable. We also identified articles from our knowledge of this research area.

4.3 Hypothermia and Neuroprotection

Cooling can impact all mechanisms of injury either directly or indirectly, depending upon the treatment parameters used (depth, duration, etc.). For instance, TH has been shown to mitigate excitotoxicity, oxidative stress, BBB breakdown, and inflammation, to name a few.[130–132] Many of these are triggers and/or are essential for plasticity responses that are key to behavioral recovery. Thus, TH may directly impact repair by impeding such processes (e.g., glutamatergic neurotransmission).[266] Indirect effects are also expected. For example, a highly neuroprotective cooling protocol would likely diminish the need for repair. Alternatively, the rescue of tissue can promote or alter the spatial pattern or timing of neuroplasticity, such as by saving enough of a circuit to enable further repair.

4.4 Plasticity Following Brain Injury

Neuroplasticity refers to molecular and anatomical modifications that are driven as a result of aging, experiences, hormones, drugs, and disease or injury to the brain. The mechanisms that enact these plastic changes, such as synaptogenesis, neurogenesis, and angiogenesis, are anatomical alterations underwritten by various molecular processes and cascades (e.g. growth factors) that are subsequently shaped and refined by internal or external factors.[261,267–270] Kolb and Gibb, Murphy and Corbett, and Carmichael comprehensively review this information on the mechanisms of plasticity.[261,271,272]

The extent of redundant connectivity within the central nervous system (CNS) provides a basis for reorganization and functional recovery following brain injury.[273] For example, imaging studies have established that corresponding contralesional brain regions are recruited to aid in behavioral recovery early after stroke. Later, as injury processes and inflammation resolves,

there is a shift of recruitment to diffuse ipsilesional areas surrounding the site of injury, at least for some stroke patients, illustrating the progression of plastic processes.[274] Similarly, traumatic brain injury patients demonstrate a wider cerebral activation pattern following their initial injury that gradually decreases in complexity as more efficient connections are made and recovery progresses.[275] These macroscopic changes are the result of a period of heightened synaptic malleability and neurogenesis following injury. Brain injury, especially stroke, induces peri-injury axonal sprouting through both alterations in the cellular environment, including upregulation of growth factors and genetic modifications favoring neuronal growth.[274] These new axonal connections, along with unmasked latent connections, are strengthened and refined through Hebbian processes, resulting in greater functional recovery.[275] Use of TH promotes neuroprotection and cellular sparing, possibly reducing the need for broad scale network recruitment while enabling restoration and retraining of salvaged tissue.

4.5 Effects of Hypothermia in Naïve Animals

There is substantial evidence that learning acquisition is heavily influenced by body temperature, with significant reductions in core temperature resulting in poor performance in serial problem-solving tasks and spatial learning tasks, such as the Morris Water Maze.[276,277] Thus, it is natural to presume that the plastic processes underlying cognitive tasks and motor skill learning are also affected by changes in core body temperature. Klahr *et al*. recently subjected naïve rats to mild focal TH treatment (30°C -31°C) in the hemisphere contralateral to the preferred paw for 5 days, followed by 5 days of normothermia, and measured learning acquisition during a skilled reaching task throughout the 10-day treatment period.[278] They found no significant difference in learning acquisition rates between the TH and normothermic

conditions, and subsequent histological analysis did not show any difference in dendritic complexity.[278] In this case, the cooling depth was mild, but clinically relevant, and it is fortunate that this dose had no obvious effects on plasticity or behavior. Presumably, by extending the duration of mild cooling, the risk to plasticity is higher. However, as demonstrated in stroke sham-operated rats, application of mild focal TH to the motor cortex for 21 days at 32°C did not negatively affect subsequent behavior, dendritic morphology, or result in cortical injury.[176] Evidence from brain slice preparations suggest that mild hypothermia (30°C -36°C) maintains ionotropic glutamate receptor function due to the reduction in metabolic demand. However, deep hypothermia (below 18°C) results in suppression of long-term potentiation (LTP), the neuroplastic process by which efficiency of synaptic transmission is upregulated via coincident bursts of high frequency stimulation between neurons.[266,279] Taken with the behavioral data, this suggests that mild TH for long durations is not inherently harmful to natural plastic processes; however, given the risk of impairing LTP, caution should be taken when cooling to greater depths or durations. In addition, these limited studies are in naïve animals, and they do not include the study of other pharmacological agents that are often given concurrently with TH, which may potentially impact plastic processes. Finally, to fully understand the effect TH has on plasticity, we must consider brain injuries such as stroke, as TH may potentially change the ability of the brain to regulate repair processes, and must be evaluated.

4.6 Focal Ischemia

Most focal ischemia animal studies report positive outcomes following application of TH, with robust reductions in lesion volume and significant improvement in behavior.[114,130,280] However, clinical studies using TH following focal ischemia are limited, and have mixed

evidence in terms of efficacy.[131] To ensure better translational success, we must also consider that factors important to neuroprotection such as cooling duration, depth, intervention delay, and cooling/rewarming rates may ultimately harm plasticity. Although the effect of these factors on neuroprotection is well-studied preclinically, the same cannot be said for plasticity. The optimal duration of TH following focal ischemia seems to depend on treatment delay and injury severity; overall, mild cooling for longer durations results in better outcomes, even when delayed, although not everyone agrees.[126,168,281–289] For example, Clark *et al.* demonstrated that initiation of 48 hours of mild systemic TH 1 hour following permanent middle cerebral artery occlusion resulted in improved behavioral scores and lesion volume compared to 12- and 24-hour durations.[280] In general, mild cooling for longer (up to a few days) appears to provide the best results; however, recent evidence suggests that this may depend upon the cell type that is assessed.[168] Mild cooling is widely used across preclinical and clinical studies due to a lower risk of adverse complications. Many have shown that cooling past approximately 32°C -34°C results in worse neuroprotection and outcome.[283,284,290,291] Therefore, caution should be taken when cooling to both a greater depth and duration as this increases the risk of complications, interference in repair processes, and impeding plasticity.

Clinical studies typically use systemic whole-body cooling due to challenges in achieving target temperature with focal cooling methods and the longer duration required to achieve equivalent protection.[292] It is difficult to deal with the complexities of inducing hypothermia in clinical practice. For example, the recent EuroHYP-1 trial ran into several issues, such as the logistical challenge of administering such a complex treatment for a full 24 hours. This trial was unfortunately discontinued due to these issues and funding expiration.[284] In addition, there is concern that systemic cooling may affect the overlapping temporal and spatial processes of

neuroprotection and plasticity occurring in the ipsilesional versus contralesional hemispheres following the focal ischemic insult, and may therefore limit the extent of benefit. The neuroplastic involvement of the contralateral hemisphere is important following focal ischemia, as it is one of the sites of synaptogenesis and remapping of cortical areas, especially following severe injury.[272,293,294] Nonetheless, there is some concern of behavioral compensation and resulting contralesional plasticity limiting true recovery.[295] Thus, it is important to discern how cooling affects the contralesional hemisphere in order to best inform clinical treatment decisions. In this regard, focal cooling in rodents is advantageous, as it can be used to evaluate the effect of cooling on a single hemisphere or area without confound. Following a motor cortex devascularization model of focal ischemic stroke, Klahr *et al.* applied focal cooling to the contralesional hemisphere and found that cooling did not affect dendritic spine complexity or skilled reaching success.[278] In addition, early cooling of the contralateral hemisphere reduced reliance on the unimpaired limb, perhaps discouraging compensatory behaviors (learned nonuse).[278] With large lesions, or in cases where cooling is less effective (e.g., insufficient depth or duration), contralateral cooling may be harmful. This is because with little tissue left in the ipsilesional hemisphere to rely upon, compensatory behaviors and contralateral reorganization may be needed, which are heightened in the days following stroke.[296] Based on this work, it appears that contralateral cooling after a focal ischemic insult does not harm plasticity and could provide benefits in certain cases by limiting compensation. However, similar work in additional focal ischemia models and clinical studies should be done to make definitive conclusions.

4.7 Intracerebral Hemorrhage

Most preclinical and clinical work investigating TH for ICH has assessed the effects on edema, inflammation, BBB dysfunction, and the mitigation of functional deficits, with little focus on neuroplastic processes.[7,160,283] A recent meta-analysis of outcomes in ICH studies following application of TH demonstrated a significant positive impact on behavioral assessment.[6] Although this benefit is concurrent with a simultaneous decrease in edema and other neuroprotective factors, it is a rough indicator that TH is not directly harming plastic processes following ICH. Supporting this conclusion, Fingas *et al*. gave rats an ICH via autologous blood infusion and focally cooled on the ipsilesional side for either 12 hours, 3 days, or 6 days poststroke and found no significant group differences in behavior or lesion size.[136] If cooling does negatively affect plasticity, the expectation would be that groups who are cooled for longer would have worse behavioral performance than those cooled for a shorter duration. Although this is not a direct measure of plasticity, the behavioral improvement paired with a lack of change in histology does indicate that plastic processes are maintained regardless of focal cooling duration after ICH. Without preclinical data on plasticity following systemic cooling, it is difficult to definitively state whether TH has any adverse effects on plasticity; however, Staykov *et al.* have shown that prolonged systemic cooling (8-10 days) reduced mortality and improved patient outcome, indicating similar conclusions to preclinical data.[7] This was a small study and not a randomized control trial. Thus, further preclinical ICH studies that directly assess plasticity following TH are needed, especially if the goal of translating this therapy clinically is to become a reality.

4.8 Adjunct Therapies in Therapeutic Hypothermia

The use of pharmacological intervention in conjunction with TH differs between clinical and preclinical settings. Whereas sedative and anti-shivering agents like meperidine, propofol, midazolam, clonidine, and morphine are common in clinical care, preclinical studies rarely use any pharmacological intervention, despite the fact that these sedatives are crucial in mitigating the stress of cooling and improving patient recovery.[196,297–304] In preclinical studies, many use general anesthetics such as isoflurane, which are typically used during short durations of cooling. As clinical sedative agents are rarely used preclinically, their effect on neuroplasticity in the context of TH is unknown. Future clinical and preclinical studies should take sedative agents and their potential impact on recovery into account.

Although these pharmacological agents have not been studied in the context of TH, especially following brain injury, there has been some study on the plastic changes that these drugs induce independently. For example, one study investigating propofol demonstrated that it can decrease synapse number and alter dendritic spine density, morphology, length, and arborization patterns.[305] This disturbance in synaptic organization resulted in atypical behavioral activity up to 6 months later. In conjunction with other work showing the effect of propofol on dendritic spine densities, this demonstrates the potential for propofol to permanently impair neuronal morphology and function, especially in developing brains.[306,307] In contrast, midazolam seems to be beneficial, as it induces changes in the signaling of sleep-induced cortical plasticity by promoting factors involved in neurogenesis and cellular sparing, and has additional neuroprotective effects.[308] Similarly, analgesics like clonidine and morphine can prevent unwanted CNS secondary neuroplastic changes like central sensitization resulting from prolonged pain stimuli.[309–311] However, some research suggests that these agents may impede

memory formation and induce unhealthy changes to brain structures and neuronal connectivity when used over days.[312,313] Finally, although reduction of complications such as shivering is important, extended sedation can delay rehabilitation and mobilization, diminishing any potentially beneficial returns. Therefore, both positive and negative effects of pharmacological intervention on plasticity, rehabilitation, and recovery must be considered for optimization of treatment, especially with the concurrent use of TH.

4.9 Current Perspectives on Therapeutic Hypothermia and Plasticity

Considering the available evidence at this time, it does not appear that TH impedes plasticity or neurogenesis. However, this comes with several considerable caveats. First, it is clear that there is a substantial need for further study on the effect of TH on neuroplasticity; the bulk of the TH literature does not consider either direct or indirect effects on neuroplasticity. Second, a more holistic battery of plasticity measures and time points are needed to accurately gauge the effects of TH. Third, most of the studies discussed here used mild TH ranging from 30°C to 35°C. Experiments in which cooling depth, duration, and delay are varied are needed in order to establish which treatment parameters will optimally augment or negatively affect plasticity, especially as local cooling methods become more commonly used. Fourth, pharmacological agents that are used clinically in conjunction with TH must be assessed in preclinical models to establish their effect on plasticity and to ensure safety or enhance efficacy. This must be done across models and pathologies in order to fully inform clinical practice. Finally, future work must be done in both sexes, multiple species, young to aged animals, replicated in multiple laboratories, and be appropriately randomized and blinded, in accordance with Stroke Treatment Academic Industry Roundtable (STAIR) guidelines.[314] Ensuring that the

above aspects are considered when planning future studies on this subject will aid translational success, as plasticity is an important driving force behind optimal functional recovery and must be assessed as part of the efficacy of TH as a potential treatment.

Chapter 5: General Discussion

5.1 Primary Findings

The purpose of this thesis was to examine aspects concerning the safety of using TH in a rat model of collagenase-induced ICH. These investigations are an effort to better predict the clinical effects of TH after ICH. Considered together, the thesis results yield the following four conclusions: 1) ICI can be safe in a hemorrhagic setting; 2) there is no significant late or re-bleeding regularly occurring in our rat model of collagenase-induced ICH; 3) coagulopathy, an isolated factor of TH, does not cause late or re-bleeding by itself; 4) mild hypothermia does not have deleterious effects on neuroplasticity.

The results in Chapter 2 show that ICI given during a time of active brain bleeding does not affect brain hematoma volume, edema, or elemental concentrations 24 hours after ICH. This suggests that although this infusion protocol appears safe, it does not improve outcome. Future research may be able to implement a co-treatment with ICI, or use different dosing regimens to improve efficacy and achieve some level of neuroprotection. In the experiments outlined in Chapter 3, warfarin was used 24 hours after ICH for 6 days to induce a state of anticoagulation. Anticoagulated animals did not have larger hematomas after the week of treatment compared to the placebo group. This finding suggests that anticoagulation, as an isolated factor of TH, does not increase bleed size on its own. Applying this further, the late re-bleeding seen in previous work is unlikely to be due to anticoagulation alone.[1] These findings further suggest that late bleeding is not regularly occurring in a rat animal model of ICH. Future studies are needed to determine which factors, or combinations thereof, can cause the re-bleeding complications seen in previous work.[1] In Chapter 4, I reviewed the literature regarding the effects of TH on neuroplasticity after stroke, finding that mild hypothermia does not appear to be harmful to

neuroplastic responses. This suggests that the possibility of TH impeding critical recovery mechanisms following injury is less of a concern than previously thought. However, future research should consider direct and indirect effects on neuroplasticity, and include measures and time points that will capture the wide range of TH effects. They should also implement varied cooling depths, durations, and delays in treatment parameters. Further pre-clinical research should also investigate the pharmacological agents used with clinical TH to establish their effect on plasticity, to ensure safety, and enhance efficacy.

In summary, TH appears to be safe for ICH; however, future investigations are needed to identify which combination of TH factors could cause harm in ICH. Moreover, studies to optimize efficacy of TH for ICH should be pursued. Addressing the many parameters of TH and balancing its risks with benefits will help inform optimal individualistic patient treatment.

5.2 Limitations and Future Directions

One limitation of this work is the use of a single rodent ICH model in Chapters 2 and 3. Although the collagenase model mimics ongoing bleeding, it is unlikely to incorporate all the nuances, comorbidities, and complications of clinical ICH. Also, young, healthy male rats were used. These rats may be biologically better equipped (i.e., with healthier vasculature and lack of other diseases) for protection against ICH than their aged counterparts. Future research should use multiple models of ICH, such as spontaneously hypertensive rats or other species, before attempting to translate findings to a clinical setting. The STAIR guidelines provide a standard for future work: research should include both sexes, multiple species, young to aged animals, and be replicated in multiple laboratories, and appropriately randomized and blinded.[314]

The work in Chapter 2 was continued by others in the lab during the writing of this thesis.[2] They followed up on the results presented here with an investigation of functional and histological outcome for a long-term (28-day post-ICH) survival. They found similar performance on functional behavioral assessments and no difference in histological lesion volume between the groups. Considered together, these findings were contrary to our initial hypothesis that ICI would worsen hematoma volume and outcome in ICH. The lack of difference between ICI and control groups suggests that, although ICI may not cause harm in ICH, it does not appear to be beneficial either. Further research is needed to determine what changes, if any, ICI causes in the course of injury in ICH. For example, ICI could be engaging collateral vasculature and flushing inflammatory mediators, and this is a possible avenue for further investigations. Additionally, ICI was performed immediately following (~1 hour) ICH initiation in our studies to reduce confounds associated with re-anesthetizing the animal. Future research could vary the timing of ICI following ICH, possibly using the awake ICH method[227] newly developed by others in our lab to avoid re-anesthetizing confounds. Lastly, our results suggest that ICI is safe for primary hemorrhage, but this may not be generalizable to other hemorrhagic scenarios or when given at different doses or time periods. For example, safety of ICI use in secondary hemorrhage in ischemic stroke should be investigated. Individual circumstances, such as co-morbidities and active pharmaceuticals, should also be considered and investigated.

Results from Chapter 3 indicate that anticoagulation alone does not cause late re-bleeding in ICH. However, late bleeding may occur when anticoagulation is combined with other factors, such as high BP, variable vasodynamics (constriction or dilation), or co-morbid diseases. More work is needed to find feasible methods of manipulating these factors in various combinations. Possibilities could include pharmacological methods, or the use of animals with co-morbid

diseases such as hypertension or diabetes. However, the avenues already explored, such as midodrine and phenylephrine, show the challenges drug studies face. These require considerable time and resources to find the right type of substance, appropriate dose and timing, and to ensure that interactions are known and managed when combined with other drugs. Milrinone is a hopeful next stage in examining the effects of vasodynamics and anticoagulation. Milrinone is used clinically in TH to counteract acidosis, afterdrop, rebound vasodilation, and vascular dysfunction associated with the rewarming phase.[259,260] If investigated pre-clinically, these types of studies could inform clinical practice with the use of these drugs, TH, and coagulopathy or high BP. Furthermore, investigations into the treatment of these combined harmful effects should be pursued.

The literature review in Chapter 4 found that, although cooling does not appear to critically impede plasticity, there is need for further studies into the effects of TH on neuroplasticity. This is because most TH literature does not consider either direct or indirect effects on neuroplasticity. For this, multiple measures, at variable times, using a range of cooling depth, duration, and delays should be used to attain a more holistic view of the effects of TH on neuroplasticity. For example, measuring cell counts, morphology, or activity can be direct measures of neuronal plasticity. Furthermore, the many pharmacological agents that are used in combination with TH clinically should be assessed for effects on neuroplasticity. In preparing the literature review presented in Chapter 4, I found 108 substances that have been associated with TH or other stroke therapies.[196,297–304,315–331] Of these, 55 substances have been investigated for use with TH. A further 42 substances have been reported as ‘on-board’ when TH is initiated (i.e., drugs that a patient is taking for other purposes, but that may interact with TH). Finally, another 11 were reported as neuroprotective substances that are hopeful for improving the efficacy of

treatment overall. However, the activity of all these substances could alter with changed temperature. Some of these activity changes are known, and some are not. It is important to continue the work on these drugs, to be informed about how these many substances may act in conjunction with TH, so patient care can be optimized. All these factors must be considered together to develop an optimal protocol of TH for individual patients.

5.3 Conclusions

This thesis evaluated aspects of safety relating to TH use for ICH. First, ICI was found to have no effect on hematoma size or edema measured 24 hours after ICH. This suggests that ICI is safe when administered during a time of active bleeding in ICH. Second, warfarin-induced anticoagulation starting 24 hours post ICH did not worsen hematoma volume assessed at 7 days post-ICH. Lastly, a review of recent literature suggests mild TH is not harmful to neuroplastic mechanisms following ICH. Considered together, TH appears to be safe for ICH; however, future investigations are needed to identify which (likely compounding) factors of TH could cause harm in ICH.

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Appendix

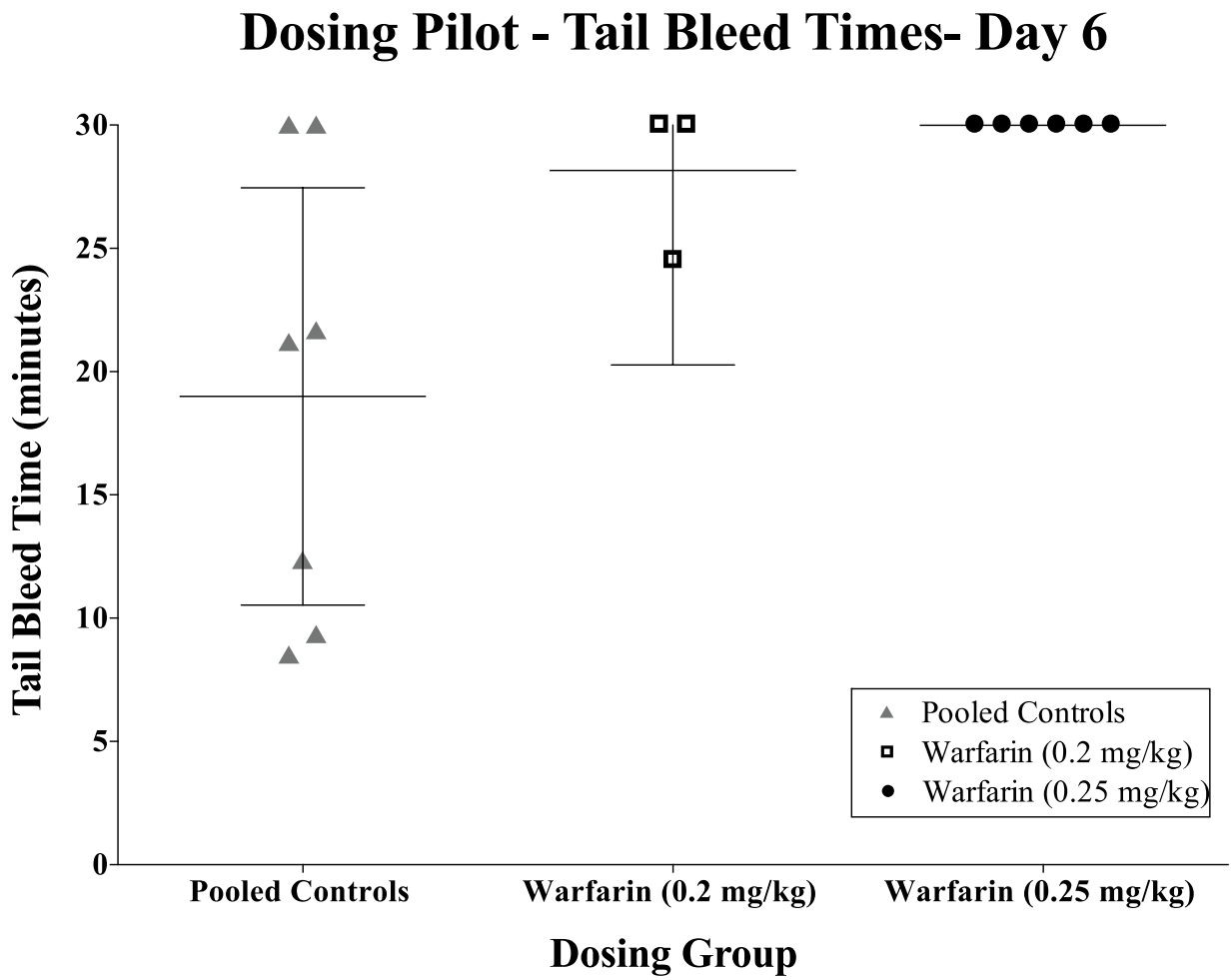

Figure. A3.1. (Warfarin Dosing Pilot) Testing tail bleed times of 3 dosing groups in naïve (no ICH) animals: Controls (saline), 0.2 mg/kg/day, and 0.25 mg/kg/day (both treatment groups had a 0.4 mg/kg loading dose on Day 1). Bars represent the average, with 95% confidence intervals. Tail bleed times of the initially tested 0.2 mg/kg maintenance dose regimen were more variable than desired. When re-tested with a 0.25 mg/kg maintenance dose, that variability reduced, and all animals bled for maximum time allowed, without spontaneous bleeding being induced. This outcome was desired.

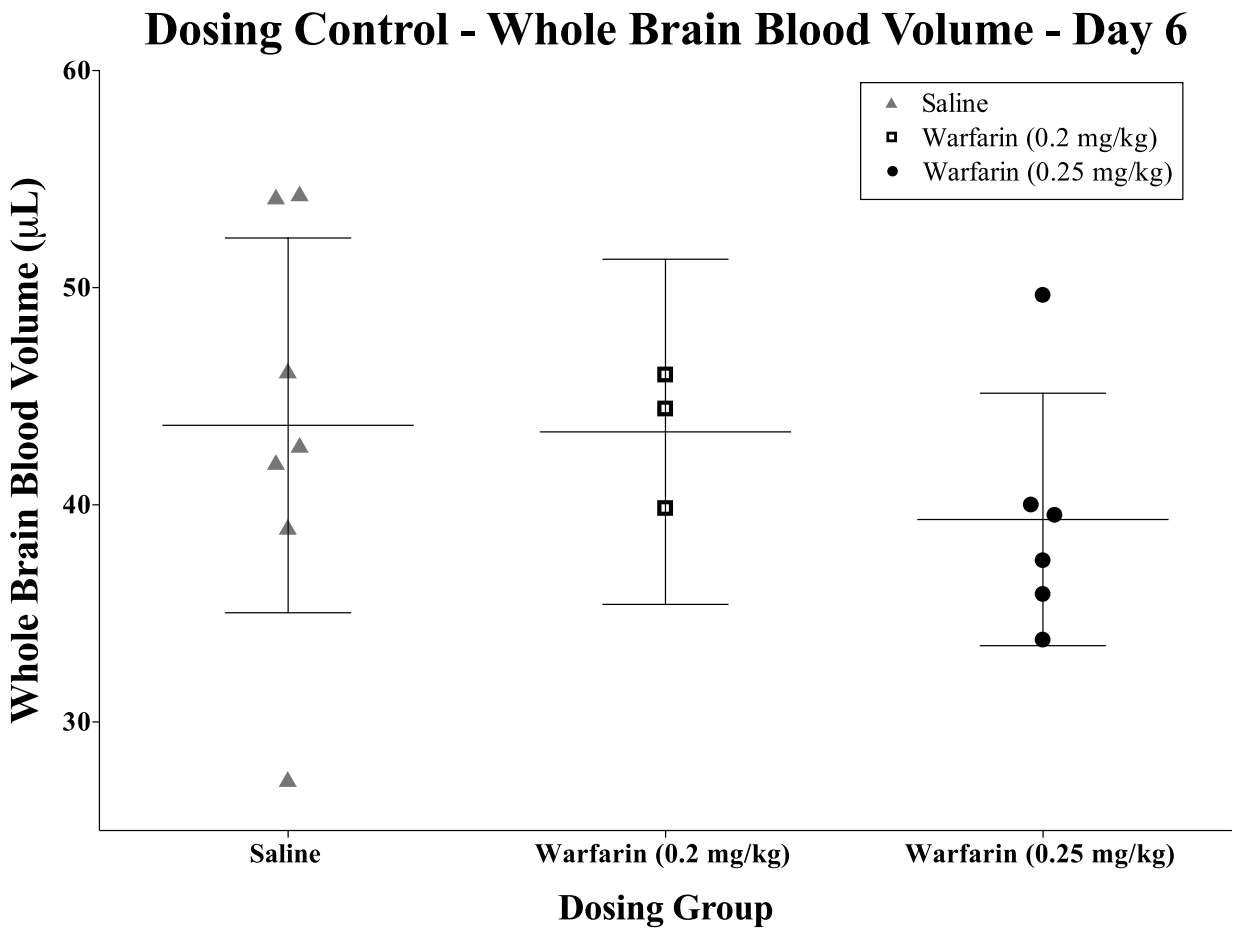

Figure. A3.2. (Warfarin Dosing Pilot) Whole brain blood volume (µL) on day 6, comparing 0.2 mg/kg and 0.25 mg/kg warfarin doses and saline treated groups in naïve (no ICH) animals. Bars are average with 95% confidence interval. One-way ANOVA with multiple comparisons shows no difference between groups ($p > 0.9$). Thus, each warfarin dose is not causing detectable spontaneous bleeding in the brain tissue.

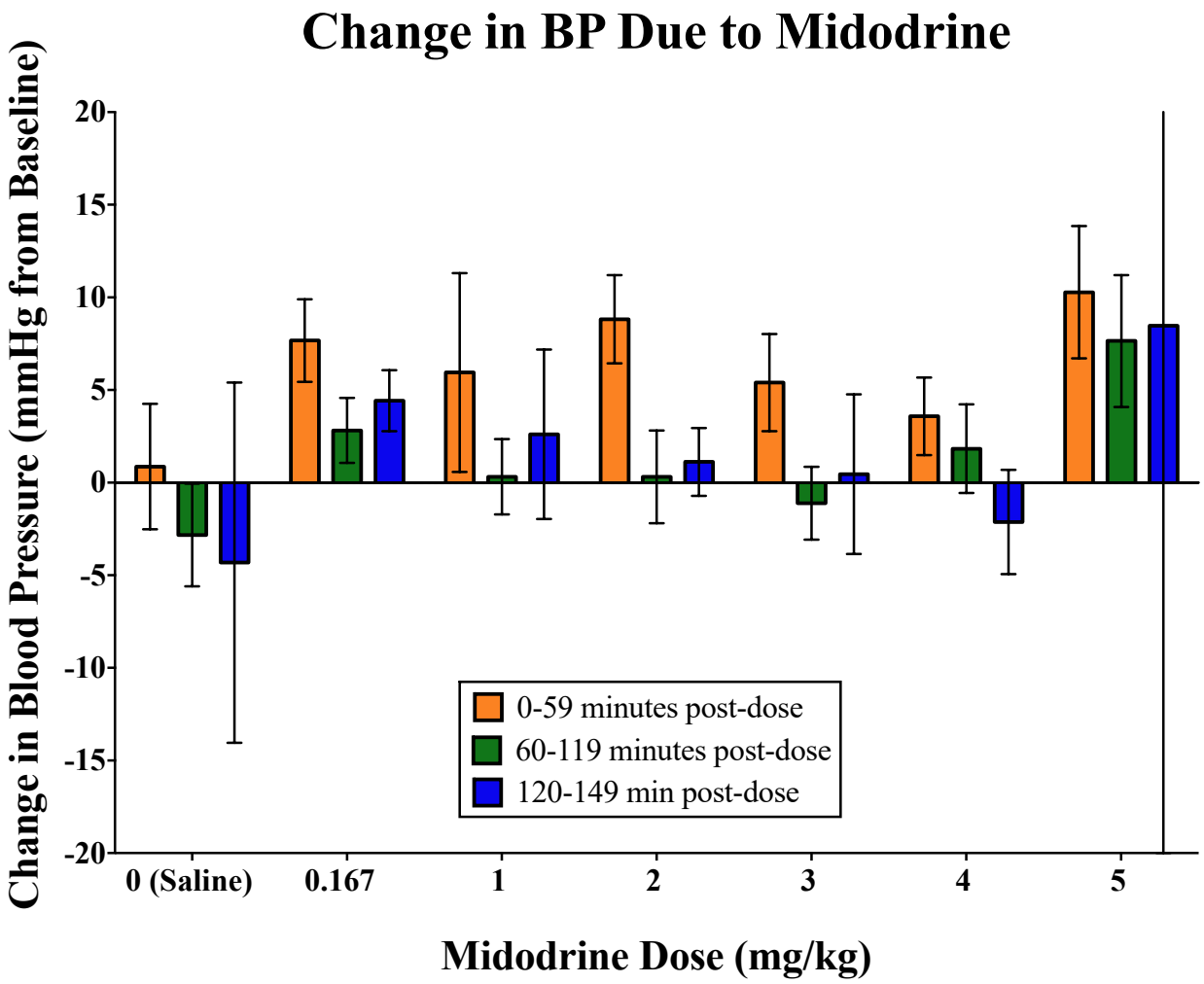

Figure. A3.3. (Midodrine Dosing Pilot) Investigation of various midodrine doses effects on blood pressure (BP) over time. Bars are average with 95% confidence interval. Extent of change and variability of the BP response was not adequate to pursue midodrine hydrochloride as an acceptable pharmacological manipulation of BP.